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Safety evaluation of the human-identical milk monosaccharide, L-fucose

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ABSTRACT

L-Fucose is a natural monosaccharide present in mammals where it is found predominantly as an *O*-glycosidically linked component of glycoproteins, glycolipids and oligosaccharides. It is also present in its free form in human breast milk (human milk monosaccharide). L-Fucose plays important roles in the development of the immune and nervous systems and is involved in cognitive function and memory formation. The human-identical milk monosaccharide L-fucose is therefore proposed for use in infant formulas to better simulate the free saccharides present in human breast milk. As part of the safety evaluation of L-fucose, a subchronic dietary toxicity study preceded by an *in utero* phase was conducted in Sprague–Dawley rats. L-Fucose was without maternal toxicity or compound-related adverse effects on female reproduction and general growth and development of offspring at a maternal dietary level up to 1%, equivalent to a dose of 1655 mg/kg body weight (bw)/day. During the subchronic phase, no compound-related adverse effects were observed in first generation rats at dietary levels of up to 1% (highest level tested), corresponding to doses of 516 and 665 mg/kg bw/day in males and females, respectively. L-Fucose was non-genotoxic in a series of *in vitro* genotoxicity/mutagenicity tests. These results support the safe use of L-fucose in infant formula and as a food ingredient at levels equivalent to those present in human breast milk.

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1. Introduction

L-Fucose (6-deoxy L-galactose) is a 6-deoxy hexose monosaccharide belonging to the rare series of L-configured sugars. Its chemical structure is shown in Fig. 1. In nature, L-fucose occurs as part of the indigestible polysaccharides (fiber) found in fruit, vegetables, plants and seaweed (Vanhooren and Vandamme, 1999; Pomin, 2012; Smith, 2013), or as part of oligosaccharides in the animal kingdom. In animals free L-fucose is biosynthesized endogenously and transferred onto oligosaccharides via its activated metabolite GDP-fucose. L-Fucose-containing oligosaccharides occur as soluble glycoproteins (e.g., mucins), membrane bound *N*- and *O*-glycoproteins, glycolipids (gangliosides and glycosphingolipids), and free oligosaccharides, which are found mainly in milk (the human milk oligosaccharides, HMOs) (Coppa et al., 1993; Bode, 2006). These fucosylated glycans play important roles in various physiological and pathological processes, including leukocyte adhesion (Becker and Lowe, 2003), host–microbe interactions (Hooper and Gordon, 2001), and neuronal development and memory formation (Murrey and Hsieh-Wilson, 2008). These substances are also prevalent in erythrocyte glycolipids where they form the LEWIS and ABO blood group antigens that distinguish specific blood types (Marionneau et al., 2001). In addition, aberrant expression of fucosylated glycoconjugates has been associated with several disease processes, including cancer and inflammation (Lowe, 2003; Dennis et al., 2009; Christiansen et al., 2014).

Abbreviations: 2-AA, 2-aminoanthracene; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; ARA, arachidonic acid; bw, body weight; CBPI, cytokinesis-blocked proliferation index; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; F_1, first generation; FOB, functional observational battery; GD, gestation day; GLP, Good Laboratory Practice; HiMMs, human-identical milk monosaccharides; HMMs, human milk monosaccharides; HMOs, human milk oligosaccharides; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; MCH, mean corpuscular hemoglobin; MMS, methyl methanesulfonate; NaN_3, Sodium azide; Neu5Ac, HiMM *N*-acetyl-D-neuraminic acid; NOAEL, no-observed-adverse-effect level; OECD, Organisation for Economic Co-operation and Development; P, parental; PND, post-natal day; S9, S9 microsomal fraction; US FDA, United States Food & Drug Administration; USDA, US Department of Agriculture.

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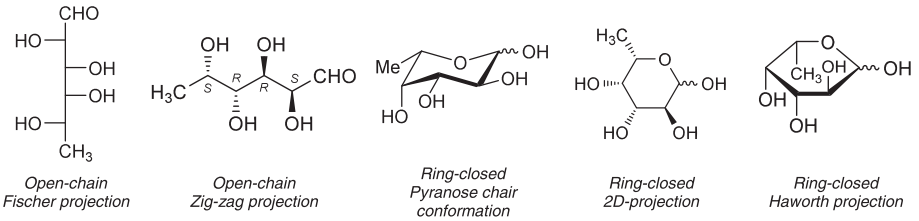

Fig. 1. Different representations of the chemical structure of L-fucose, in open-chain and ring-closed forms. The predominant form, under physiological conditions, is ring-closed, which is in equilibrium with the open-chain form.

Of great interest is the role of L-fucose in human breast milk in infant development. This is due particularly in light of the results of a number of studies indicating a role for fucosylation in cognitive function, learning, and memory Matthies et al., 1996; Murrey and Hsieh-Wilson, 2008; Mountford et al., 2014). L-Fucose is highly enriched at neuronal synapses (Murrey et al., 2006), where the majority of the fucosylated glycoconjugates exist as complex *N*-linked glycoprotein structures. A review of the literature demonstrates a wide range of studies supporting a role for such fucosylated oligosaccharides in the regulation of neuronal growth and synapse formation (Murrey and Hsieh-Wilson, 2008). It is also suggested that L-fucose within human breast milk may play a role in the development of the infant immune system (Newburg et al., 2004).

In human breast milk, L-fucose is present as an important component of the human milk monosaccharides (HMMs), as well as the HMOs (Coppa et al., 1999; Kunz et al., 2000; Thurl et al., 2010). The HMO fraction is the third largest constituent of human breast milk after lactose (~70 g/L) and lipids (~38 g/L). Colostrum is reported to contain 20–23 g/L HMOs and mature breast milk is reported to contain 12–15 g/L HMOs (Montreuil and Mullet, 1960; Viverge et al., 1985, 1990; Coppa et al., 1999; Bode, 2006; Thurl et al., 2010). HMOs consist of a highly complex mixture of individual compounds, with more than 200 identified to date (Ward et al., 2006), at least 115 of which have been fully elucidated (Bode, 2006; Kobata, 2010). HMOs are highly fucosylated. In contrast, fucosylation in bovine milk is nearly absent and rare in porcine milk (Kunz et al., 2000; Bode, 2006; Ninonuevo et al., 2006; Tao et al., 2008, 2010). As a result, bovine-based infant milk formula is lacking in important HMOs that are potentially beneficial to neonatal development (Bode, 2006). As mentioned, L-fucose also occurs in the free form in human breast milk at concentrations in the range of 20–30 mg/L (Heyns et al., 1956; Newburg and Neubauer, 1995; Smilowitz et al., 2013). Its presence in human breast milk is likely in part due to the activity of sugar cleaving enzymes, particularly α-L-fucosidase (Wiederschain and Newburg, 1995, 1996, 2001) and is not an analytical artifact of hydrolysis during sample preparation (Heyns et al., 1956).

Given the discussion above, there is considerable interest in the commercial development of HMOs, and their constituent monosaccharides (i.e., human-identical milk monosaccharides or HiMMs), including L-fucose, for addition/incorporation into infant formulas. Such incorporation may benefit the development of the nervous (Murrey and Hsieh-Wilson, 2008) and immune systems (Bode, 2006; Oriquat et al., 2011; Lee et al., 2012) in formula-fed neonates in which the biological need for L-fucose may not be sufficiently met by endogenous production While L-fucose is an endogenous compound present in human breast milk, it was considered prudent to establish the safety of HiMM L-fucose for use in infant formula in part through the conduct of a series of toxicological studies.

The objective of the current series of experiments was, therefore, to investigate the effects of HiMM L-fucose on female reproduction and general growth and development of Sprague–Dawley rats following *in utero* and lactational exposure. The *in utero* and lactational phases were followed by a 13-week dietary exposure period in the first generation (F_1) generation in order to evaluate reversibility, progression, or delayed appearance of any observed changes. After the 13-week exposure period, F_1 animals were observed in a recovery period for 1 month. This study design has been generally accepted by regulatory authorities world-wide for assessing the safety of infant formula ingredients associated with neural development, such as docosahexaenoic acid (DHA) and arachidonic acid (ARA) (Burns et al., 1999; Hempenius et al., 2000; Lina et al., 2006; Casterton et al., 2009; Fedorova-Dahms et al., 2011). A series of genotoxicity tests also were performed that consisted of a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus assay.

2. Materials and methods

All studies[1] were conducted in compliance with the United States Food & Drug Administration (US FDA) regulations on Good Laboratory Practice (GLP) for Nonclinical Laboratory Studies (Title 21 of the *Code of Federal Regulations*, Part 58) (US FDA, 2013) and the Organisation for Economic Co-operation and Development (OECD) Principles of GLP (OECD, 1998a). The protocol for the animal study was reviewed and approved by the Institutional Animal Care and Use Committee before animal receipt. The animal study conducted does not unnecessarily duplicate any previous experiment. Study protocols were similar to those applied in the safety evaluation of the HiMM *N*-acetyl-D-neuraminic acid (Neu5Ac) as described in Choi et al. (2014).

2.1. Test materials

L-Fucose (purity 98.8 %) was provided by Glycom A/S (Kongens Lyngby, Denmark) as a fine white powder. 2-Aminoanthracene (2-AA), 2-nitrofluorene (2-NF), 9-aminoacridine (9-AA), methyl methanesulfonate (MMS), mitomycin C, vinblastine, cyclophosphamide, and cytochalasin B were obtained from Sigma Aldrich Chemical Co (Saint Louis, Missouri, USA). Sodium azide (NaN_3) was obtained from Alfa Aesar (Ward Hill, MA) and dimethyl sulfoxide (DMSO) was obtained from EMD Chemicals (Gibbstown, NJ, USA). Sterile distilled water was obtained from Life Technologies (Grand Island, NY) and from Mediatech (Manassas, VA). The S9 microsomal fraction (S9) used as the metabolic activation system was prepared by and purchased from Moltox (Boone, North Carolina, USA). The S9 was prepared from male Sprague–Dawley rats induced with a single intraperitoneal injection of 500 mg/kg body weight Aroclor 1254 and euthanized 5 days later.

2.2. Post-weaning 13-week dietary toxicity study with an in utero phase

The study consisted of a repeated dose 13-week dietary toxicity phase preceded by an *in utero* phase. The 13-week toxicity study

[1] The animal study was conducted at MPI Research and the genotoxicity studies were conducted at BioReliance.

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