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Isotopic labeling of milk disialogangliosides (GD3)

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ABSTRACT

The most abundant ganglioside group in both human milk and bovine milk during the first postnatal week is ganglioside GD3. This group of disialogangliosides forms up to 80% of the total ganglioside content of colostrum. Although dietary gangliosides have shown biological activity such as improvement of cognitive development, gastrointestinal health, and immune function, there is still a gap in our understanding of the molecular mechanisms governing its uptake and the metabolic processes affecting its bioavailability. The use of isotopically labeled ganglioside to track the bioavailability, absorption, distribution, and metabolism of gangliosides may provide key information to bridge this gap. However, isotope labeled

GD3 is not commercially available and its preparation has not been described. We report for the first time the preparation of labeled GD3 with stable isotopes. Using alkaline hydrolysis, we were able to selectively remove both acetyl groups from the tetrasaccharide portion of GD3 without promoting significant hydrolysis of the ceramide portion of the molecule to generate N-deacetyl-GD3 (Neu5α2-8Neu5-GD3). The N-deacetyl-GD3 was then chemoselectively re-acetylated in aqueous medium using deuterated acetic anhydride in the presence of Triton X 100 to produce $^{2}H_{6}$-GD3 {GD3[(Neu5Ac-11-$^{2}H_{3}$)-(Neu5Ac-11-$^{2}H_{3}$)]}. This method provided $^{2}H_{6}$-GD3 with approximately 60% yield. This compound was characterized by proton nuclear magnetic resonance (^{1}H NMR) and liquid chromatography mass spectrometry (LC–MS). The oral absorption of the $^{2}H_{6}$-GD3 was demonstrated using a Sprague-Dawley weaning rats. Our results indicate that some ingested labeled milk gangliosides are absorbed and transported into the bloodstream without modification.

1. Introduction

Gangliosides are a class of glycosphingolipids that include at least one sialic acid in the carbohydrate moiety of their molecular structure (McJarrow et al., 2009). They represent a significant component of the cell surface glycans found on neuronal cells and play important roles in cell adhesion (Hakomori et al., 1998), signal transduction (Hakomori, 1990), and neural development (Blum and Barnstable, 1987). It is believed that individual dietary gangliosides may be required at different stages of neonatal development as there is a chronological change in the composition of gangliosides in human milk. Ganglioside GD3 (Fig. 1) is most abundant in human colostrum and ganglioside GM3 is most abundant in mature milk (Pan and Izumi, 1999; Rueda et al., 1996; Nakano 1998; Takamizawa et al., 1986).

Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; $^{2}H_{6}$-GD3, Neu5Ac-$^{2}H_{3\alpha}$2-8Neu5Ac-$^{2}H_{3}$-GD3; GD3, disialoganglioside; Gal, galactose; Glu, glucose; Sph, sphingosine; FA, fatty acid; H1Gal, anomeric hydrogen from galactose; H1Glc, anomeric hydrogen from glucose; FAαH, α hydrogen from fatty acid portion; H3-Sia, hydrogen at sialic acid carbon 3; HPLC, high performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; N-deacetyl-GD3, Neu5α2-8Neu5-GD3; SPE, solid phase extraction; HPTLC, high performance thin layer chromatography; UV, ultraviolet.

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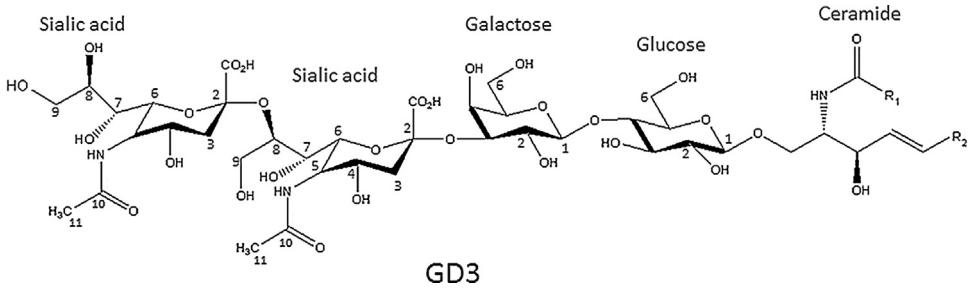

Fig. 1. General structure of GD3 (1). For R1 and R2 description see Table 3.

Evidence of the benefit of the consumption of milk gangliosides on cognitive, gastrointestinal, and immune function during neonatal life has recently been reported (Ryan et al., 2013). Ganglioside-enriched infant formula increased cognitive development scores in infants aged 0–6 months and was associated with increased serum ganglioside levels (Gurnida et al., 2012). In another study, infant formula supplemented with ganglioside was reported to modify the intestinal ecology of preterm newborns, increasing bifidobacterial levels and lowering coliform levels (Rueda et al., 1998). Moreover, by way of animal models, dietary gangliosides have been associated with the development of the intestinal immune system (Vazquez et al., 1999).

In infants, more than 80% of dietary gangliosides survive the passage through the stomach, and are absorbed in the intestine (Ryan et al., 2013; McJarrow et al., 2009; Larson et al., 1990). Breast-fed infants have higher brain gangliosides and glycoprotein sialic acid concentrations than do formula-fed infants (Wang et al., 2003). While the number of studies involving dietary ganglioside supplementation is limited, those involving animal experiment and clinical trial support this hypothesis. Park et al. (Park et al., 2005a) administrated a diet with 20% fat to 18-day-old Sprague-Dawley rats over two weeks, of which 0.1% of the fat corresponded to a mixture of gangliosides (80% GD3, 9% GD1b, 5% GM3 and 6% others). An increase was observed in the total amount of gangliosides in the intestinal mucosa, plasma, and brain (16%), with respect to the control group. In subsequent studies, using the same conditions, Park et al., detected an increase in gangliosides in the rat enterocyte membrane microdomain (Park et al., 2005b) and in the rat retina (Park et al., 2005c). Gustavsson et al. (2010) investigated the effect of oral supplementation of the maternal diet (with increased levels of GD3) on brain composition in the offspring of Wistar rats. The diet administrated to the mother, which contained GD3 with a small proportion GM3 and with only traces of other gangliosides influenced brain composition immediately post birth. It has been shown that gangliosides GM3 and GD3 are able to be transported across the human placenta (Mitchell et al., 2007). Gurnida et al. showed that infant fed with infant formula supplemented with gangliosides have increased serum ganglioside level (Gurnida et al., 2012). While, Reis et al. showed that dietary supplementation with complex lipids rich in GD3 affected the biosynthesis of specific piglet brain gangliosides (Reis et al., 2016).

Notwithstanding the advances in this area, there is still a gap in our understanding of the mechanisms that govern the uptake of milk gangliosides. The use of isotopically labeled gangliosides to track the bioavailability, absorption, distribution, and metabolism of milk gangliosides in biological studies may provide key information in this area.

Gangliosides are complex molecules composed of different types of functional groups (*i.e.* fatty acid, sphingosine and sugars) that can be associated with its biological activity as well as physicochemical properties that are important to bioavailability and absorption. Thus for nutritional studies using animal or *in vitro* models, it is desirable that the labeled compound resemble, as much as possible the original ganglioside of interest. This becomes even more important when evaluating gangliosides from natural sources such as those from bovine milk. Bovine milk is composed of a mixture of gangliosides varying on composition of the fatty acid on the ceramide portion that are different in proportion from those found human milk (Bode et al., 2004).

Stable isotopic labelling coupled with mass spectrometry has been shown to be a valuable methodology to assess bioavailability, absorption, distribution, and metabolism of substances (Schellenkens et al., 2011). It also allows monitoring of the labeled molecules as well labeled derivatives and has been widely used in pharmacology, as well in nutritional studies. The advantages of stable isotope labeling over other tracers include: (a) these are non-toxic as they are not radioactive; (b) the structure of the labeled ganglioside is the same as the natural gangliosides of interest, *i.e.* there is no replacement or addition of functional groups as in the case of fluorescently labeled derivatives; (c) this results in a low difference in the molecular structure, i. e. the physicochemical properties are not largely affected as compared to that observed for fluorescently labelled derivatives. Therefore, stable isotope compounds are more representative of the naturally occurring analogue and therefore more appropriate for nutritional studies of natural compounds.

Several research groups have developed successful methods for the total synthesis of unlabeled GD3 and its derivatives using chemical and enzymatic methods (Castro-Palomino et al., 2001; Gilbert et al., 2000). Milk GD3 is a heterogeneous mixture, with the ceramide group containing a range of fatty acids that vary in both chain length and saturation. These structural heterogeneities make preparation of this complex mixture by total synthesis challenging. A more attractive approach for labeling ganglioside mixtures could be *via* chemical or enzymatic modifications of natural mixture of gangliosides (Sonnino et al., 1996). Four strategies have been applied for the preparation of labeled gangliosides using semisynthetic methods: (i) selective oxidation and reduction of hydroxyl groups; (ii) N-deacetylation and N-acetylation at the sialic acid residue; (iii) N-deacylation and N-acylation with a labeled fatty acid at the ceramide moiety and (iv) reduction of the double bonds in the ceramide. Although these strategies have been applied to different gangliosides (Sonnino et al., 1996), to the best of our knowledge, the labeling of GD3 structures with stable isotope atoms has not yet been described, although the preparation of fluorescent derivatives of GD3 has been described by Saver et al. (2012).

This prompted us to develop a method for preparing labeled GD3 using bovine milk GD3 as a starting material. Our strategy was focused on labeling the N-acetyl groups in both sialic acids using selective N-deacetylation. This labeled structure has the potential to be used to investigate uptake of gangliosides as intact structure; evaluation of direct glycosylation of milk GD3, which would produce gangliosides more complex structures; or if would occur a stepwise degradation (GM3, N-acetylsialosyl-lactosylceramide