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Toxicological evaluation of 3′-sialyllactose sodium salt

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ABSTRACT

The safety of 3′-sialyllactose (3′-SL) sodium salt was evaluated by testing for gene mutations, *in vivo* and *in vitro* clastogenic activity, and animal toxicity in beagle dogs and rats. The results of all mutagenicity and genotoxicity tests were negative, indicating that 3′-SL does not have any mutagenic or clastogenic potential. The mean lethal dose (LD_{50}) of 3′-SL sodium salt was well above 20 g/kg body weight (bw) in rats. A dose escalation acute toxicity study in Beagle dogs also indicated no treatment-related abnormalities. Subsequent 28-day and 90-day toxicity studies in Sprague- Dawley (SD) rats involved dietary exposure to 500, 1,000, and 2000 mg/kg bw of 3′-SL sodium salt and a water (vehicle) control. There were no treatment-related abnormalities on clinical observations, body weight, food consumption, behavior, hematology, clinical chemistry, organ weights, relative organ weights, urinalysis parameters, or necropsy and histopathological findings. The No Observed Adverse Effect Level (NOAEL) of 3′-SL sodium salt was determined to be higher than 2000 mg/kg bw/day in an oral subchronic toxicity study in rats, indicating that the substance is an ordinary carbohydrate with the lowest toxicity rating. Results confirm that 3′-SL sodium salt has a toxicity profile similar to other non-digestible carbohydrates and naturally occurring human milk oligosaccharides (HMOs) and support its safety for human consumption in foods.

1. Introduction

3′-sialyllactose (3′-SL) is a human milk oligosaccharide (HMO) and unconjugated complex carbohydrate (Jantscher-Krenn et al., 2013). With a degree of polymerization (DP) units of 3, 3′-SL is a trisaccharide that resists digestion in the small intestine and reaches the colon intact, where it is fermented by colonic bacteria. It is categorized as a dietary fiber and defined as a non-digestible carbohydrate (AACC, 2001). The National Academy of Medicine and many authoritative agencies have recommended that dietary fiber intake should be increased for health benefits that include reduced risk for cardiovascular diseases, diabetes, and obesity (Cho et al., 2013).

HMOs are a family of structurally diverse unconjugated glycans (Bode, 2012) involved in many biological functions that influence infant health (Vazquez et al., 2017). Their structural and chemical diversity derives from α-glycosidic linkages of fucose (neutral oligosaccharides) and/or acidic oligosaccharides (SA) to respective core HMO molecules (Boehm and Moro, 2008). Among HMOs, approximately 50–70% are fucosylated, 10–30% are sialylated, and less than 10% are neither fucosylated nor sialylated (Ninonuevo et al., 2006).

Sialyllactose (SL) has a combined structure of lactose and *N*-acetylneuraminic acid (also called sialic acid) (Kunz et al., 2000). The most abundant sialylated oligosaccharides in human milk are 6′-sialyllactose (6′-SL), disialyllactose-*N*-tetraose (DSLNT), sialyllacto-*N*-tetraose and 3′-SL (ten Bruggencate et al., 2014). The concentrations of 3′-SL ranges from 42 to 840 mg/L with mean concentrations at earlier stages of lactation being higher than those at later stages. The bovine milk-based typical infant formula is estimated to contain 17–19 mg/L of 3′-SL.

SL and sialylated oligosaccharides are thought to have significant health benefits for neonates (ten Bruggencate et al., 2014; Wu et al., 2011). These include the establishment and maintenance of healthy intestinal bacterial microflora that support development of the neonatal immune system and the maintenance of normal cognitive, learning, and memory functions of the brain (Bode, 2012; Hester et al., 2013; ten Bruggencate et al., 2014). Charbonneau et al. (2016) found significantly less abundant sialylated HMO content in the milk of mothers with severely stunted infants.

More than 100 HMOs have been fully characterized from human milk (Urashima et al., 2012; Austin et al., 2016). Several studies have characterized the types and levels of some oligosaccharides during lactation (Kunz et al., 2000; Thurl et al., 2010) but few have focused on sialyloligosaccharides (Austin et al., 2016; Bao et al., 2007; Sakaguchi et al., 2014). Neutral oligosaccharides (i.e., galacto-oligosaccharides and fructo-oligosaccharides) that are used in infant formula reduce the

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incidence of atopic dermatitis in infants, and stimulate the growth of bifidobacteria and lactobacilli (Ben et al., 2008; Moro et al., 2006; ten Bruggencate et al., 2014). Despite potentially important health benefits to neonates, acidic oligosaccharides, such as SL, have yet to be used as a component for fortification in infant formula (ten Bruggencate et al., 2014). The addition of 3′-SL sodium salt is consistent with efforts to produce products that closely match the nutrient composition of human milk, but additional preclinical and clinical studies are required to demonstrate efficacy (Jantscher-Krenn and Bode, 2012).

To date, the safety of 3′-SL supplementation has not been documented. We carried out toxicological evaluations in experimental animals to provide guidelines for selecting a safe dose in humans. The dietary safety of 3′SL was separately assessed by comprehensive 28- and 90-day oral toxicity studies in rats and Beagle dogs, *in vivo* mutagenicity assays (bacterial reverse mutation assays), and *in vivo* clastogenicity assays (mammalian erythrocyte micronucleus and mammalian chromosomal aberration). This is the first systemic study on the safety of 3′-SL sodium salt for human use as a food additive.

2. Materials and methods

2.1. Test articles

3′-SL sodium salt (purity 98.8%), produced by enzymatic synthesis was supplied by GeneChem Inc., Republic of Korea. The molecular formula of 3′-SL is $C_{23}H_{38}NO_{19}Na$, with a molecular weight of 655.54 g/mol. The identity of 3′-SL sodium salt was confirmed by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). Quantitation was done by high-performance anion exchange-chromatography (HPAEC). Impurities which may potentially remain in the 3′-SL product include lactose (0.25%), 3′-sialylgalactose (0.46%) and sialic acid (0.49%).

2.2. Animals and organisms

Chinese hamster lung (CHL/IU) mammalian cells were obtained from American Type Culture Collection (ATCC) *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* (WP2*uvrA* [pKM101]) strains were obtained from Molecular Toxicology, Inc., Boone, North Carolina, USA. The bacterial strains were maintained as frozen stocks at - 80 ± 3 °C. In the *in vivo* mouse micronucleus test, we used healthy 8-week-old male Institute of Cancer Research (ICR) mice supplied by OrientBio, Republic of Korea. In single dose, 28-day and 90-day toxicity studies, we used 6-week-old Sprague-Dawley rats (males weighing 188.3–209.4 g; females weighing 140.8–174.1 g) supplied by OrientBio, Republic of Korea. Their health status was confirmed by a veterinarian at the start of acclimatization. We used 4 male Beagle dogs, 5–6 months old, with a weight range from 6.65 to 6.80 kg, and 4 female Beagle dogs of the same age with a weight range of 5.39–5.66 kg for the dose escalating study. The dogs were supplied by Woo Jung BSC, Republic of Korea. All animals were kept under conventional conditions, in individual stainless steel mesh cages, and acclimated to laboratory conditions for one week.

Room temperature was maintained at 22 ± 3 °C and the relative humidity ranged from 30% to 70%. The animal room received a minimum of 12 air changes per hour. Filtered water suitable for human consumption was provided *ad libitum*. Rats received an *ad libitum* pelleted diet (Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C). Batches of the diet were periodically analyzed for contamination. Samples of the drinking water were periodically tested for bacteriological or chemical contamination. All the animal studies were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Biotoxtech Co., Ltd. based on Animal Protection and Welfare Act (Enactment May 31, 1991, No. 4379, Revision Feb. 29, 2008, No. 8852). Exception was the acute oral toxicity study which was approved by Yantai University Animal Care Committee based on Animal Welfare guidelines.

2.3. Guidelines

Standard operating procedures were used to perform mutagenicity, genotoxicity, and animal toxicity studies. These were conducted according to FDA Redbook 2000 guidelines and in compliance with the Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) as revised in 1997 and adopted on November 26, 1997 by decision of the OECD Council [c(97) 186/Final]: Bacterial reverse mutation study -OECD 471; *in vitro* chromosome aberration test-OECD 473, *in vivo* mouse micronucleus test -OECD 473; acute oral toxicity study in rats - FDA Redbook 2000; 28 day and 90-day oral toxicity studies in rats - OECD 408.

2.4. Experimental design

2.4.1. Bacterial reverse mutation test of 3′-SL sodium salt

In vitro bacterial mutagenicity assays were performed to evaluate the mutagenic potential of 3′-SL sodium salt in 4 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and 1 strain of *E. coli* WP2*uvrA* (pKM101) in the absence and presence of metabolic activation. The high dose of the test substance was 5000 μg/mL. We also used lower dose levels of 2500, 1000, 500, 250, 100, 50, 10 and 5 μg/mL. Sodium azide, 2-nitrofluorene, 2-aminoanthracene, 9-aminoacridine, and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide were the positive controls. The vehicle of the test substance (3′-SL) was the negative control. S9 (rat liver homogenate-Lot No.: 09041001) and Cofactor A (Lot No.: A09040701) were purchased from Orient Yeast Co. in Japan and stored in the deep freezer (−70 ± 10 °C). For the preparation of S9 mix, S9 and Cofactor A were mixed at a ratio of 1 mL: 9 mL prior to use. The formulated S9 mix consists of S9, NADH, NADPH, glucose-6-phosphate, KCl, and $MgCl_2$ in PBS buffer.

2.4.2. In vitro chromosome aberration test of 3′-SL sodium salt using cultured mammalian cells

The cytotoxicity of 3′-SL sodium salt and its potential to induce chromosomal aberrations were assessed in Chinese Hamster Lung (CHL/IU) mammalian cells in the presence or absence of metabolic activation (Sofuni et al., 1990). The high dose of the test substance was 5000 μg/mL. Lower dose levels were 2500, 1000, 500, 250, 100, 50, 10 and 5 μg/mL.

Acute cytotoxicity was not evident in the presence or absence of metabolic activation, or continuous treatment without metabolic activation. We selected 5000 μg/mL as the high dose. It was sequentially diluted by the geometric ratio of 2 to produce 3 additional lower dose levels. Mitomycin C and benzo[a]pyrene groups were used as positive controls. The vehicle of the test substance was used as the negative control.

In the study with and without metabolic activation, each well was washed with phosphate-buffered saline (PBS) after 6 h of treatment. A fresh medium was then added and cultured for an additional 18 h. Cells were incubated for 24 h during continuous treatment. Slides were prepared after completion of incubation. The deposition of the test substance was observed after addition of the test substance and at the end of incubation. S9 and Cofactor A are from the same source described in the 2.4.1.

2.4.3. In vivo micronucleus test of 3′-SL sodium salt in mice

3′-SL sodium salt was tested for its ability to induce micronuclei in polychromatic erythrocytes (PCE) of the bone marrow of treated Imprinting Control Region (ICR) mice. The doses of 3′SL sodium salt used in the study were 500, 1000, and 2000 mg/kg body weight (bw). Fifty-four male and female mice aged 8 weeks were treated by oral gavage with 3′-SL sodium salt dissolved in saline over 3 consecutive days. Saline was used as a vehicle control. Mitomycin C (2 mg/kg, i.p.)