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Toxicological and pharmacokinetic properties of sucralose-6-acetate and its parent sucralose: *in vitro* screening assays

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ABSTRACT

The purpose of this study was to determine the toxicological and pharmacokinetic properties of sucralose-6-acetate, a structural analog of the artificial sweetener sucralose. Sucralose-6-acetate is an intermediate and impurity in the manufacture of sucralose, and recent commercial sucralose samples were found to contain up to 0.67% sucralose-6-acetate. Studies in a rodent model found that sucralose-6-acetate is also present in fecal samples with levels up to 10% relative to sucralose which suggest that sucralose is also acetylated in the intestines. A MultiFlow[®] assay, a high-throughput genotoxicity screening tool, and a micronucleus (MN) test that detects cytogenetic damage both indicated that sucralose-6-acetate is genotoxic. The mechanism of action was classified as clastogenic (produces DNA strand breaks) using the MultiFlow[®] assay. The amount of sucralose-6-acetate in a single daily sucralose-sweetened drink might far exceed the threshold of toxicological concern for genotoxicity (TTC_{genotox}) of 0.15 µg/person/day. The RepliGut[®] System was employed to expose human intestinal epithelium to sucralose-6-acetate and sucralose, and an RNA-seq analysis was performed to determine gene expression induced by these exposures. Sucralose-6-acetate significantly increased the expression of genes associated with inflammation, oxidative stress, and cancer with greatest expression for the metallothionein 1 G gene (MT1G). Measurements of transepithelial electrical resistance (TEER) and permeability in human transverse colon epithelium indicated that sucralose-6-acetate and sucralose both impaired intestinal barrier integrity. Sucralose-6-acetate also inhibited two members of the cytochrome P450 family (CYP1A2 and CYP2C19). Overall, the toxicological and pharmacokinetic findings for sucralose-6-acetate raise significant health concerns regarding the safety and regulatory status of sucralose itself.

KEYWORDS



Sucralose; sucralose-6-acetate; genotoxicity; gene expression; intestinal barrier

Introduction

Background: Discovery and synthesis

Sucralose is a chlorinated artificial sweetener that is used worldwide as a sugar substitute in thousands of food, beverage, and pharmaceutical products (Schiffman and Rother 2013). The sweet taste of sucralose was discovered at Queen Elizabeth College in London (Hough and Phadnis 1976) as part of a program to chemically modify sucrose (table sugar) for possible industrial applications. One structural modification was a chlorinated version of a novel disaccharide fructogalactose in which three hydroxyl groups were replaced by chlorine atoms with the chemical name 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-

D-galactopyranoside. This compound was originally called trichlorogalactosucrose (TGS) but was subsequently given the name sucralose. The sweetness potency of sucralose is approximately 385 to 650-fold greater than sucrose by weight depending on the specific application (DuBois et al. 1991; Schiffman, Sattely-Miller, and Bishay 2008). The manufacturing process involves the synthesis of sucralose-6-acetate in multiple steps from sucrose with subsequent deacetylation to produce sucralose (Hao 2011; Mufti and Khan 1983; Wang et al. 2011). The chemical structures of sucralose-6-acetate and sucralose are presented in Figure 1. The sucralose-6-acetate intermediate generated during the manufacturing process is retained as an impurity in commercial sources of sucralose (Catani et al. 2006; OpAns

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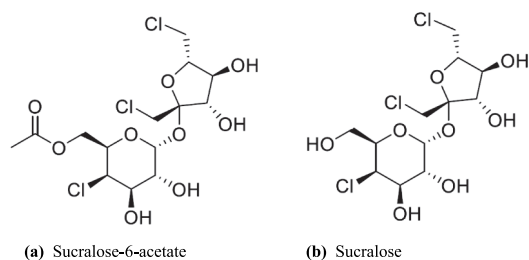


Figure 1. Chemical structures of sucralose-6-acetate (molecular weight 439.7, CAS number 105066-21-5) and sucralose (molecular weight 397.6, CAS number 56038-13-2).

2021; United States Food and Drug Administration US FDA 2021; Werness 2021).

Historical safety claims

Sucralose has regulatory approval in North America, Europe, and Asia along with the establishment of Acceptable Daily Intake (ADI) levels (Canada Gazette 1991; European Union EU 2004; Japanese Ministry of Health and Welfare JMHW 1999; Scientific Committee on Food SCF 2000; United States Food and Drug Administration US FDA 1998, 1999). Regulatory approval and establishment of ADIs were based upon historical studies performed during the 1980s and early 1990s in rats, mice, dogs, rabbits, and humans and ultimately published in part in the year 2000 (Baird et al. 2000; Goldsmith 2000; Grice and Goldsmith 2000; John, Wood, and Hawkins 2000a, 2000b; Kille et al. 2000a, 2000b; Roberts et al. 2000; Sims et al. 2000; Wood, John, and Hawkins 2000). These historical studies made the following 6 claims regarding sucralose and constituted the rationale for its regulatory approval:

- 1) Stability *in vivo*: Passes through the intestine unchanged.
- 2) Gut Microflora: No effect on gut microflora.
- 3) Intestinal Barrier: No effect on intestinal tissue.
- 4) Bioaccumulation: Does not bioaccumulate.
- 5) Metabolism: No effect on metabolism including blood glucose or insulin.
- 6) Biological/Toxicological Impact: Not genotoxic with no associated biologically significant consequences, and is heat stable.

Based on these historical claims, an ADI of 15 mg/kg body weight/day for humans was established by the Joint FAO/WHO Expert Committee on Food Additives JECFA (1991) and by the Scientific Committee on Food SCF (2000). A lower ADI of 5

mg/kg body weight/day was set by the United States Food and Drug Administration US FDA (1998).

Independent research contrary to historical safety studies

Many scientific research investigations since regulatory approval, however, do not corroborate any of the 6 early historical claims regarding the biological fate or safety of sucralose.

Stability *in vivo*

Two acetylated sucralose biotransformation products were found in urine and feces of rats dosed with sucralose (Bornemann et al. 2018), and this finding is inconsistent with the historical claim that sucralose is stable and excreted unchanged (i.e., not metabolized) in the intestine. The more abundant acetylated metabolite was identified as sucralose-6-acetate (Werness and Schiffman 2020), and its relative contribution to the biological consequences of sucralose exposure has not yet been determined.

Gut microflora

Ingestion of sucralose by humans and/or animals within approved ADI levels was found to disrupt the microbiome in the gastrointestinal tract (GIT) (Abou-Donia et al. 2008; Bian et al. 2017; Méndez-García et al. 2022; Suez et al. 2022; Zhang et al. 2022). Maternal ingestion of sucralose in pregnant and lactating mice also impacted their progeny's microbiome (Dai et al. 2020, 2021; Olivier-Van Stichelen, Rother, and Hanover 2019). Sucralose has also been detected in human breast milk samples from lactating women which indicates it is ingested by nursing infants (Sylvetsky et al. 2015).

Intestinal barrier

Sucralose also impacts the intestinal tissue. Sucralose ingestion induced histopathological changes including lymphocytic infiltrates into the intestinal epithelium, glandular disorganization, and epithelial scarring (Abou-Donia et al. 2008), increased bacterial infiltration into the ileal lamina propria in Crohn's disease – like ileitis (Rodriguez-Palacios et al. 2018), elevated % CD3⁺T cells, CD19⁺B cells, and IgA⁺ plasma cells in Peyer's patches (Rosales-Gómez et al. 2018), significantly increased levels of fecal chymotrypsin and trypsin and reduced fecal β -glucuronidase

(Li et al. 2016), initiated lymphocyte aggregation in the ileum and colon (Zheng et al. 2022), and promoted inflammation and colitis-associated colorectal cancer risk (Guo et al. 2021; Li et al. 2020; Wang et al. 2019). Further, maternal ingestion of sucralose inhibited intestinal development, disrupted barrier function, and induced Paneth cell defects in offspring (Dai et al. 2020, 2021). *In vitro* studies demonstrated that sucralose enhanced biofilm formation along with bacterial invasion into gut epithelial cells (Shil and Chichger 2021) and disrupted tight junctions and barrier function in an intestinal epithelial model (Shil et al. 2020).

Bioaccumulation

Sucralose was found to bioaccumulate in adipose tissue of rats and was present two weeks after cessation of a 40-day feeding period even though it had disappeared from the urine and feces (Bornemann et al. 2018). An *in vitro* study reported that sucralose increased lipid accumulation and expression of adipocyte differentiation genes in cultured adipocytes (Azad et al. 2020).

Metabolism

Consumption of sucralose was noted to alter glucose and/or insulin concentrations in the plasma of some human subjects when delivered in liquids or capsules (Lertrit et al. 2018; Méndez-García et al. 2022; Pepino et al. 2013; Romo-Romo et al. 2018; Schiffman and Rother 2013; Suez et al. 2022) and when accompanied by carbohydrate (Dalenberg et al. 2020) or another non-caloric sweetener (Young et al. 2017). Maternal ingestion of sucralose during pregnancy impacted the progeny's metabolism including downregulation of hepatic detoxification mechanisms and changes in bacterial metabolites (Olivier-Van Stichelen, Rother, and Hanover 2019). Additional studies reported that sucralose might affect incretin hormones including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) as well as the sodium-dependent glucose co-transporter-1 (SGLT-1) (Kreuch et al. 2018; Lertrit et al. 2018; Margolskee et al. 2007; Sun et al. 2017; Young et al. 2017). Further, sucralose was demonstrated to blunt thyroid function (Pałkowska-Goździk, Bigos, and Rosołowska-Huszcz 2018). Chronic sucralose ingestion at levels that have regulatory approval in the

United States and Europe also modify the fecal metabolome (Bian et al. 2017), liver proteome (Liu et al. 2019), and induce the expression of two intestinal proteins involved in first-pass metabolism, specifically P-glycoprotein (P-gp) and cytochrome P450 (CYP3A4) (Abou-Donia et al. 2008).

Biological/Toxicological impact

Additional independent research investigations since regulatory approval reported toxicological findings including genotoxicity and cancer risk following sucralose exposure. Sucralose, administered in feed beginning prenatally through the lifespan, induced hematopoietic neoplasias such as leukemias in male mice (Soffritti et al. 2016). Significant elevation in the number and size of colorectal tumors was detected in a murine model of colorectal cancer after sucralose treatment (Li et al. 2020). Four different studies utilizing a comet assay found that sucralose damaged DNA (Pasqualli et al. 2020; Raya et al. 2020; Sasaki et al. 2002; Van Eyk 2015). Sucralose also enhanced antimicrobial resistance and mutation frequency of *E. coli* (Qu et al. 2017). Further, heating sucralose with glycerol or lipids was found to generate chloropropanols, a potentially toxic class of compounds (Rahn and Yaylayan 2010), and this finding was supported by subsequent investigations that also reported thermal instability of sucralose accompanied by generation of hazardous polychlorinated compounds even in mild temperature conditions (De Oliveira, de Menezes, and Catharino 2015; Eisenreich, Gürtler, and Schäfer 2020).

Rationale for additional safety studies

It has not yet been established if adverse biological findings reported since regulatory approval are due to exposure to sucralose itself, to acetylated sucralose (e.g., sucralose-6-acetate), or both. Exposure to sucralose-6-acetate might occur during sucralose ingestion or result from metabolism of sucralose in the intestine. The amount of sucralose-6-acetate retained as an impurity in recent commercial sucralose samples varied with levels up to 0.67% (Werness 2021). McNeil Specialty Products Company acknowledged the presence of sucralose-6-acetate in batches of sucralose in their Food Additive Petition to the US FDA prior to approval (stated as 0.8% but <1.3%) (United States

Food and Drug Administration US FDA 2021). However, the concentration of sucralose-6-acetate relative to sucralose in feces of rats dosed with sucralose was significantly greater than amounts in commercial sucralose samples, with fecal sucralose-6-acetate levels up to 10% (Bornemann et al. 2018; Werness and Schiffman 2020). This significant rise of the ratio of sucralose-6-acetate to sucralose in fecal samples may result from acetylation of sucralose by commensal bacteria in the GIT. Commensal bacteria in the intestine are known to acetylate xenobiotic compounds (Delomenie et al. 2001; Dull, Salata, and Goldman 1987). Previously Labare and Alexander (1994) noted that microorganisms including bacteria were found to metabolize sucralose in sewage and soil samples. Further, Sun et al. (2017) reported that a bacterium, *Bacillus amyloliquefaciens*, is capable of both acetylating and deacetylating sucralose. Overall, these findings indicate that biological exposure to sucralose-6-acetate may occur from ingestion of commercial impure sucralose as well as from acetylation of sucralose in the intestine.

The potential adverse health effects of exposure to sucralose-6-acetate are not yet known because this chemical has not been tested individually with a toxicology battery of tests to assess human risks. A comprehensive and systematic search of the scientific literature including government websites, chemical databases, patent literature, and scientific publications did not produce any apparent information on genotoxicity and cytotoxicity attributed to sucralose-6-acetate.

Additional studies undertaken

In this report, a series of 8 experiments were performed to screen for toxicological and pharmacokinetic properties of sucralose-6-acetate along with its structural parent sucralose (control). The following tests were utilized in these 8 experiments.

- (1) An *in vitro* MultiFlow[®] assay, a rapid high-throughput screening tool that predicts clastogenicity (induced DNA strand breakages) and aneugenicity (alterations in chromosome numbers), was used to determine genotoxic potential (Bryce et al. 2017; 2018).
- (2) A traditional *in vitro* Mammalian Cell Micronucleus Assay in thymidine kinase 6

(TK6) cells was performed to assess potential cytogenetic/chromosomal damage (Organisation for Economic Cooperation and Development OECD 2016).

- (3) The *in silico* Model Applier Leadscope[®] program was employed to detect structural chemical features with the potential to induce genetic mutations (Dearfield et al. 2017).
- (4) A traditional Bacterial Reverse Mutation Test (Ames test) was performed to assess mutagenic potential (Organisation for Economic Cooperation and Development OECD 2020).
- (5) Transepithelial Electrical Resistance (TEER) and permeability of human transverse colon were assessed using the RepliGut[®] system to investigate potential impairment and integrity of the intestinal barrier (Allbritton et al. 2021; Altis Biosystems, Durham, North Carolina, USA).
- (6) RNA-seq (RNA-sequencing) was utilized to investigate gene expression and identify differentially expressed genes in human intestinal epithelium (Marioni et al. 2008; Wang, Gerstein, and Snyder 2009).
- (7) Liver Microsome Stability Assays were employed to assess potential hepatic biotransformation (Houston 1994).
- (8) A Cytochrome P450 (CYP450) Inhibition Study was conducted to assess potential blunting of detoxification enzymes that might lead to drug-drug interactions (Obach et al. 2006).

These toxicological and pharmacokinetic tests were conducted in independent analytical labs that were selected based upon their expertise in specific *in vitro* techniques. This test battery of multiple assays was undertaken because no single test is able to conclusively identify the numerous potential toxicological and pharmacokinetic properties of a chemical compound.

Methods

Test articles

Two test articles, sucralose-6-acetate and sucralose, were utilized in 8 experiments to determine their toxicological and pharmacokinetic properties. Sucralose-6-acetate (4,1,6-trichloro sucralose-6-acetate) was synthesized by Jiangyin PharmaAdvance,

Inc., 6 Dongsheng, West Road, Building D1, Jiangyin, Jiangsu Province, P. R. China 214431. The sucralose-6-acetate conformed to structure with a purity of 99.7% and was certified by ^1H NMR spectrum, mass spectrum and high-performance liquid chromatography with an evaporative light scattering detector (HPLC-ELSD). Sucralose, used for control and comparison in several studies, was obtained from Sigma-Aldrich. It contained 0.5% sucralose-6-acetate as determined by HPLC-MS/MS (OpAns, Durham, North Carolina, USA) which is consistent with commercial food-grade sucralose.

Experiment 1: *In vitro* MultiFlow[®] DNA damage assay in TK6 cells

A rapid high-throughput flow cytometric assay (*in vitro* MultiFlow[®] Assay) was performed to assess the genotoxic potential of sucralose-6-acetate and sucralose in human TK6 cells using a 96-well format. This screening tool for DNA damage predicts whether compounds are clastogens, aneugens, or non-genotoxic based upon increases in two clastogen-sensitive biomarkers ($\gamma\text{H2A}\times$ and p53) and two aneugen-sensitive biomarkers (p-H3 and polyploidy) (Bernacki et al. 2016; Bryce et al. 2014, 2016, 2017, 2018). The phosphorylated histone $\gamma\text{H2A}\times$ is an indicator of double-strand DNA breaks, and translocation of tumor protein p53 to the nucleus is a marker of DNA damage response. Phospho-histone 3 (p-H3) accumulates in cells exposed to aneugens, and polyploidization is a consequence of aneugenic activity. The methodology for the MultiFlow[®] assay that assessed these endpoints of DNA damage response pathways was described previously (Bryce et al. 2017; Hung et al. 2020). The assay was conducted by BioReliance (Rockville, MD) under their protocol entitled *In Vitro* Clastogenic, Aneugenic, or Non-Genotoxic (CAN) FlowScreen Assay in TK6 Cells (BioReliance 2020a, 2021). High-performing mathematical algorithms were used to predict the mode of action (MoA) based upon the signatures of biomarkers for clastogenicity and aneugenicity using established Global Evaluation Factors (GEFs) that provide cutoff values indicating significant fold increases for each biomarker (Bryce et al. 2017).

Materials

Materials for liberation of nuclei, staining of chromatin, and immunological labeling of specific nuclear epitopes (MultiFlow[®] DNA Damage Kit – p53, $\gamma\text{H2A}\times$, Phospho-Histone H3 kit) were purchased from Litron Laboratories, Rochester, NY. The components and reagents in the proprietary kit included: Nuclei Release Solution with Counting Beads (lyses cells and provides absolute bead count), DNA Stain (propidium iodide) that labels free nuclei for identification in flow cytometric analysis, RNase Solution that removes RNA, p53 Antibody FITC that detects nuclear translocation of the protein p53, $\gamma\text{H2A}\times$ Antibody Alexa Fluor[®] 647 that detects double-strand breaks, and Phospho-Histone H3 Antibody PE that detects mitotic cells. Multiflow[®] analyses were performed at two time points (4 and 24 hr) after treatment initiation with test articles. Latex microsphere counting beads were utilized to calculate nuclei density and cytotoxicity metrics. The Multiflow[®] reagent solution was prepared from these components according to the instruction manual for the kit.

An exogenous metabolic activation system (MutazymeTM, a Phenobarbital/ β -Naphthoflavone (PB/NF) induced liver S9 derived from male Sprague Dawley rats) was obtained from Moltox[®], Boone, NC (www.moltox.com). Four genotoxic compounds with either a clastogenic or aneugenic MoA were employed as controls. Methyl methanesulfonate (MMS) and carbendazim (100, 50, 25, or 12.5 μM for both compounds) were utilized as positive controls for the treatment without S9 (–S9) activation. Cyclophosphamide (80, 40, 20 or 10 μM) and benzo (a)pyrene (100, 50, 25, or 12.5 μM) were used as positive controls for the treatment with S9 (+S9) activation. The positive controls were employed to ensure responsiveness of the test system and appropriate clastogenic and/or aneugenic MoA prediction but not to provide a standard for comparison with test articles. Dimethyl sulfoxide (DMSO) was used as the solvent for positive controls.

TK6 cell culture test system

TK6 cells, lymphoblastoid cells of human origin, were obtained from the American Type Culture Collection (repository number CRL-8015), Manassas, VA. The TK6 cell line is p53 proficient,

sensitive to different mechanisms of genotoxicity with a doubling time of 12–14 hr. The spontaneous frequency of mutations and chromosome alterations in TK6 cells does not differ significantly from primary human cells (Schwartz et al. 2004). The TK6 cells were cultured in T -75 cm^2 flasks in RPMI 1640 medium with L-glutamine (Sigma-Aldrich) supplemented with 10% heat-inactivated horse serum and penicillin-streptomycin complete culture medium (CCM). Cultures were incubated at 37°C, 5% CO_2 and $\geq 85\%$ humidity. After 22–26 hr incubation, cell density in the T -75 cm^2 flasks was calculated after cell counting. Target cell stocks required for the Multiflow® tests in both absence and presence of S9 were calculated; an appropriate volume of cell suspension was transferred to 50 ml tubes and centrifuged at $150 \times g$ for 6 min. The culture medium was aspirated, and cell densities adjusted with the CCM solution to 2×10^5 cells/ml in the absence of S9, and to 2.2×10^5 cells/ml in the presence of S9, immediately prior to use.

Treatments, and flow cytometric analysis

The TK6 cells were exposed to 20 concentrations of sucralose-6-acetate (maximum 4.5489 mM or 2000 $\mu\text{g}/\text{ml}$) or 20 concentrations of sucralose (maximum 10 mM or 3980 $\mu\text{g}/\text{ml}$) with a dose spacing of 1.4142 (square root of 2) in the presence (+S9) and absence (–S9) of metabolic activation along with vehicle control alone using 96-well plates. The test articles were prepared using DMSO with a final DMSO concentration in the cell suspension below 1%. In the +S9 condition, cells were exposed to S9 (Mutazyme™) for 4 hr after which S9 was washed out, centrifuged twice (5 min at $340 \times g$), and re-incubated in fresh culture media. Aliquots were taken from +S9 treatment wells at 4 hr (prior to the wash step) and at 24 hr incubation and subsequently transferred to new plates that were pre-loaded with 50 μl MultiFlow®-kit reagent solution. In the -S9 condition, aliquots were also taken at 4 and 24 hr exposure and transferred to new plates that were also pre-loaded with MultiFlow®-kit reagent solution. Cells were incubated in the reagent mix according to instructions in the kit in order to simultaneously digest the cytoplasmic membranes in the harvested cells, liberate the nuclei, stain the chromatin with the fluorescent nucleic

acid dye, and label $\gamma\text{H2A}\times$, p-H3, and p53 with fluorescent antibodies. Fluorescent microspheres in reagent mix were used to obtain nuclei-to-bead ratios as a simple cytotoxicity index at 4 and 24 hr. Analysis was performed by flow cytometry utilizing a BD FACSCanto II Flow Cytometer with BD FACSDiva™ software (BD Biosciences), and fold-shifts in biomarkers determined.

Analysis of cytometric results

High-performing mathematical algorithms (Bernacki et al. 2016; Bryce et al. 2016, 2017) were used to predict MoA based upon multi-endpoints of biomarkers for clastogenicity and aneugenicity using Global Evaluation Factors (GEFs) that provided cutoff values representing significant fold elevation for each biomarker. Conditions for making MoA calls were established for 3 cases. First, a clastogenic call was made for S9 treatments (+S9) by fold-increases in two consecutive concentrations that met or exceeded GEFs for at least two out of 4 clastogen-sensitive biomarkers with $\gamma\text{H2A}\times$ required for at least one response:

- (a) ≥ 1.44 -fold 4-hr $\gamma\text{H2A}\times$,
- (b) ≥ 1.31 -fold 24-hr $\gamma\text{H2A}\times$,
- (c) ≥ 1.23 -fold 4-hr nuclear p53,
- (d) ≥ 1.12 -fold 24-hr nuclear p53.

Second, a clastogenic call was made for 24-hr treatments (–S9) by fold elevation in two consecutive concentrations that met or exceeded cutoffs for at least two out of 4 clastogen-sensitive biomarkers with $\gamma\text{H2A}\times$ required for at least one response:

- (a) ≥ 1.51 -fold 4-hr $\gamma\text{H2A}\times$,
- (b) ≥ 2.11 -fold 24-hr $\gamma\text{H2A}\times$,
- (c) ≥ 1.40 -fold 4-hr nuclear p53,
- (d) ≥ 1.45 -fold 24-hr nuclear p53.

Third, an aneugenic signature was demonstrated by fold increases in two consecutive concentrations that met or exceeded cutoffs for at least two of the following aneugenic responses:

- (a) ≥ 1.71 -fold 4-hr phospho-histone H3,
- (b) ≥ 1.52 -fold 24-hr phospho-histone H3,
- (c) ≥ 5.86 -fold 24-hr polyploidy,
- (d) ≥ 1.45 -fold 24-hr nuclear p53.

The call was nongenotoxic under the test conditions if less than two clastogen-sensitive or two aneugen-sensitive biomarkers did not meet or exceed the above GEFs. Cytotoxicity was based upon reduction of nuclei counts for individual cultures.

Experiment 2: *In vitro* mammalian cell micronucleus test in TK6 cells

An *in vitro* mammalian cell micronucleus (MN) test in TK6 cells was used to determine if MN are present in the cytoplasm of cells that were exposed to sucralose-6-acetate. Micronuclei are small extranuclear structures that are produced by DNA breakage (clastogens) or are induced by numerical chromosomal aberrations (aneugens) (OECD 487, 2016). A rise in MN frequency is a biomarker of cytogenetic/chromosomal damage. The MN assay was performed by BioReliance (2020b) using TK6 cells according to standard protocol guidelines of the Organisation of Economic Cooperation and Development (OECD 487, 2016).

TK6 cells and treatment

The assay was conducted by treating TK6 cells with a range of concentrations of the test article (sucralose-6-acetate) as well as with positive and vehicle controls. The procedure for preparation of TK6 cells was described above for the Multiflow® test, and cell density was adjusted to 2.5×10^5 cells/ml in the absence and presence of exogenous metabolic activation (S9). DMSO was the vehicle for the sucralose-6-acetate and served as the vehicle control for each treatment type. The potential of sucralose-6-acetate and/or its metabolites to induce MN in TK6 cells was assessed in the presence (4-hr treatment) and absence (27-hr treatment) with S9. After the 4-hr incubation, cells were centrifuged to remove the treatment medium, resuspended in CCM, and incubated for an additional 23 hr. Sucralose-6-acetate was assessed at the following concentrations. For the 4-hr incubation, the concentrations of sucralose-6-acetate were 2000, 1500, 1000, 750, 700, 600, 500, 400, 350, 300, 200, and 100 µg/ml. For the 27-hr exposure, the levels of sucralose-6-acetate were 2000, 1500, 1250, 1000, 750, 500, 250, 125, 100, 80, 40, and 20 µg/ml. Cyclophosphamide (2.5, 3 or 4 µg/ml prepared in

water) was employed as a positive control for the 4-hr treatment, and vinblastine (10 or 12 ng/ml prepared in water) served as the positive control in the 27-hr treatment.

Micronucleus scoring and statistical analysis

Micronucleus scoring was performed after exposure to sucralose-6-acetate for a minimum of 2000 mononuclear cells and a minimum of 200 binucleated/multinucleated cells at 4 concentrations in the 4-hr and at 5 concentrations for the 27-hr treatment. Scoring included the number and frequency of MN in binucleated and multinucleated cells as well as mononuclear cells as increases in binucleated cells were evident at higher concentrations of sucralose-6-acetate in this study. Scoring was performed for 300, 500, 700 and 1000 µg/ml for the 4-hr exposure and 100, 250, 500, 750 and 1000 µg/ml for 27-hr incubation.

Significance was assessed with a Fisher's Exact Test (Fisher 1954) relative to solvent control. The Cochran-Armitage trend test was performed to determine if there was a trend in the number of micronucleated cells across increasing concentrations of sucralose-6-acetate (Agresti 2002; Armitage 1955; Cochran 1954). The criterion for significance was set at $p < .05$. Calculations were made in Excel (Microsoft Corporation). A MN test for sucralose itself was not undertaken in the current study as it was conducted and reported previously (United States Food and Drug Administration US FDA 1998).

Experiment 3: *In silico* assessment of mutagenic potential by Leadscope®

Leadscope®, a quantitative structure activity tool (Leadscope® 2019), was used to predict the genotoxic potential of sucralose-6-acetate and sucralose *in silico* based upon the chemical structure and performed by Aclairo Pharmaceutical Development Group (Aclairo 2019). The Leadscope® model utilizes a large mutagenic toxicology database called SAR Genetox based on International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH M7) (United States Food and Drug Administration US FDA 2018) with validated structures to generate computational structure-

activity predictions (Hevener 2018). This tool also provides bacterial mutation alerts for the active/primary portion or molecular fragment(s) of these molecules. The alert knowledge base was constructed from a systematic analysis of available bacterial mutagenicity data and scientific literature. The chemical structures of sucralose-6-acetate and its parent sucralose (control) were both entered into the Leadscape® model applicer as MOL files, a file format that includes attributes associated with entire chemical structure (CTFile Formats 2005).

Experiment 4: Bacterial reverse mutation test (Ames Test)

A Bacterial Reverse Mutation Test was performed by BioReliance (2020c) according to OECD guidelines (Organization of Economic Cooperation and Development OECD 2020). This classic mutagenicity test was employed to evaluate the potential of the sucralose-6-acetate, sucralose, and/or their metabolites to induce reverse mutations at loci of TA98, TA100, TA1535, and TA1537 strains of *Salmonella typhimurium* and the WP2 *uvrA* strain of *Escherichia coli* in the presence and absence of an exogenous metabolic activation system (S9). The test article, sucralose-6-acetate was prepared in DMSO, and sucralose was prepared in sterile deionized water. Both test articles were evaluated via the plate incorporation method at 8 concentrations 1.5, 5, 15, 50, 150, 500, 1500, and 5000 µg/plate. The final DMSO concentration in the cell suspension was at or below 1% v/v. The positive controls in the Bacterial Reverse Mutation Test for each bacterial strain are presented in Table 1.

Experiment 5: Assessment of transepithelial electrical resistance (TEER) and permeability in human transverse colon epithelium

A high throughput *in vitro* intestinal stem cell platform (RepliGut®, Altis Biosystems, Durham, NC USA) was utilized to screen the effects of sucralose-6-acetate and sucralose on human transverse colon. The assay was performed by Altis Biosystems (2020, 2021). The RepliGut® system is comprised of polarized intestinal cells grown on transwells that mimic normal intestinal barrier function (Allbritton et al. 2021). The effect of sucralose-6-acetate and sucralose on TEER and permeability was assessed in two separate trials. TEER is a measure of monolayer resistance which is an indirect measure of barrier function and integrity of tight junctions (Elbrecht, Long, and Hickman 2016; Srinivasan et al. 2015). In Trial 1, TEER was determined after exposure of the RepliGut® system to a single effective concentration of test article (10 mM sucralose-6-acetate or 10 mM sucralose). In Trial 2, TEER as well as permeability were assessed at a range of effective concentrations of sucralose-6-acetate (0.3125 mM to 10 mM) and sucralose (5 mM to 160 mM). In both trials there was an additional no-treatment (control) condition.

Transverse colon cells were plated directly onto RepliGut® transwell plates coated with a thin hydrogel. Cultures were monitored for cell confluence by eye using a bright field microscope. Epithelial cells became confluent after 4 days. Once confluent, media was changed to a proprietary Altis Differentiation Media (ADM). Cells were then grown for 2 days in the ADM. To monitor cell confluence while cells were grown in ADM, TEER was measured using an EVOM2 Epithelial Volt/

Table 1. Positive controls for each bacterial strain with and without metabolic activation.

Strain	Activation	Positive Control
TA98	None	2-nitrofluorene 1 µg per plate
TA98	S9	2-aminoanthracene 2 µg per plate
TA100	None	Sodium azide 1 µg per plate
TA100	S9	2-aminoanthracene 2 µg per plate
TA1535	None	Sodium azide 1 µg per plate
TA1535	S9	2-aminoanthracene 2 µg per plate
TA1537	None	9-aminoacridine 75 µg per plate
TA1537	S9	2-aminoanthracene 2 µg per plate
WP2 <i>uvrA</i>	None	Methyl methanesulfonate 1000 µg per plate
WP2 <i>uvrA</i>	S9	2-aminoanthracene 15 µg per plate

Ohm Meter with an STX2 Electrode (World Precision Instruments).

After 48 hr in ADM, TEER was measured on each transwell. Subsequently, the test compound was added to both apical and basal sides of each transwell. Transwells were incubated with the test compound for 24 hr, and TEER was measured in all transwells in Trials 1 and 2. In Trial 2, a permeability assay using 40 kDa fluorescein isothiocyanate labeled dextran (FITC) was also performed on each transwell to measure flux of the dextran over 4 hr. While TEER is an indicator of ionic conductance, 40 kDa FITC is an indicator of paracellular permeability (Utami et al. 2018).

In Trial 1, apical and basal supernatants were collected from all transwells prior to cell collection and transferred to tubes and stored at -80°C . The apical and basal supernatants were submitted for chromatographic analysis to determine if there was any conversion to sucralose-6-acetate in sucralose-exposed transverse colon cells or deacetylation to sucralose in cells exposed to sucralose-6-acetate (OpAns, Durham, NC USA). After cell collection, 500 μl RNA Lysis Buffer was added to the apical side of each transwell. Lysates were pipetted up and down 10 times and complete lysis was confirmed by using a bright field microscope. Lysates were transferred to individual tubes and stored at -80°C for RNA extraction and RNA-seq analysis.

Experiment 6: RNA-seq and gene expression in transverse colon

RNA was isolated, quantified, and quality checked by Altis Biosystems using the transverse colon cells exposed to sucralose-6-acetate and sucralose (along with the no-treatment control) in the RepliGut[®] system in Trial 1 of Experiment 5. There were 12 samples, 4 each from sucralose, sucralose-6-acetate, and untreated controls. RNA was isolated using a RNAqueous-Micro Total RNA Isolation Kit (Invitrogen Cat#AM1931) and stored at -80°C . RNA concentration was determined using a Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat#Q32852) and a Qubit 3.0 Fluorometer. RNA Integrity (RIN) values were determined utilizing an RNA 6000 Pico Kit (Agilent, Cat#5067-1513) on a Bioanalyzer 2100 machine. Subsequently, RNA concentrations and RIN values were determined.

RNA-seq (RNA-sequencing) was employed to analyze the transcriptome and determine changes in gene expression due to exposure to sucralose-6-acetate and sucralose, each relative to control (no treatment). RNAseq was conducted at the North Carolina State University (NCSSU) Genomic Sciences Laboratory, and samples were run as 150bp paired-end reads on the NovaSeq 6000 (Illumina, Inc., San Diego, CA, USA). Raw reads were trimmed for adapter and quality using Trim Galore version 0.6.1 (Babraham Bioinformatics 2019) with the two-color flag set. Trim Galore calls cutadapt (v2.1) (Martin 2011) for adapter trimming. Quality cutoff was set to 20 and minimum sequence length for both reads was set to 20bp. All reads passed the initial quality and length filters. Bases kept post-trimming ranged from 95.7 to 98% of total bases analyzed. A second round of trimming to account for poly-G runs that may have been introduced during sequencing was performed with fastp (version 0.19.10) (Chen et al. 2018) with adapter trimming disabled and polyG tail trimming enforced with 98.77 to 99.36% reads retained post-trim.

Trimmed reads were aligned to the human genome as represented in the GRCh38 (Genome Reference Consortium Human Build 38, National Center for Biotechnology Information, USA) no alt analysis set using HISAT2 (v2.1.0) with the `-dtacufflinks` flag set for downstream processing (Kim et al. 2019; Kim, Langmead, and Salzberg 2015; Pertea et al. 2016). Alignment rates ranged from 95.92 to 97.62%. The resulting SAM (Sequence Alignment Map) files were sorted and converted to BAM (Binary Alignment Map) binary files using samtools (Li et al. 2009). Stringtie2 (Pertea et al. 2015) was deployed for the individual BAM files for each replicate to assemble the alignments into potential transcripts and resulting replicate gene transfer format (GTF) files were merged into a single expressed transcriptome. Coverage tables were produced with the Stringtie `-eB` command to create a Ballgown object directory.

The Ballgown R package (Frazee et al. 2015) was deployed to access count data. Hierarchical clustering using Euclidean distances in gene expression between different replicates indicated that one of the 4 controls (the Control-2 replicate) was an outlier. It was therefore removed from further analysis. All remaining

replicates passed quality control based upon PCA/MDS and hierarchical clustering analysis. Gene expression values were passed through a low-abundance filter such that any gene with a variance of less than 1 in expression across all samples was removed from further analysis. Four different comparisons were then made for the data: Sucralose-6-Acetate vs Control, Sucralose vs Control, Sucralose vs Sucralose-6-Acetate, and a three-way comparison for all samples. Significance was determined by a false-discovery-rate q -value of 0.05. Over-representation testing of significant genes was accomplished using gProfiler's Gost service using default parameters, version: e101_eg48_p14_baf17f0 (Raudvere et al. 2019).

Experiment 7: Liver microsome stability assays for half-life ($T_{1/2}$) determination

A microsomal stability assay was performed by BioDuro-Sundia (Shanghai, China) using a standardized protocol to determine the *in vitro* half-life $T_{1/2}$ (min) of the two test articles, sucralose-6-acetate and sucralose, in the presence of liver microsomes that contain membrane-bound metabolizing enzymes including cytochrome P450 (CYP450). The goal of this assay was directed at determining Phase I metabolism in 5 different species (human, monkey, dog, rat, and mouse) using dihydronicotinamide-adenine dinucleotide phosphate/NADPH (ACROS Cat#328742500) as an enzyme co-factor. Microsome stability was assessed using a singlet incubation of 100 μM of each test article at 5 time points (0, 5, 15, 30, and 60 min). Samples were assessed using liquid chromatography/tandem mass spectrometry (LC/MS/MS) at peak area ratios to determine $T_{1/2}$ along with an estimation of intrinsic clearance (Cl_{int}). The fraction of each test article removed by the liver (extraction ratio) was calculated for each of the 5 different species based upon physiological variables reported by Houston (1994) and Davies and Morris (1993). Subsequent liver stability assays were also conducted

by Cyprotex (Watertown, MA USA) in human liver microsomes both with and without NADPH using standardized protocols (Cyprotex 2022).

Experiment 8: Inhibition of cytochrome P450 (CYP450) xenobiotic detoxification enzymes in human liver microsomes

A cytochrome P450 (CYP450) inhibition assay was conducted by BioDuro-Sundia (Shanghai, China) to determine if sucralose-6-acetate or sucralose are inhibitors of CYP enzymes that metabolize exogenous as well as endogenous compounds. CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 were selected for assessment as recommended by the United States Food and Drug Administration (2020). The probe substrates for each CYP isoform along with the inhibitors used as positive controls are provided in Table 2. The CYP isoform-specific substrates were incubated with human liver microsomes along with sucralose-6-acetate or sucralose according to standardized protocols of BioDuro-Sundia. In Trial 1, sucralose-6-acetate was incubated in duplicate at 0, 0.137, 0.412, 1.23, 3.7, 11.1, 33.3 and 100 μM , and sucralose was incubated in duplicate at 0, 4.12, 12.3, 37, 111, 333, and 1000 μM (BioDuro-Sundia 2021a). In Trial 2, sucralose-6-acetate was reassessed to compare the results with Trial 1 and incubated in duplicate at 0, 0.098, 0.39, 1.56, 6.3, 25, and 100 μM (BioDuro-Sundia 2022). At the end of each incubation, the amount of probe substrate remaining was monitored by LC/MS/MS, and IC_{50} (half-maximal inhibitory concentration) values for sucralose and sucralose-6-acetate were computed.

Results

Experiment 1: *In vitro* MultiFlow® DNA damage assay in TK6 cells

The results of the MultiFlow® assay in TK6 cells for sucralose-6-acetate indicated that sucralose-6-

Table 2. CYP450 inhibition study.

CYP Isoform	Probe Substrates	Positive Control Inhibitors
1A2	Phenacetin	β -Naphthoflavone
2C9	Diclofenac	Sulfaphenazole
2C19	Mephenytoin	Tranylcypromine
2D6	Dextromethorphan	Quinidine
3A4/5	Midazolam	Ketoconazole

Positive controls and substrate controls used by BioDuro-Sundia (2021a, 2022).

acetate exhibited a prototypical clastogenic signature for both +S9 and -S9 conditions. The fold-increases in γ H2A \times and nuclear p53 relative to control for the +S9 and -S9 treatments are shown in Tables 3 and 4 respectively. A clastogenic call was made for the +S9 treatment in Table 3 because fold-elevation in 3 consecutive concentrations of sucralose-6-acetate met or exceeded the GEF cutoffs for the 24-hr γ H2A \times (1.31) and the 24-hr nuclear p53 (1.12) biomarkers. The lowest observed concentration of genotoxicity with S9 for sucralose-6-acetate was 353 μ g/ml (803 μ M). A clastogenic call was detected for 24-hr treatment without S9 in Table 4 as fold-increases in 2 consecutive concentrations exceeded the cutoffs for fold 4-hr nuclear p53 (1.40) and 24-hr nuclear p53 (1.45), and one concentration exceeded the cutoff for 24-hr γ H2A \times (2.11). The lowest observed concentration for genotoxicity of sucralose-6-acetate without S9 was 707 μ g/ml (1607 μ M or 1.607 mM). Thus, clastogenicity call for sucralose-6-acetate occurred at a lower concentration with S9 metabolic activation than it did without S9 activation. Sucralose-6-acetate did not display an aneugenic signature.

The MultiFlow[®] assay for sucralose with concentrations up to 3980 μ g/ml yielded no marked predictions of genotoxicity, with or without S9, in TK6 cells. However, there were 4 successive increasing concentrations of sucralose beginning at 994 μ g/ml (2.5 mM) in the non-activated treatment that resulted in a greater than 2-fold rise in γ H2A \times , a marker for DNA breaks.

Experiment 2: In vitro mammalian cell MN test in TK6 cells

The results of the *in vitro* mammalian cell micronucleus test in TK6 cells shown in Tables 5 and 6 indicated that sucralose-6-acetate was positive in the 27-hr treatment without S9 but not with S9. That is, sucralose-6-acetate (-S9) elevated the MN frequency which is a biomarker of cytogenetic/chromosomal damage. The occurrence of binucleated cells was also enhanced at higher concentrations. For this reason, the MN frequency was determined in combined mono-, bi-, and multinucleated cells. When all populations of cells were combined and assessed (mono-, bi-, and multi-

nucleated), there was a significant rise at 1000 μ g/ml in the 27 hr -S9 treatment. A Cochran Armitage Trend Test of the top 3 concentrations of 500, 750 and 1000 μ g/ml (1137, 1705, and 2274 μ M) showed a significant concentration-dependent elevation within that concentration range, and the MN frequency was outside of the historical vehicle control limit. Data indicate the sucralose-6-acetate is genotoxic which is consistent with the MultiFlow[®] assay.

A MN test for sucralose itself was not performed in the current study as a previous MN test submitted during regulatory assessment was inconclusive (United States Food and Drug Administration US FDA 1998) and the MultiFlow[®] assay was not positive.

Experiment 3: In silico assessment of mutagenic potential by Leadscope[®]

The consensus call by the Leadscope[®] quantitative structure activity tool predicted that sucralose-6-acetate may be mutagenic and induce permanent transmissible genetic variations. Leadscope[®] also provided a bacterial mutation alert for the secondary alkyl halide as the active/primary portion or molecular fragment of concern for sucralose-6-acetate (Figure 2). The program recommended a follow up with a bacterial reverse mutation test using standard strains of *Salmonella typhimurium* and/or *E. coli*. These findings for sucralose were ambiguous.

Experiment 4: Bacterial reverse mutation test (Ames Test)

Data from the bacterial reverse mutation tests in Tables 7 and 8 indicated that sucralose-6-acetate and sucralose were both negative (non-mutagenic) under the conditions, and according to the criteria, of the study protocol. Sucralose-6-acetate and sucralose, and/or their metabolites, did not induce reverse mutations in 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) or in the WP2 *uvrA* strain of *Escherichia coli* in the presence and absence of an exogenous metabolic activation system (S9). These results do not confirm the *in silico* prediction by Leadscope[®] and suggest that sucralose-6-acetate and sucralose are

Table 3. MultiFlow® assay for the sucralose-6-acetate (+S9) condition.

Concentration of Sucralose-6-Acetate		Clastogen Responsive Biomarkers and GEF Cutoffs			Pan-Genotoxic Responsive Biomarker and Cutoff		Clastogenic Signature
µM	µg/ml	YH2AX (4 Hr) ≥ 1.44	YH2AX (24 Hr) ≥ 1.31	p53 (4 Hr) ≥ 1.23	p53 (24 Hr) ≥ 1.12		
1607	707	0.74	1.83*	1.08	1.28*		Yes
1136	500	0.82	1.48*	1.04	1.19*		Yes
803	353	0.87	1.35*	1.03	1.12*		Yes
568	250	0.97	1.12	1.05	1.10		No
402	177	1.01	1.00	1.05	1.02		No
284	125	1.03	1.02	1.03	1.03		No
201	88	0.93	1.03	0.98	1.02		No
142	62	0.88	0.95	0.94	1.07		No
100	44	0.95	1.00	0.97	1.02		No
71	31	1.08	1.02	1.02	1.00		No
50	22	1.13	1.10	1.05	0.96		No
35	16	1.07	1.11	1.00	0.97		No
25	11	1.06	1.13	1.01	0.96		No
18	8	1.18	1.06	1.06	1.02		No
13	6	1.06	1.12	1.02	0.96		No
9	4	1.11	1.04	1.03	0.98		No
6	3	1.02	1.06	0.98	0.94		No

* Met or exceeded the cutoffs. Fold changes in biomarkers along with GEF cutoffs and predictions of genotoxicity for each concentration of sucralose-6-acetate.

Table 4. MultiFlow® assay for the sucralose-6-acetate (–S9) condition.

Concentration of Sucralose-6-Acetate		Clastogen Responsive Biomarkers and GEF Cutoffs		Pan-Genotoxic Responsive Biomarker and Cutoff		Clastogenic Signature
µM	µg/ml	YH2AX (4 Hr) ≥ 1.51	YH2AX (24 Hr) ≥ 2.11	p53 (4 Hr) ≥ 1.40	p53 (24 Hr) ≥ 1.45	
2274	1000	0.57	3.59*	1.68*	1.73*	Yes
1607	707	0.59	1.08	1.47*	1.46*	Yes
1136	500	0.62	0.97	1.30	1.21	No
803	353	0.66	1.18	1.17	1.21	No
568	250	0.77	2.14*	1.07	1.22	No
402	177	0.89	2.79*	0.97	1.27	No
284	125	0.90	2.47*	1.00	1.19	No
201	88	0.92	2.15*	0.96	1.18	No
142	62	0.89	1.54	0.99	1.14	No
100	44	0.87	1.25	0.96	1.10	No
71	31	1.00	1.09	1.01	1.08	No
50	22	1.06	1.07	1.00	1.05	No
35	16	1.00	1.13	1.01	1.06	No
25	11	1.09	1.06	1.00	1.06	No
18	8	1.01	1.08	1.01	1.06	No
13	6	1.01	1.12	1.00	1.05	No
9	4	1.05	1.11	1.01	1.08	No
6	3	1.00	1.09	1.01	1.08	No

*Exceeded cutoff.
Fold changes in biomarkers along with GEF cutoffs and predictions of genotoxicity for each concentration of sucralose-6-acetate.

Table 5. Micronucleus test: 27-hr treatment without S9.

Condition	S6A ^a (μ M)	S6A (μ g/ml)	Total Micronucleated Cells	Total Cells Counted	Micronucleus Frequency (%)
Vehicle control (DMSO)	0	0	12	2261	0.53
Test article	227	100	18	2272	0.79
Test article	569	250	21	2235	0.94
Test article	1137	500	13	2242	0.58
Test article	1705	750	18	2270	0.79
Test article	2274	1000	24	2278	1.05*
Positive control (VB ^b , 12 ng/mL)			114	2400	4.75*

^aS6A = Sucralose-6-acetate; ^bVB= Vinblastine.

Results of the micronucleus frequency in combined mono-, bi-, and multi-nucleated cells. * $p \leq .05$; Fisher's Exact Test, relative to the solvent control.

Table 6. Micronucleus test: 4-hr treatment with S9.

Condition	S6A ^a (μ M)	S6A (μ g/mL)	Total Micronucleated Cells	Total Cells Counted	Micronucleus Frequency (%)
Vehicle control (DMSO)	0	0	22	2254	0.98
Test article	682	300	16	2214	0.72
Test article	1137	500	25	2269	1.10
Test article	1592	700	20	2244	0.89
Test article	2274	1000	18	2258	0.80
Positive Control (CP ^b , 4 μ g/ml)			59	2248	2.62*

^aS6A = Sucralose-6-acetate; ^bCP = Cyclophosphamide.

Results of the micronucleus frequency in combined mono-, bi-, and multi-nucleated cells. * $p \leq .05$; Fisher's Exact Test, relative to the solvent control.

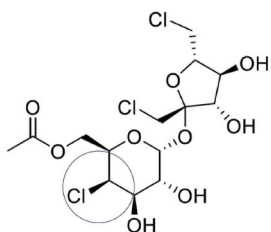


Figure 2. The secondary alkyl halide (encircled) of sucralose-6-acetate that generated a bacterial mutation alert by Leadscope[®].

not mutagenic. These observations indicate that although sucralose-6-acetate was genotoxic in both the MultiFlow[®] and MN test, DNA damage initiated by sucralose-6-acetate may not lead to permanent alterations in further generations of cells because it is not mutagenic.

Experiment 5: Assessment of transepithelial electrical resistance (TEER) and permeability in human transverse colon epithelium

Both sucralose-6-acetate and sucralose altered TEER and permeability in human colonic epithelial monolayers at mM concentrations in the absence of bacteria. The results in Figures 3a,b below illustrate that a reduction in TEER from a single

treatment for 24 hr with sucralose-6-acetate begins at 5 mM with a complete collapse at 10 mM. In Figure 3c, the relative permeability to 40 kDa FTIC-dextran significantly increased after exposure to 10 mM sucralose-6-acetate.

Figures 4a,b show that a reduction in TEER from a single treatment for 24 hr with sucralose begins at 40 mM with a complete collapse at 80 mM. Figure 4c illustrates that the relative permeability to 40 kDa FTIC-dextran was significantly elevated after incubation with 80 mM and 160 mM sucralose.

Experiment 6: RNA-seq and gene expression in transverse colon

Differential gene expression analysis was used to determine changes in gene expression in human transverse colon induced by sucralose-6-acetate and sucralose, each relative to control (no treatment). A total of 12,553 genes were analyzed after low-abundance filtering. Four comparisons using the R package Ballgown's "stattest" function were: Sucralose-6-Acetate vs Control, Sucralose vs Control, Sucralose vs Sucralose-6-Acetate, and a three-way comparison for all samples (Sucralose vs Sucralose-6-Acetate vs Control).

Table 7. Bacterial reverse mutation test for sucralose-6-acetate.

Tester Strains	Without metabolic activation (μg per plate)		Without metabolic activation (μg per plate)	
	Precipitate	Toxicity*	Precipitate	Toxicity*
TA98	None	None	None	None
TA100	None	None	None	None
TA1535	None	None	None	None
TA1537	None	None	None	5000
WP2 <i>uvrA</i>	None	None	None	None

Precipitate and toxicity results.

*Toxicity is defined as a > 50% reduction in revertants compared to the negative controls.

Table 8. Bacterial reverse mutation test for sucralose.

Tester Strains	Without metabolic activation (μg per plate)		Without metabolic activation (μg per plate)	
	Precipitate	Toxicity*	Precipitate	Toxicity*
TA98	None	None	None	None
TA100	None	None	None	None
TA1535	None	None	None	None
TA1537	None	None	None	5000
WP2 <i>uvrA</i>	None	None	None	None

Precipitate and toxicity results.

*Toxicity is defined as a > 50% reduction in revertants compared to the negative controls.

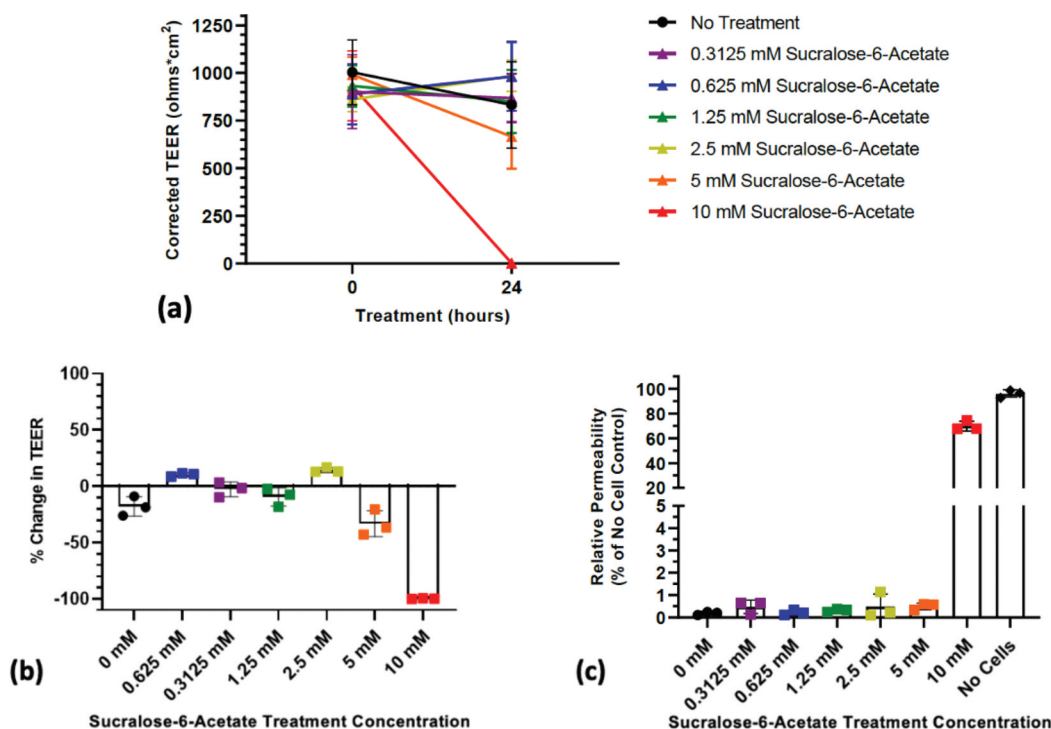


Figure 3. Response of human transverse colon epithelium to sucralose-6-acetate: (a) Corrected TEER of transverse colon monolayers showing that a reduction began to occur at 5 mM with a total loss at 10 mM; (b) Percent change in TEER of transverse colon computed from corrected TEER; (c) Change in permeability to 40 kDa FTIC-dextran transitions at 10 mM.

Sucralose-6-acetate vs control

Thirty-four (34) genes were differentially expressed between sucralose-6-acetate and control samples, and 23 of these were identified. The expression of 16 of the

identified genes significantly increased in the sucralose-6-acetate samples as compared to controls (Table 9), and the expression of 7 of the identified genes significantly decreased in the sucralose-6-acetate

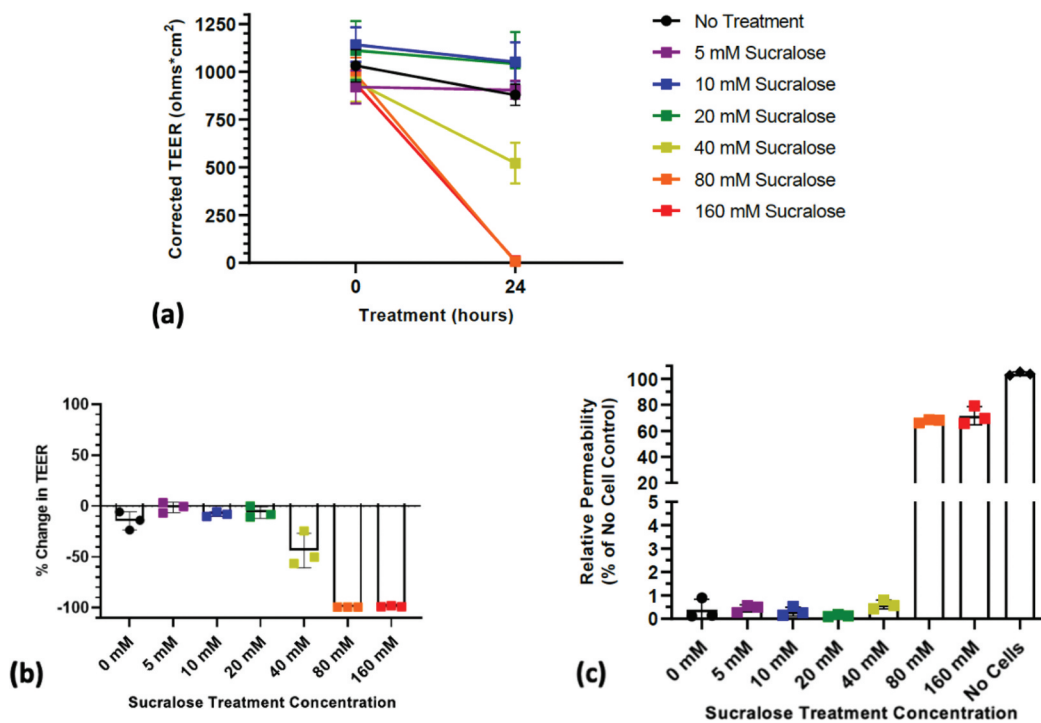


Figure 4. Response of human transverse colon epithelium to sucralose: (a) Corrected TEER of transverse colon monolayers (note: results for 80 mM and 160 mM overlap); (b) Percent change in TEER of transverse colon; (c) Change in permeability to 40 kDa FITC-dextran transitions at 80 mM.

samples compared to controls (Table 10). In Table 9, three additional named but uncharacterized genes including LOC399900, LOC105371483, and LOC107986058 also exhibited significantly increased expression in sucralose-6-acetate than control. Twenty (20) of the 23 identified genes encode proteins while 2 of these are non-coding RNAs and 1 is a pseudogene. A brief description of each gene is presented in Tables 9 and 10 along with fold change and significance values. The fold changes for sucralose-6-acetate relative to control for three genes, metallothionein 1 G (MT1G), serine hydroxymethyltransferase (SHMT2), and activating transcription factor 3 (ATF3) were exceptionally large at 253.82, 81.23, and 54.49 respectively.

Sucralose vs control

Only two protein coding genes were differentially expressed between sucralose vs control samples. A brief description of these two genes is presented in Table 11 along with fold change and significance values. For COX10, gene expression was higher in

sucralose than control. For FAM166A, gene expression was lower in sucralose than control.

Sucralose vs sucralose-6-acetate

There were 186 genes that were differentially expressed in the sucralose vs sucralose-6-acetate comparison. For 62 genes, expression in sucralose samples was higher than in sucralose-6-acetate samples with an average fold elevation of 4.63 ± 4.53 . For 126 genes, expression was higher in sucralose-6-acetate than in sucralose but with a lower average fold change of only 0.28 ± 0.2 . The Farnesyl-Diphosphate Farnesyltransferase gene (FDFT1) that encodes the first specific enzyme in cholesterol biosynthesis (Genecards 2023; The Human Protein Atlas 2023) displayed the largest change in expression in sucralose with a 30.93-fold rise relative to sucralose-6-acetate (P (q) value $3.6E-4$ (0.02)). Other genes for which the expression was 3-fold higher or more in sucralose than in sucralose-6-acetate include: TFRC (cellular iron uptake), PFKP (glycolysis regulation), RHOT1 (mitochondrial trafficking), MRPL16 (protein synthesis within the mitochondrion), PRDX4 (protection

Table 9. Differential gene expression for sucralose-6-acetate with fold change >1.

Gene Symbol and name	Brief description (GeneCards 2023; The Human Protein Atlas 2023)	Fold Change	P(q) value
MT1G (Metallothionein 1 G)*	Binds heavy metals; regulated by heavy metals and glucocorticoids; cancer-related gene	253.82	2.07E-05 (0.02)
SHMT2 (Serine hydroxymethyltransferase 2)*	Encodes mitochondrial form of a pyridoxal phosphate-dependent enzyme; involved in glycine synthesis	81.23	8.02E-05 (0.04)
ATF3 (Activating Transcription Factor 3)*	Encodes member of activation transcription factor/cAMP responsive element-binding (CREB) protein family; involved in cellular stress response	54.49	1.22E-04 (0.04)
CHST3 (Carbohydrate Sulfotransferase 3)*	Encodes an enzyme that catalyzes sulfation of chondroitin; involved in cell migration and differentiation	9.26	1.08 E-04 (0.04)
LOC399900	Uncharacterized	9.02	2.22E-05 (0.02)
ATE1-AS1 (ATE1 Antisense RNA 1)**	Pseudogene	5.89	1.61E-04 (0.04)
MCM2 (Minichromosome Maintenance Complex Component 2)*	Involved in initiation of eukaryotic genome replication; cancer related gene	5.14	1.54E-04 (0.04)
KCNQ1DN (KCNQ1 Downstream Neighbor)***	Affiliated with the lncRNA class	3.57	2.01E-05 (0.02)
ZSCAN10 (Zinc Finger and SCAN Domain Containing 10)*	Embryonic stem (ES) cell-specific transcription factor; metal-binding	3.07	1.21 E-04 (0.04)
ARMC10 (Armadillo Repeat Containing 10)*	Decreases the transcriptional activity of the tumor suppressor protein p53	2.78	1.98E-04 (0.04)
ELP5 (Elongator Acetyltransferase Complex Subunit 5)*	Component of the elongator complex that is required for multiple tRNA modifications	2.78	9.94E-06 (0.02)
LOC105371483	Uncharacterized	2.49	1.28E-04 (0.04)
UVRAG-DT (UVRAG Divergent Transcript)***	Affiliated with the lncRNA class	2.38	1.85E-04 (0.04)
EWSR1 (EWS RNA Binding Protein 1)*	Involved in various cellular processes, including gene expression, cell signaling, and RNA processing and transport; cancer related gene	2.28	3.44E-05 (0.02)
MTMR9 (Myotubularin Related Protein 9)*	Adapter for myotubularin-related phosphatases	2.04	7.03E-05 (0.04)
LOC107986058	Uncharacterized	1.77	1.37E-04 (0.04)
TNFSF14 (TNF Superfamily Member 14)*	Encodes member of the tumor necrosis factor (TNF) ligand family that stimulates proliferation of T cells and mediates apoptosis.	1.78	1.97E-04 (0.04)
SET (SET Nuclear Proto-oncogene)*	Multitasking protein; inhibits acetylation of nucleosomes; cancer-related gene	1.70	1.73E-04 (0.04)
CASKIN1 (CASK Interacting Protein 1)*	Predicted to be involved in signal transduction; may link scaffolding protein CASK to intracellular effectors	1.25	1.07E-04 (0.04)

Ranked by fold change including significance and brief description from gene and protein databases.

*Protein coding gene, **Pseudogene, ***RNA coding gene.

Table 10. Differential gene expression for sucralose-6-acetate with fold change <1.

Gene Symbol and name	Brief description (Genecards 2023; The Human Protein Atlas 2023)	Fold Change	P(q) value
DHX35 (DEAH-Box Helicase 35)*	May be involved in pre-mRNA splicing	0.54	1.10E-04 (0.04)
RABL2B (RAB, Member Of RAS Oncogene Family Like 2B)*	Small GTPase required for ciliation	0.54	2.16E-06 (0.01)
ABO (Alpha 1-3-N-Acetylgalactosaminyltransferase and Alpha 1-3-Galactosyltransferase)*	Encodes proteins that are the basis of the ABO blood group system	0.26	2.11E-04 (0.04)
PIGN (Phosphatidylinositol Glycan Anchor Biosynthesis Class N)*	Involved in glycosylphosphatidylinositol-anchor biosynthesis	0.26	1.14E-05 (0.04)
FRMD8 (FERM Domain Containing 8)*	Involved in positive regulation of tumor necrosis factor production	0.15	1.15E-04 (0.04)
ADCY6 (Adenylate Cyclase 6)*	Encodes a member of the adenyl cyclase family of proteins, which are required for the synthesis of cyclic AMP	0.06	1.03E-04 (0.04)
SMARCC2 (SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin Subfamily C Member 2)*	Involved in transcriptional activation and repression of select genes by chromatin remodeling	0.01	1.51E-04 (0.04)

Ranked by fold change including significance and brief description from gene and protein databases.

*Protein coding gene.

Table 11. Differential gene expression for sucralose.

Gene Symbol and name	Brief Description (Genecards 2023; The Human Protein Atlas 2023)	Fold Change	P (q) value
COX10 (cytochrome c oxidase assembly factor heme A: farnesyltransferase COX10)*	Encodes heme A:farnesyltransferase which is required for the expression of functional cytochrome c oxidase	1.26	2.12E-05 (0.04)
FAM166A (family with sequence similarity 166 member A)*	Protein located in ciliary basal body; broad expression in testis	0.61	9.16E-06 (0.04)

Fold change, significance, and brief description from gene and protein databases.

*Protein coding gene.

against oxidative stress), PLCB3 (production of the secondary messengers diacylglycerol and inositol 1,4,5-triphosphate), ABO (production of ABO blood group proteins), FAM3D (insulin regulation), ACADVL (energy from fats), OGDH (biochemical conversions during the Krebs cycle), ACTR1A (microtubule-based vesicle motility), VPS13A (lipids transfer between membranes), and PTPRA (cell adhesion and proliferation)

Three-way comparison: Sucralose vs Sucralose-6-Acetate vs Control

There were 464 genes identified as differentially expressed. There was an over-representation in 7 Gene Ontology (Ashburner et al. 2000; The Gene Ontology Consortium 2019) categories listed under “Cellular Component.” In addition, 43 total regulatory motifs from TRANSFAC (Wingender 2008) were significantly over-represented as well as 33 terms from the Human Protein Atlas (The Human Protein Atlas 2023; Uhlén et al. 2015). The Cellular components were cytoplasm, cytosol, integral component of Golgi membrane, intracellular, intracellular membrane-bound organelle, intrinsic

component of Golgi membrane, and membrane-bound organelle. The Human Protein Atlas indicated expression in 33 different tissue types originating in the small intestine, bronchus, colon, appendix, duodenum, salivary gland, pancreas, rectum, urinary bladder, stomach, lung, prostate, endometrium and kidneys. Transcription factor binding sites were associated with 266 of the genes. The 23 transcription factors identified were as follows: AP-2gamma: Elk-1, AP-2gamma, BEN, Churchill, E2F-1, E2F-2, E2F-3:HES-7, E2F-3, E2F-4, E2F-7, E2F, ETF, IRX-1, MAZ, MOVO-B, Sp1, TCF-1, TR4, WT1, ZF5, ZIC4, p300, pax-6.

Genes in common for the four comparisons

The Venn diagram in Figure 5 illustrates the overlap of the loci (both named and unnamed genes) that were found in common for the 4 comparisons: Sucralose-6-Acetate vs Control, Sucralose vs Control, Sucralose vs Sucralose-6-Acetate, and the three-way comparison for all samples (Sucralose vs Sucralose-6-Acetate vs Control). The two genes in the Sucralose vs Control comparison were not found in any of

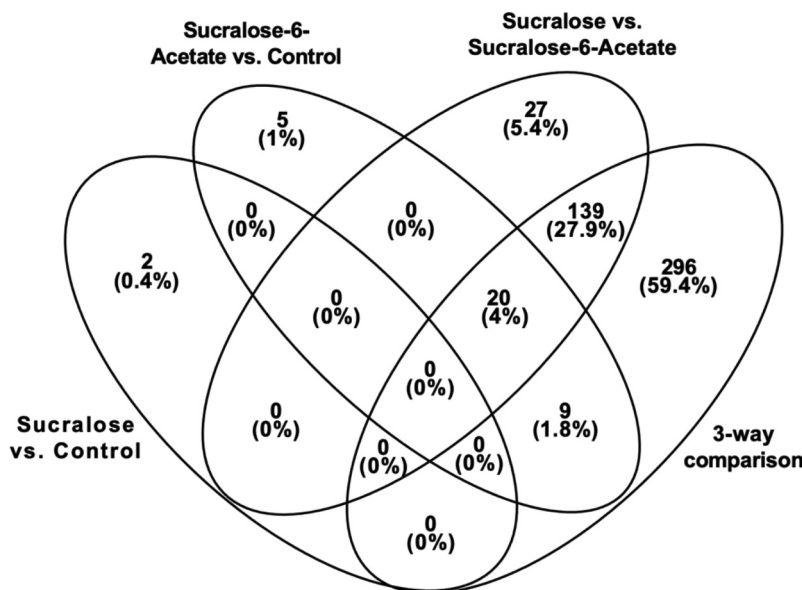


Figure 5. Venn diagram that shows the overlap of loci (including both named and unnamed genes) that were found in common for the four comparisons: Sucralose-6-Acetate vs. Control, Sucralose vs. Control, Sucralose vs. Sucralose-6-Acetate, and the three-way comparison for all samples (Sucralose vs. Sucralose-6-Acetate vs. Control).

the three other comparisons. Twenty loci were in common between comparisons that contain sucralose-6-acetate. Of those 20, the 16 named genes (ABO, ATE1-AS1, CASKIN1, CHST3, ELP5, EWSR1, KCNQ1DN, MCM2, MT1G, MTMR9, TNFSF14, UVRAG-DT, ZSCAN10) along with LOC399900, LOC105371483, and LOC107986058 are included in [Table 9](#).

Experiment 7: Liver microsome stability assays for half-life ($T_{1/2}$) determination

The results of the liver microsome stability assays in human, monkey, dog, rat, and mouse microsomes with NADPH are shown in [Table 12](#) for both sucralose-6-acetate and sucralose. The fraction of sucralose-6-acetate removed by the liver (extraction ratio) was calculated for sucralose-6-acetate using data from [Houston \(1994\)](#) and [Davies and Morris \(1993\)](#). A subsequent study in human liver microsomes obtained a value of $T_{1/2}$ of 37.6 min (with and without NADPH) for sucralose-6-acetate and >180 for sucralose (with and without NADPH) ([Cyprotex 2022](#)). Data indicate that NADPH is not necessary for extraction of sucralose-6-acetate.

Experiment 8: Inhibition of cytochrome P450 (CYP450) xenobiotic detoxification enzymes in human liver microsomes

Sucralose-6-acetate was found to be an inhibitor of CYP1A2 and CYP2C19 in human liver microsomes while no significant inhibition was detected for CYP2C9, CYP2D6, or CYP3A4/5. No marked inhibitory effect on any CYP450 enzymes was noted for sucralose. The inhibition study was initially performed in duplicate for both sucralose-6-acetate and sucralose and repeated in duplicate 8 months later for sucralose-6-acetate to confirm the results. The mean IC_{50} (μ M) values for the initial and replication investigations for sucralose-6-acetate are given in [Tables 13 and 14](#). In the initial study, the mean IC_{50} values for CYP1A2 and CYP2C19 were 42.9 μ M and 89.3 μ M. In the repetition study, the mean IC_{50} values for CYP1A2 and CYP2C19 were 65.1 μ M and 46.3 μ M. [Figures 6a and 6b](#) report the patterns of response of CYP1A2 and CYP2C19 from sucralose-6-acetate that were used to determine IC_{50} (μ M). [Table 15](#) indicates that no marked CYP inhibition occurred for sucralose.

Table 12. $T_{1/2}$ (min), intrinsic clearance (CL_{int}), hepatic clearance (CL_{hep}), and extraction ratios determined in microsome stability assays with NADPH (BioDuro-Sundia 2021b).

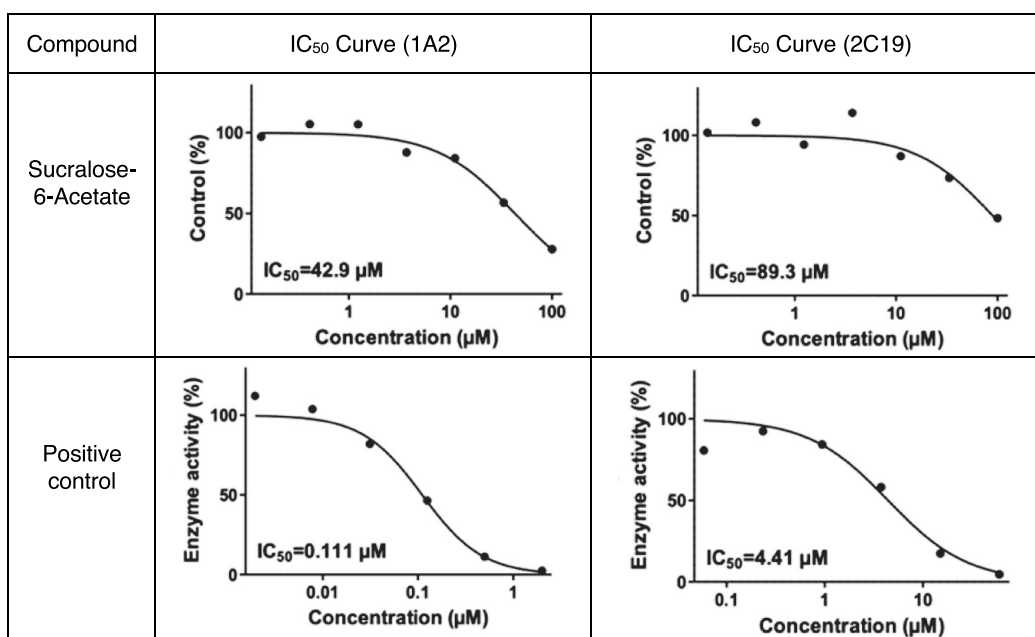
Species	Compound	$T_{1/2}$ (min)	<i>In vitro</i> CL_{int} ($\mu\text{l}/\text{min}/\text{mg}$ protein)	CL_{hep} (ml/min/kg)	Extraction Ratio
Human	Sucralose-6-Acetate	36.6	37.9	14.1	0.68
	Sucralose	>186.4	<7.4	<6.1	
Monkey	Sucralose-6-Acetate	6.2	224.2	38.1	0.87
	Sucralose	>186.4	<7.4	<8.2	
Dog	Sucralose-6-Acetate	36.3	38.2	19.8	0.64
	Sucralose	>186.4	<7.4	<8.0	
Rat	Sucralose-6-Acetate	108.9	12.7	16.2	0.29
	Sucralose	>186.4	<7.4	<10.8	
Mouse	Sucralose-6-Acetate	32.0	43.3	58.9	0.65
	Sucralose	>186.4	<7.4	<22.1	

Table 13. CYP450 inhibition initial study: IC_{50} (μM) for sucralose-6-acetate (BioDuro-Sundia 2021a).

Compound	Mean IC_{50} (μM)				
	1A2	2C9	2C19	2D6	3A4/5
Sucralose-6-acetate	42.9	>100	89.3	>100	>100
Positive Control	0.111	0.450	4.41	0.0586	0.0235

Table 14. CYP450 inhibition replication study: IC_{50} (μM) for sucralose-6-acetate (BioDuro-Sundia 2022).

Compound	Mean IC_{50} (μM)				
	1A2	2C9	2C19	2D6	3A4/5
Sucralose-6-acetate	65.1	>100	46.3	>100	>100
Positive Control	0.130	0.380	9.27	0.0522	0.0134

**Figure 6(a).** CYP450 inhibition – initial study: IC_{50} (μM) curves for sucralose-6-acetate for CYP1A2 and CYP2C19. Results from BioDuro-Sundia (2021a).

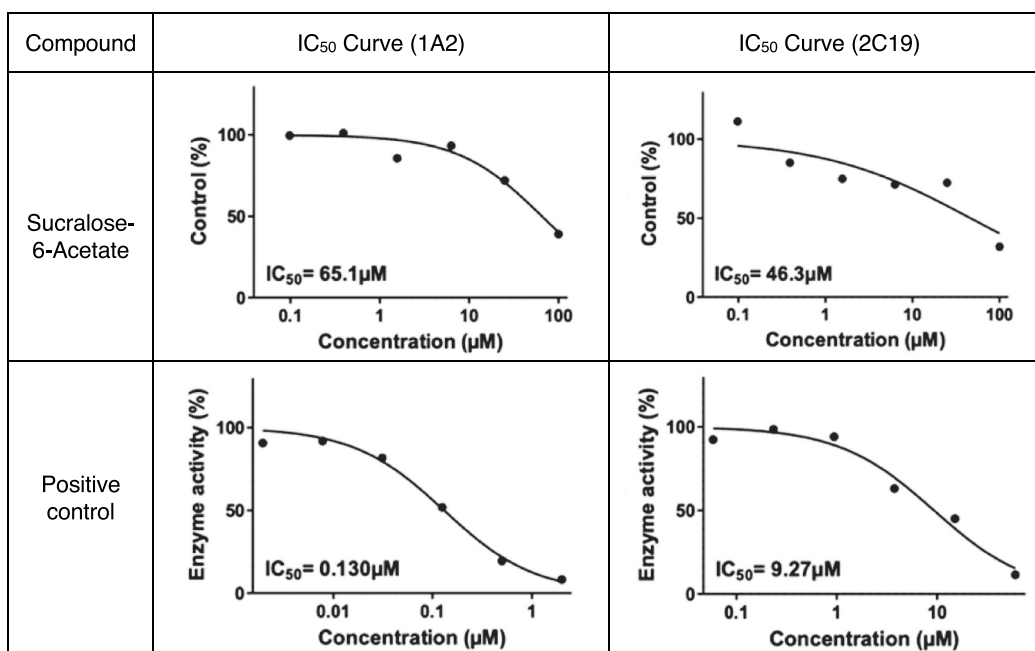


Figure 6(b). CYP450 inhibition – repeated study: IC₅₀ (μM) curves for sucralose-6-acetate for CYP1A2 and CYP2C19. Results from BioDuro-Sundia (2022).

Table 15. CYP450 inhibition study: IC₅₀ (μM) for sucralose study (BioDuro-Sundia 2021a).

Compound	Mean IC ₅₀ (μM)				
	1A2	2C9	2C19	2D6	3A4/5
Sucralose	>1000	>1000	>1000	>1000	>1000
Positive Control	0.167	0.575	7.28	0.040	0.0305

Discussion

The experiments conducted in this study found that sucralose-6-acetate was genotoxic, and the MoA was classified as clastogenic (induced DNA strand breaks). Further, exposure of human intestinal epithelium to sucralose-6-acetate as well as sucralose itself damaged tight junctions and impaired intestinal barrier function at mM concentrations. The transcriptome from human intestinal tissue determined using RNA-seq noted that sucralose-6-acetate significantly increased expression of genes associated with inflammation, oxidative stress, and cancer. Sucralose-6-acetate also inhibited two members of the CYP450 family at low μM concentrations that might potentially interfere with metabolism of endogenous and exogenous chemicals including medications.

Genotoxicity

The sucralose impurity and metabolite, sucralose-6-acetate, was found to be genotoxic in human lymphoblastoid cells in both the MultiFlow® high throughput assay as well as the standard MN test. In the MultiFlow® test, the lowest observed concentration for genotoxicity for sucralose-6-acetate without metabolic activation was 1.607 mM while the lowest observed level for genotoxicity with metabolic activation was 803 μM. The finding that metabolic activation resulted in a lower observed concentration for genotoxicity suggests that sucralose-6-acetate may be converted into additional DNA reactive metabolites. The MoA was concluded to be clastogenic (induction of DNA strand breaks) utilizing the MultiFlow® assay. These observations for sucralose-6-acetate genotoxicity by the Multiflow® assay were corroborated by cytogenetic/chromosomal damage in the Mammalian Cell Micronucleus Assay.

Table 16. Three examples of estimated sucralose-6-acetate content in common beverages.

Product	Volume	Sucralose content	Sucralose-6-acetate content (0.67%)
Flavored water, juice, soft drink*	330 ml (11.16 oz)	99 mg (99,000 µg)	663 µg
Flavored water, juice, soft drink**	330 ml (11.16 oz)	49.5 mg (49,500 µg)	332 µg
One Energy Drink***	57 ml (1.93 oz)	114 mg (114,000 µg)	763 µg

*Maximum usable dose of 300 mg/l (European Union (EU) 2004).

**One half of maximum usable dose of 300 mg/l (European Union (EU) 2004).

***Personal notes.

The potential adverse health effects attributed to genotoxicity of sucralose-6-acetate have not yet been addressed in archival scientific publications. Further, there has been no apparent systematic post-marketing surveillance of health effects from sucralose exposure by the manufacturer since this sweetener entered the food supply. Table 16 provides three examples of the level of exposure to sucralose-6-acetate that might occur following ingestion of a single beverage sweetened with sucralose. The exposure is based upon the presence of sucralose-6-acetate in current commercial sucralose samples of levels up to 0.67% (Werness 2021).

While some regulatory agencies hold that there is no acceptable level of genotoxic exposure, other institutions evaluated the genotoxic risk associated with potential exposure and MoA. A threshold of toxicological concern for genotoxicity (TTC_{genotox}) of 0.15 µg/person/day (0.0025 µg/kg bw/day for a 60 kg person) was suggested for chemicals at low levels in the diet (European Food Safety Authority EFSA 2016; Gooderham et al. 2020; Kroes et al. 2004; Serafimova, Coja, and Kass 2021). Data in Table 16 indicate that single servings of sucralose-containing drinks may contain levels of sucralose-6-acetate that exceed a TTC_{genotox} of 0.15 µg/person/day by 4 orders of magnitude or more.

The actual exposure to sucralose-6-acetate, however, likely far exceeds levels in Table 16 due to repeated dosing, biotransformation of sucralose to sucralose-6-acetate in the intestine, and potential of bioaccumulation. Repeated daily dosing enhances exposure to sucralose-6-acetate because this impurity was reported to persist in the body for at least 11 days after cessation of intake of sucralose (Bornemann et al. 2018). The systemic persistence of sucralose-6-acetate occurred even though this compound was shown in the present study to undergo partial extraction in the liver.

Biotransformation of sucralose to sucralose-6-acetate was demonstrated to occur in the intestinal tract, elevating the % beyond that in commercial sucralose samples by a factor of approximately 20 (Bornemann et al. 2018; Werness 2021). Further, sucralose-6-acetate is more lipophilic than sucralose itself which may favor bioaccumulation through enhanced intestinal absorption and transport across cellular barriers.

Previous questions regarding sucralose genotoxicity were raised but the contribution of sucralose-6-acetate to these effects was not apparently investigated. Early tests of potential sucralose genotoxicity in a mouse MN test and a chromosomal aberration test in cultured human lymphocytes were inconclusive (United States Food and Drug Administration US FDA 1998). Four different comet assays (two *in vivo* and two *in vitro*) subsequently found that sucralose-initiated DNA damage in blood cells (Pasqualli et al. 2020), colon cell lines (Van Eyk 2015), gastrointestinal organs (Sasaki et al. 2002), and brain, kidneys, and liver tissues (Raya et al. 2020). The *in vitro* comet assays employed by Pasqualli et al. (2020) and Van Eyk (2015) detected DNA damage in the µM and mM range respectively. Our current finding of the fact that sucralose-6-acetate is genotoxic may partially explain these prior observations regarding sucralose in comet tests. Dietary exposure to sucralose from the 12th day of fetal life through the lifespan was found to initiate a significant dose-related elevated incidence of malignant tumors and a significant dose-related increased incidence of hematopoietic neoplasias in male mice (Soffritti et al. 2016). The mechanism by which sucralose exposure beginning prenatally produces hematopoietic neoplasias is not known but compounds such as sucralose-6-acetate (with a molecular weight <500 Daltons and lipophilic

properties) readily diffuse across lipid membranes of the placenta (Griffiths and Campbell 2015) and mammary tissue in lactating mothers (Sylvetsky et al. 2015) and might bioaccumulate over time.

Recent epidemiological studies found that early onset of colorectal and other cancers of the digestive system are rapidly increasing in many middle- and high-income nations, and this elevated incidence of human cancer was associated with dietary choices and dysbiosis (Ugai et al. 2022). The global rise in colorectal cancers parallels the burden of inflammatory bowel disease (IBD) that is also rising globally (Alatab et al. 2020). Sucralose consumption was noted as a causative factor in IBD as well as a risk factor for colorectal cancer in animal models (Guo et al. 2021; Li et al. 2020; Rosales-Gómez et al. 2018; Wang et al. 2019) but the potential contribution of sucralose-6-acetate to this finding is not yet known. Questions have been raised in the scientific literature whether sucralose consumption may also contribute to IBD in humans (Qin 2011).

Intestinal barrier function effects

The assessment of transepithelial electrical resistance (TEER) and permeability in human transverse colon epithelium in the current *in vitro* study found that sucralose-6-acetate and sucralose both disrupt gastrointestinal epithelial tight junctions and mucosal barrier function at mM concentrations in the absence of bacteria. A significant collapse of TEER occurred after a single 24-hr exposure to 40 mM sucralose which is only 6.7-fold greater than the concentration of sucralose currently approved by the European Union (2004) for use in a single syrup-type food supplement at 2400 mg/kg (6 mM). Integrity of the intestinal epithelial barrier is dependent upon tight junctions, the specialized complexes which connect adjacent cells and provide a physical and functional barrier that limits or regulates passive diffusion of ions, solutes, macromolecules, and cells from the lumen through the paracellular space. Sucralose-6-acetate and sucralose reduced the transepithelial resistance and enabled ions and macromolecules to pass from the apical (luminal) to the basolateral side of intestinal epithelium through the paracellular pathways. Enhanced intestinal permeability

(leaky gut) that enables passage of microorganisms and metabolites into the body plays a major role in IBD (Lee 2015; Welcker et al. 2004), chronic liver disease (Mohandas and Vairappan 2017), as well as pathogenesis of colorectal cancer (Sánchez-Alcoholado et al. 2020). Further, elevated intestinal permeability in conjunction with repeated ingestion and retention of colonic contents over days may increase intraluminal concentration, absorption, and systemic exposure to sucralose and sucralose-6-acetate resulting long-term in bioaccumulation and toxicity.

Previous studies showed that factors, in addition to the direct interaction of sucralose and sucralose-6-acetate with tight junctions, also play a role in disruption of the intestinal barrier from exposure to sucralose (Schiffman and Rother 2013). These factors include dysbiosis of microbial gut flora as well as inflammation associated with oxidative stress and elevated presence of proinflammatory cytokines. Abou-Donia et al. (2008) first reported that strain-specific decrements in commensal anaerobic bacteria were associated with histopathological changes in the colon including lymphocytic infiltrates into the epithelium, epithelial scarring, atrophy/disorganization/architectural disruption, inflammation of glands, submucosal (and/or lamina propria) lymphoid aggregates, lymphoid follicles, mild depletion of goblet cells, and loss of superficial mucin. These effects occurred after 90 days of consumption of sucralose by rats at levels approved by regulatory agencies. Subsequent studies also demonstrated alterations in gut bacteria from sucralose exposure (Bian et al. 2017; Méndez-García et al. 2022; Suez et al. 2014, 2022; Zhang et al. 2022). These investigations do not support the historical claim in the Food Additive Petition for regulatory approval by the US FDA that “sucralose does not inhibit either aerobic or anaerobic microorganisms” (United States Food and Drug Administration US FDA 2021). Maternal ingestion of sucralose in mice during pregnancy also impacted their progeny’s microbiome (Dai et al. 2020, 2021; Olivier-Van Stichelen, Rother, and Hanover 2019). Gut microbes and their metabolites were shown to modulate the expression of tight junction proteins in both *in vivo* and *in vitro* models (Anderson et al. 2010; Bansal et al. 2010; Ewaschuk et al. 2008; Ukena et al. 2007).

Inflammation is also associated with dysbiosis along with disruption of gut barrier integrity (Al Bander et al. 2020), and numerous studies reported that inflammatory biomarkers are induced by sucralose ingestion (Bian et al. 2017; Farid et al. 2020; Li et al. 2020; Rosales-Gómez et al. 2018; Shil and Chichger 2021). Intestinal inflammation might also induce genotoxicity in extraintestinal tissues (Westbrook et al. 2011).

Another conclusion that may be drawn from the current study of human transverse colon in the absence of bacteria is that bioconversion of sucralose to sucralose-6-acetate observed by Bornemann et al. (2018) was mediated by bacteria and not host metabolism. Chromatographic analysis of the apical and basal supernatants illustrated no apparent conversion of sucralose to sucralose-6-acetate in the RepliGut® System (OpAns 2021). Thus, enrichment of the sucralose-6-acetate to sucralose ratio in fecal samples reported by Bornemann et al. (2018) most likely results from bacterial metabolism rather than metabolism by acetyl coenzyme A (acetyl CoA) in host intestinal epithelium. Acetylation of sucralose in the gut may serve as a detoxification mechanism for bacteria (Koppel, Rekdal, and Balskus 2018) because it facilitates excretion of sucralose from microbial cells by diminishing its polarity. Sucralose-6-acetate may also contribute to the blunted growth of anaerobic bacteria in the gut that was reported after ingestion of sucralose by Abou-Donia et al. (2008). Acetylation of pharmaceutical compounds such as 5-aminosalicylate drugs previously were found to inhibit growth of anaerobes (Delomenie et al. 2001).

Gene expression

The RNA-seq and gene expression analysis in the current study indicates that sucralose-6-acetate upregulated expression of genes in transverse colon that are associated with biological responses to harmful chemicals and conditions. The metallothionein 1 G (MT1G) displayed the largest change in expression in sucralose-6-acetate with a 253.82-fold rise relative to untreated control. Metallothionein (MT) genes encode proteins that are biomarkers of inflammation, oxidative stress, and cancer as well as cellular toxicity from insecticides, herbicides, metals, and other xenobiotic

compounds (Bauman et al. 1991; Dai et al. 2021; Migliaccio et al. 2020; Ostrakhovitch et al. 2006; Rodrigo et al. 2020; Ruttkay-Nedecky et al. 2013; Si and Lang 2018; Tong et al. 2020). MT1G upregulation accelerates the G1/S transition in the growth phase of acute promyelocytic leukemia cells (Hirako and Takahashi 2021). Enhanced expression of MTs was also reported in IBD (Brüwer et al. 2001; Dooley et al. 2004). As noted previously, hematopoietic neoplasias (Soffritti et al. 2016) and IBD (Li et al. 2016; 2020; Wang et al. 2019; Guo et al. 2021; Rodriguez-Palacios et al. 2018) occur in sucralose-fed rodent models. However, the potential contribution of sucralose-6-acetate to the genesis of hematopoietic neoplasias or IBD has not apparently been investigated.

Three additional genes, SHMT2, ATF3 and carbohydrate sulfotransferase 3 (CHST3), were also markedly expressed by sucralose-6-acetate with 81.23, 54.49, and 9.26-fold elevation relative to untreated control. SHMT2 encodes a key mitochondrial enzyme, serine hydroxymethyltransferase-2, that catalyzes the reaction of serine to glycine that is found in high concentrations in intestinal epithelial cells. SHMT2 initiates lymphoma development through epigenetic tumor suppressor silencing (Parsa et al. 2020), drives the progression of colorectal cancer (Cui et al. 2022; Liu et al. 2021), potentiates the aggressive process of oral squamous cell carcinoma (Zheng et al. 2022) and promotes tumorigenesis in rhabdomyosarcoma (Nguyen et al. 2021). ATF3 encodes a member of the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family of transcription factors. ATF3 is a marker of oxidative stress (Ketola et al. 2012) and plays a role in modulation of metabolism, immunity, and oncogenesis (Yin et al. 2008; Ku and Cheng 2020). The carbohydrate sulfotransferase 3 (CHST3) gene encodes an enzyme (chondroitin 6-O-sulfotransferase 1 or C6ST-1) that plays a role in the formation of chondroitin 6-sulfate (MedlinePlus 2023). Chondroitin 6-sulfate is involved in development and maintenance of the skeleton as well as naïve T lymphocytes (Uchimura et al. 2002). Chondroitin 6-sulfate expression is upregulated in human glioma cells, and this upregulation is correlated with glioma malignancy (Pan et al. 2020).

Other genes for which expression was greater for sucralose-6-acetate than control were also implicated in cancer in some tissues. Minichromosome maintenance complex component 2 (MCM2) is a prognostic marker of poor prognosis in squamous cell/adenosquamous carcinoma and adenocarcinoma of the gallbladder (Liu et al. 2016) and hepatocellular carcinoma (Tang et al. 2022). Elevated expression of zinc finger and SCAN domain containing 10 (ZSCAN10) in glioma tissues was associated with poor prognosis in glioma (Jiang et al., 2019). EWS RNA Binding Protein 1 (EWSR1) is a prognostic marker of unfavorable outcomes liver cancer (Jiang et al. 2021). Tumor necrosis factor superfamily member 14 (TNFSF14) is upregulated and a prognostic marker of poor outcome in renal cell cancer (Xu et al. 2020) and SET nuclear proto-oncogene is an unfavorable prognostic marker in liver cancer (Van Nguyen et al. 2021). Elongator acetyltransferase complex subunit 5 (ELP5) plays a role in tumorigenicity of melanoma cells (Close et al. 2012).

Only two genes were differentially expressed between sucralose and control samples. COX10 displayed higher numerical expression in the sucralose incubation as compared to control. The COX10 gene encodes a component of the mitochondrial electron transport chain and is required for natural killer (NK) cell expansion (Mah-Som et al. 2021). FAM166A which plays a role in spermatogenesis (The Human Protein Atlas, 2023) exhibited a higher numerical expression in control than sucralose. This finding of lower expression of FAM166A in the sucralose exposure relative to control did not occur with sucralose-6-acetate such that the reduction in expression of this spermatogenesis-associated gene appears to be initiated by sucralose itself. The effects of diminished expression of the spermatogenesis gene FAM166A by sucralose are not known. A historical 28-day gavage study on the glycolytic activity of sucralose in the rat spermatozoa showed no marked effect (Kille et al. 2000b). However, the 28-day test period may be insufficient to assess the entire duration of spermatogenesis in rats which is 52 days (Clouthier et al. 1996). A four-week feeding study found that a commercial artificial sweetener containing sucralose as well as acesulfame-K altered spermatogenesis in mice (Al-Qudsi and Al-Dossary, 2020).

Human feeding studies of the influence of sucralose on spermatogenesis were not undertaken but exposure to organochlorine compounds is associated with altered semen quality, DNA fragmentation, and chromosome aneuploidy in human males (Giulioni et al. 2022).

Within the current study, results of the sucralose vs sucralose-6-acetate comparison of gene expression are consistent with the sucralose-6-acetate vs control comparison that indicate sucralose-6-acetate impaired normal cellular functioning. The expression of genes for essential and fundamental cellular functions were lower in sucralose-6-acetate relative to sucralose. The Farnesyl-diphosphate farnesyltransferase (FDFT1) gene that encodes the first specific enzyme in cholesterol biosynthesis was markedly expressed by sucralose relative to sucralose-6-acetate with a fold change of 30.93. Cholesterol is not only essential for stability of cell membranes but also for tight junction formation (Shigetomi et al. 2023). Taken together data obtained from the sucralose vs sucralose-6-acetate comparison along with increased expression of MT1G, SHMT2, ATF3, and CHST3 in the sucralose-6-acetate vs control comparison indicated that exposure of human intestinal epithelium to sucralose-6-acetate disrupts essential cellular processes.

Previous studies reported changes in gene expression related to inflammation after exposure to sucralose. Increased gene expression of hepatic inflammatory markers (MMP-2 and iNOS) was detected in sucralose-treated mice (Bian et al. 2017) although it is not known if this was due to direct stimulation of liver cells by sucralose or sucralose-6-acetate, or to functional/metabolic alterations subsequent to sucralose exposure. Sucralose also enhanced adipogenesis and antioxidant gene expression in an *in vitro* study of human adipose tissue (Kundu et al. 2020). The upregulation of the antioxidant gene GPX3 was interpreted as a compensatory response to elevated intracellular accumulation of reactive oxygen species (ROS). Another *in vitro* investigation in adipose tissue found that sucralose upregulated PPAR γ , a suppressor of NF- κ B-mediated pro-inflammatory responses (Azad et al. 2020). Consumption of diet soda sweetened with sucralose as well as acesulfame-K (another artificial sweetener) altered inflammatory transcriptome pathways including

NF- κ B signaling in subcutaneous adipose tissue (Sylvetsky et al. 2020).

Pharmacokinetics

The study of the half-life ($T_{1/2}$) in liver microsomes indicates that sucralose-6-acetate is extracted to a certain extent by the liver with a greater effect in humans than rats, but absorption and metabolism of sucralose-6-acetate have not yet been fully characterized. The extraction does not appear to involve Phase 1 metabolism in human liver microsomes since it is independent of NADPH. Although the extraction rate is higher for sucralose-6-acetate than for sucralose, sucralose-6-acetate was detected in urine for 5 days longer than sucralose after discontinuation of sucralose intake. This might be attributed to greater lipophilicity and hence elevated bioaccumulation potential of sucralose-6-acetate.

The investigation of xenobiotic detoxification enzymes in human liver microsomes noted that sucralose-6-acetate is an inhibitor of two members of the cytochrome P450 (CYP450) family (CYP1A2 and CYP2C19). Inhibition of these enzymes may potentially affect bioavailability of drugs and levels of endogenous substrates. No significant inhibitory effect on CYP450 enzymes was found for sucralose. Inhibition of CYP1A2 and CYP2C19 might reduce metabolism of endogenous and exogenous chemicals and precipitate potentially adverse metabolic effects.

CYP1A2 metabolizes many endogenous compounds such as retinols, melatonin, steroids (including estradiol), estrogens, uroporphyrinogen and arachidonic acids. Inhibition of CYP1A2 may potentially increase estradiol levels, and there is an association between estradiol and breast cancer (Cummings et al. 2002; DrugBank 2022; PubChem 2022). Inhibition of CYP1A2 may also elevate plasma levels of caffeine (a substrate of CYP1A2), potentially exacerbating anxiety, sleep problems, and even high blood pressure.

Inhibition of CYP1A2 by sucralose-6-acetate may also potentially increase plasma concentrations of pharmaceuticals that are CYP1A2 substrates. Representative CYP1A2 substrates (along with their biological functions/indications) include alosetron (irritable bowel syndrome), axitinib (renal cell carcinoma), caffeine (CNS stimulant), clozapine (antipsychotic), flutamide (prostate

cancer), frovatriptan (migraine), melatonin (sleep-wake cycle), mexiletine (heart arrhythmias), mirtazapine (antidepressant), olanzapine (antipsychotic), rasagiline (Parkinson's disease), tacrine (Alzheimer's disease), theophylline (bronchodilator), tizanidine (muscle relaxer), and triamterene (diuretic). Inhibition of CYP2C19 by sucralose-6-acetate might potentially elevate plasma concentrations of CYP2C19 substrates. Representative CYP2C19 substrates that are known to be significantly affected by CYP2C19 inhibitors (along with their biological functions/indications) include abrocitinib (atopic dermatitis), cannabidiol (seizures), carisoprodol (muscle relaxant), cilostazol (claudication), citalopram (antidepressant), clobazam (sedative), clopidogrel (blood thinner), diazepam (anxiety), esomeprazole (gastroesophageal reflux), methadone (narcotic addiction), omeprazole (gastroesophageal reflux), phenytoin (seizures), and tofacitinib (rheumatoid arthritis) (DrugBank 2022; PubChem 2022).

The inhibition of CYP1A2 and CYP2C19 by sucralose-6-acetate reported here was observed *in vitro* in human liver microsomes in the absence of bacteria. However, additional pharmacokinetic effects, including inhibition or induction, attributed to sucralose-6-acetate may occur *in vivo* because intestinal flora and inflammation are well-known to alter the expression and activity of CYP450 metabolizing enzymes as well as transporters (Claus et al. 2011; Kuno et al. 2016; Toda et al. 2009; Selwyn et al. 2016; Togao et al. 2020; Collins and Patterson 2020; Hu et al. 2021; Lenoir et al. 2021). Further, oxidative stress was noted to induce cytochrome P450 3A4 (CYP3A4) (Nagai et al. 2004; Strolin-Benedetti et al. 1999) as well as the transporter P-glycoprotein/P-gp (Abu-Qare, Elmasry, and Abou-Donia 2003; Callaghan et al. 2008; Feng et al. 2019; Shchulkin et al. 2021), and induction of both CYP3A4 and P-gp were reported *in vivo* after 12 weeks of sucralose ingestion (Abou-Donia et al. 2008).

Conclusions

The 8 projects performed in this study add to the large and growing scientific literature that report adverse biological impacts attributed to exposure to sucralose. In the current investigation sucralose-6-

acetate, a sucralose impurity and metabolite, was found to be genotoxic with a clastogenic MoA associated with induction of breaks in DNA. Exposure of intestinal epithelium *in vitro* to mM concentrations of both sucralose-6-acetate and sucralose in the absence of intestinal bacteria impaired the integrity of intestinal barrier function. Sucralose-6-acetate induced expression of genes in intestinal epithelium associated with inflammation, oxidative stress, and cancer including MT1G and SHMT2. Sucralose-6-acetate also blocked two members of the cytochrome P450 family (CYP1A2 and CYP2C19) that metabolize both endogenous and xenobiotic compounds that might consequently lead to adverse toxicological exposures. These findings raise health and safety concerns regarding the continued presence of sucralose in the food supply and indicate that a regulatory status review needs to be undertaken.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

Data that support the findings of this study are available from the corresponding author, SSS, upon reasonable request.

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