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Safety evaluation of the human-identical milk monosaccharide sialic acid (*N*-acetyl-D-neuraminic acid) in Sprague-Dawley rats

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ABSTRACT

N-Acetyl-D-neuraminic acid (Neu5Ac) is the predominant form of sialic acid (Sia) in humans, while other mammals express Sia as a mixture with *N*-glycolyl-D-neuraminic acid (Neu5Gc). Neu5Ac occurs in highest levels in the brain and in breast milk, and is therefore, coined a human-specific milk monosaccharide, and is thought to play an important nutritional role in the developing infant. Synthesized human-identical milk monosaccharide (HiMM) Neu5Ac is proposed for use in infant formulas to better simulate the free saccharides present in human breast milk. As part of the safety evaluation of HiMM Neu5Ac, a subchronic dietary toxicity study preceded by an *in utero* phase was conducted in Sprague-Dawley rats. Neu5Ac was without maternal toxicity or compound-related adverse effects on female reproduction and on the general growth and development of offspring at a maternal dietary level of up to 2%, equivalent to a dose of 1895 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 2% (highest level tested), corresponding to doses of 974 and 1246 mg/kg bw/day in males and females, respectively. Neu5Ac also was non-genotoxic in a series of *in vitro* genotoxicity/mutagenicity tests. These results support the safe use of Neu5Ac both in infant formula and as a food ingredient at levels equivalent to those found naturally in human breast milk.

1. Introduction

Sialic acids (Sia) comprise a family of over 50 nine-carbon acidic monosaccharides [reviewed by Angata and Varki (2002) and Schauer (2004)]. These compounds are *N*- and/or *O*-substituted derivatives of D-neuraminic acid (5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid) and are biosynthesized endogenously in all vertebrates, particularly in mammals. However, by far the most prevalent forms of Sia are *N*-acetyl-D-neuraminic acid (Neu5Ac), which is the main Sia present in humans, and *N*-glycolyl-D-neuraminic acid (Neu5Gc), which is biosynthesized from Neu5Ac. While all other mammals have the capacity to produce Neu5Gc, humans are unique among extant mammals in regards to a homozygous deficiency to produce Neu5Gc from Neu5Ac due to an, evolutionary recent, inactivating mutation of the gene that encodes for the enzyme responsible for the conversion (Hayakawa et al., 2006). Neu5Gc is therefore virtually absent from the human body, while Neu5Ac is present as the predominant Sia. In fundamental contrast to Neu5Ac, Neu5Gc elicits an immune response and the production of antibodies in humans, what is suspected to be involved in adverse health effects connected to dietary exposure (Samraj et al., 2014), recombinant glycoprotein therapeu-

Abbreviations: 2-AA, 2-aminoanthracene; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; ARA, arachidonic acid; bw, body weight; DHA, docosahexaenoic acid; F_1, first generation; FOB, functional observational battery; GD, gestation day; GLP, Good Laboratory Practice; ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; HiMM, human-identical milk monosaccharide; MMS, methyl methanesulfonate; NaN_3, Sodium azide; Neu5Ac, *N*-acetyl-D-neuraminic acid; Neu5Gc, *N*-glycolyl-D-neuraminic acid; NOAEL, no-observed-adverse-effect level; OECD, Organisation for Economic Co-operation and Development; P, parental; PND, post-natal day; Sia, sialic acids; S9, S9 microsomal fraction; US FDA, United States Food & Drug Administration.

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tics (Ghaderi et al., 2010), and xenotransplantation (Padler-Karavani and Varki, 2011; Scobie et al., 2013).

Sia residues occur predominantly as the outermost unit of cell surface oligosaccharide chains of cell membrane-bound glycolipids and glycoproteins, and are therefore, involved in endogenous (cell–cell communication) and exogenous (infection) recognition processes. In humans, Neu5Ac, colloquially referred to by the family name *sialic acid* [for nomenclature see Blix et al. (1957) and McNaught (1996)] due to the history of its discovery (Klenk et al., 1941; Blix et al., 1952, 1957), occurs ubiquitously throughout the body, with the highest levels detected in the brain, specifically in the grey matter (Klenk et al., 1941; Papadopoulos, 1960; Wang et al., 1998). In the human brain, Neu5Ac residues are present mainly within specific glycolipids known as gangliosides (*i.e.*, glycosphingolipids, each composed of a ceramide and a Sia-containing oligosaccharide) and glycoproteins, such as the neural cell adhesion molecules.

Neu5Ac has also been detected at high levels in human breast milk (Carlson, 1985) in which it occurs not only as a component of glycoproteins and gangliosides (Rueda et al., 1995; Puente et al., 1996), but in considerably higher amounts at the terminal end of free oligosaccharides known as human milk oligosaccharides. Importantly, Neu5Ac also has been found to occur in the free form in human breast milk (Sabharwal et al., 1991; Hayakawa et al., 1993; Thurl et al., 1996; Wang et al., 2001; Wiederschain and Newburg, 2001; Martín-Sosa et al., 2004; Oriquat et al., 2011; Galeotti et al., 2012). The exclusive presence of the *N*-acetyl form in human milk is a distinct human-specific feature, and in this sense, pure Neu5Ac is coined a human-specific milk monosaccharide. Neu5Ac plays both structural and functional roles at the cellular level. Neu5Ac is involved in a multitude of cell signaling events and is incorporated into molecules involved in neural development, synaptic transmission, cognition, and memory function, as well as in immune function. The presence of Neu5Ac in human breast milk, together with the knowledge that Neu5Ac is an important factor in biological events linked to brain development have led to increased interest in designing infant formulas that better simulate the free saccharide structures present in human breast milk. As part of ongoing efforts in this field, the safety of the synthetic but human-identical milk monosaccharide (HiMM) Neu5Ac for use in infant formula and as an ingredient in food and/or food supplements (*i.e.*, dietary supplements), is addressed in the present work. It should be noted that the complete absence of any trace levels of Neu5Gc in the synthesized HiMM Neu5Ac offers a clear advantage over natural (non-human) sources of Neu5Ac, which unavoidably are contaminated with Neu5Gc. Pre-clinical investigations, designed in part to mimic human infant exposure as early as directly after birth, were carried out with the objective of determining any effects of the new HiMM food ingredient on female reproduction and on the 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 toxicity phase conducted with the F_1 (first generation) offspring to assess for systemic toxicity. 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/mutagenicity tests also were performed that consisted of a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus assay. These tests were selected in accordance with the European Food Safety Authority Scientific Committee's most recent guidance on genotoxicity testing strategies for food safety assessments (EFSA, 2011).

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 care and use of laboratory animals were in compliance with the US Department of Agriculture's Animal Welfare Act (Title 9 of the Code of Federal Regulations, Parts 1, 2, and 3) and in accordance with the Institute of Laboratory Animal Resources' Guide for the Care and Use of Laboratory Animals (ILAR, 2011). The protocol for the animal study was reviewed and approved by the Institutional Animal Care and Use Committee before animal receipt.

2.1. Test materials

N-Acetyl-D-neuraminic acid dihydrate [Neu5Ac*$(H_2O)_2$] (Lot No. L12058K; purity 98.6%) was provided by Glycom (Kongens Lyngby, Denmark) as a white, free flowing crystalline 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 (Saint Louis, MO). Sodium azide (NaN_3) was obtained from Alfa Aesar (Ward Hill, MA). The S9 microsomal fraction (S9) used as the metabolic activation system was prepared by and purchased from Moltox (Boone, NC). The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of 500 mg/kg body weight (bw) 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 was conducted in accordance with OECD Guideline Test No. 408 (OECD, 1998b), US FDA Redbook guideline for Subchronic Toxicity Studies with Rodents (US FDA, 2003), and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline M3(R2) (ICH, 2009).

2.2.1. Animals and housing conditions

Six-week old male and female (nulliparous and nonpregnant) CD® [Crl:CD® (SD)] rats (114 of each sex) were obtained from Charles River Laboratories International (Portage, MI), and were allowed to acclimatize to the study conditions for 1 week. Parental (P) animals that were in good general health were selected for the study, and were randomly assigned by sex and weight to one of four groups each consisting of 26 males (weighing 192–224 g) and 26 females (weighing 149–186 g).

Animals, including F_1 pups after weaning, were housed individually in suspended, stainless steel, wire-mesh cages (Suburban Surgical, Wheeling, IL) during the acclimation and study periods, except during mating, near parturition, and during lactation. During pairing, one male and one female were housed per cage. On gestation day (GD) 20, pregnant P females were individually housed in plastic cages (Allentown Caging, Allentown, NJ) with wood chip bedding (Harlan, Madison, WI) and were allowed to deliver their litters naturally. Housing conditions were maintained

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