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N-butyldeoxygalactonojirimycin reduces neonatal brain ganglioside content in a mouse model of GM1 gangliosidosis

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Abstract

GM1 gangliosidosis is a glycosphingolipid (GSL) lysosomal storage disease caused by a genetic deficiency of acid β-galactosidase (β-gal), the enzyme that catabolyzes GM1 within lysosomes. Accumulation of GM1 and its asialo form (GA1) occurs primarily in the brain, leading to progressive neurodegeneration and brain dysfunction. Substrate reduction therapy aims to decrease the rate of GSL biosynthesis to counterbalance the impaired rate of catabolism. The imino sugar *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) is a competitive inhibitor of the ceramide-specific glucosyltransferase that catalyzes the first step in GSL biosynthesis. Neonatal C57BL/6J (B6) and β-gal knockout (–/–) mice were injected daily from post-natal day 2 (p-2) to p-5 with either vehicle or *N*B-DGJ at 600 mg or 1200 mg/kg body weight. These drug concentrations significantly reduced total brain ganglioside and GM1 content in the B6 and the β-gal (–/–) mice. Drug treatment had no significant effect on viability, body weight, brain weight, or brain water content in the B6 and β-gal (–/–) mice. Significant elevations in neutral lipids (GA1, ceramide, and sphingomyelin) were observed in the *N*B-DGJ-treated β-gal (–/–) mice, but were not associated with adverse effects. Also, *N*B-DGJ treatment of B6 and β-gal (–/–) mice from p-2 to p-5 had no subsequent effect on brain ganglioside content at p-21. Our results show that *N*B-DGJ is effective in reducing total brain ganglioside and GM1 content at early neonatal ages. These findings suggest that substrate reduction therapy using *N*B-DGJ may be an effective early intervention for GM1 gangliosidosis and possibly other GSL lysosomal storage diseases.

Keywords: β-galactosidase, gangliosidosis, glycosphingolipids, GM1, neurodegenerative disease, substrate reduction therapy.

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GM1 gangliosidosis is an autosomal recessive glycosphingolipid (GSL) storage disease caused by defects in the acid β-galactosidase (β-gal) that hydrolyzes ganglioside GM1 (Suzuki *et al*. 1995). This enzymatic defect causes GM1 and its asialo derivative (GA1) to accumulate primarily in the brain, leading to progressive neurodegeneration and brain dysfunction in humans (Suzuki *et al*. 1995). Three clinical forms of the disease are recognized including infantile-, juvenile-, and adult-onset forms. The β-gal activity in affected individuals correlates with disease onset, ranging from little or no activity in the infantile- and juvenile-onset forms to measurable activity in the adult-onset form (Suzuki *et al*. 1995). Infantile-onset patients usually succumb to the disease in the first few years of life.

Mouse models of GM1 gangliosidosis, lacking a functional β-gal gene, have been generated using homologous recombination and embryonic stem cell technology (Hahn *et al*. 1997; Matsuda *et al*. 1997). These mice express low residual β-gal activity and accumulate GM1 and GA1 in the CNS, thus mimicking the neurochemical features of the infantile/juvenile disease forms. Unlike the human disease where neurobehavioral abnormalities manifest at young ages, neurobehavioral abnormalities are not apparent in affected mice until adult ages. Also, GA1 accumulation is greater in the mouse models than in the human disease, possibly due to

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Abbreviations used: β-gal, β-galactosidase; GA1, asialo form of GM1; GlcCer, glucosylceramide; GlcT, glucosyltransferase; GSL, glycosphingolipid; *N*B-DGJ, *N*-butyldeoxygalactonojirimycin; *N*B-DNJ, *N*-butyldeoxynojirimycin; SRT, substrate reduction therapy.

a more active mouse sialidase that cleaves GM1 to GA1 (Sango *et al*. 1995; Hahn *et al*. 1997; Matsuda *et al*. 1997).

Most therapies for the GSL lysosomal storage diseases focus on increasing lysosomal enzyme levels to compensate for the underlying enzyme deficiency. These approaches include enzyme replacement therapy, bone marrow transplantation, gene therapy, and stem cell therapy (Jeyakumar *et al*. 2002; Schiffmann and Brady 2002; Takaura *et al*. 2003). Substrate reduction therapy (SRT) is an alternative GSL lysosomal storage disease therapy that proposes to reduce GSL synthesis and accumulation to counterbalance impaired rates of catalysis (Liu *et al*. 1999; Tifft and Proia 2000; Platt *et al*. 2001; Butters *et al*. 2003a).

The imino sugar, *N*-butyldeoxygalactonojirimycin (*N*B-DGJ), is a competitive inhibitor of the ceramide-specific glucosyltransferase (GlcT), the enzyme that catalyzes the first step in GSL biosynthesis (Butters *et al*. 2003b; Fig. 1). GSLs are synthesized in the endoplasmic reticulum and Golgi apparatus and are degraded in the lysosomes, where catabolism occurs in a stepwise manner (Sandhoff and Conzelmann 1984; Suzuki *et al*. 1995). Glucosylceramide (GlcCer) is the precursor for GSL biosynthesis, including the gangliosides of the 'a' and 'b' metabolic pathways (Fig. 1). Because *N*B-DGJ inhibits the formation of GlcCer, *N*B-DGJ can be used to inhibit GSL biosynthesis through substrate reduction.

SRT using *N*-butyldeoxynojirimycin (*N*B-DNJ), the glucose analog of *N*B-DGJ, has been evaluated in mouse models for the treatment of the GM2 gangliosidoses, including Tay-Sachs and Sandhoff diseases (Platt *et al*. 1997; Jeyakumar *et al*. 1999, 2002). These studies showed that oral administration of *N*B-DNJ reduced GM2 accumulation in the brains of both Tay-Sachs and Sandhoff disease mice at adult ages. Furthermore, a combination of SRT and bone marrow transplantation synergistically extended life-expectancy in Sandhoff disease mice (Jeyakumar *et al*. 2001). *N*B-DNJ has been approved for clinical use in Europe, Israel, and the USA for the treatment of type 1 Gaucher disease, a GSL lysosomal storage disease characterized by non-neuronal GlcCer accumulation (Cox *et al*. 2000, 2003; Lachmann 2003; Moyses 2003).

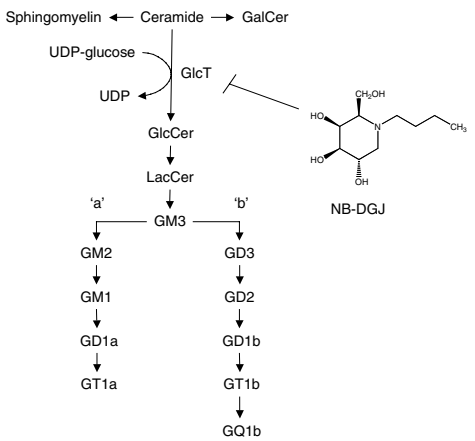

Fig. 1 Influence of *N*B-DGJ on GSL biosynthesis in neonatal mice. *N*B-DGJ inhibits GlcT, the enzyme that catalyzes the first step in GSL biosynthesis. GalCer, galactosylceramide; GlcT, ceramide-specific glucosyltransferase; GlcCer, glucosylceramide; LacCer, lactosylceramide; the 'a' and the 'b' ganglioside metabolic pathways.

Available treatments for the infantile-onset forms of the GSL lysosomal storage diseases have minimal efficacy. Previous studies showed that *N*B-DGJ treatment of normal mouse embryos inhibited ganglioside biosynthesis by approximately 90% without impairing viability, growth or morphogenesis (Brigande *et al*. 1998). Furthermore, *N*B-DGJ may be better suited than *N*B-DNJ for evaluating treatment in young mice as adverse side-effects, in particular weight loss associated with *N*B-DNJ treatment, does not occur with *N*B-DGJ treatment (Andersson *et al*. 2000).

In this study, we evaluated SRT using *N*B-DGJ at neonatal ages in normal B6 mice and in a mouse model of GM1 gangliosidosis. Our results show that *N*B-DGJ significantly reduces total brain ganglioside and GM1 content without producing noticeable adverse effects. We suggest that *N*B-DGJ may be effective in slowing progression of infantile-onset GM1 gangliosidosis.

Materials and methods

Mice

The C57BL/6J (B6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). B6/129Sv mice, heterozygous for the GM1 β-galactosidase gene (β-gal +/–) were obtained from Dr Alessandra d'Azzo (Saint Jude Children's Research Hospital, Nashville, TN, USA). These mice were derived by homologous recombination and embryonic stem cell technology as previously described (Hahn *et al*. 1997). Homozygous β-gal (–/–) mouse pups were derived from crossing β-gal (–/–) mice. As adult β-gal (–/–) female mice are poor mothers, the β-gal (–/–) pups were cross-fostered to DBA/2J females shortly after birth. Genotypes were determined by measuring β-gal-specific activity in tail tissue using a modification of the Galjaard procedure (Galjaard 1980; Hauser *et al*. 2004). All mice were propagated in the Boston College Animal Care Facility and were housed in plastic cages with filter tops containing Sani-Chip bedding (P.J. Murphy Forest Products Corp., Montville, NJ, USA). The room was maintained at 22°C on a 12-h light/dark cycle. Food (PROLAB R/M/H/3000 Laboratory Chow, Agway, St Louis, MO, USA) and water were provided *ad libitum*. Nursing females were provided with cotton nesting pads for the duration of the experiment. All animal experiments were carried out with ethical committee approval in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care Committee

*N*B-DGJ treatment

*N*B-DGJ was purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). *N*B-DGJ solutions were prepared in

sterile saline to yield a final concentration of either 0.13 mg or 0.27 mg/μL. Neonatal B6 and β-gal (–/–) mice were injected daily (i.p.) from post-natal day 2 (p-2) to p-5 with either vehicle (0.9% saline) or *N*B-DGJ at 600 mg or 1200 mg/kg body weight. Daily injections were performed using a Hamilton syringe (26 gauge, point style 2, 0.5 inch needle length) and volumes ranged from approximately 6–12 μL/mouse. Body weights were measured and brains were collected on dry ice approximately 4 h after the final injection on p-5 and were stored at − 80°C. In addition, cerebral cortex and cerebellum from B6 and β-gal (–/–) mice, treated from p-2 to p-5 with 600 mg/kg *N*B-DGJ, were collected at p-21.

Ganglioside isolation, purification, and quantification

Gangliosides were isolated and purified from lyophilized brain tissue using modifications of previously described procedures (Seyfried *et al.* 1978b; Hauser *et al*. 2004). Briefly, total lipids were extracted with 5 mL chloroform ($CHCl_3$) and methanol (CH_3OH) 1 : 1 by vol and 0.5 mL dH_2O. The solution was placed on a magnetic stirrer at room temperature for at least 8 h and then centrifuged for 10 min at 1200 *g*. The supernatant was removed and the pellet was washed with 2 mL $CHCl_3$: CH_3OH (1 : 1 by vol) and centrifuged as before. The supernatants were combined and the volume was adjusted to 7 mL with $CHCl_3$: CH_3OH (1 : 1 by vol). $CHCl_3$ (3.5 mL) and water (2.1 mL) was added and the mixture was inverted, vortexed, and centrifuged at 1200 *g* to partition gangliosides into the upper phase (Folch *et al*. 1957; Seyfried *et al.* 1978a). The upper aqueous phase was removed and the lower organic phase was washed once with 4.5 mL of the Folch 'pure solvent upper phase' ($CHCl_3$: CH_3OH : dH_2O, 3 : 48 : 47 by vol). The combined upper phases were then converted to a $CHCl_3$: CH_3OH : dH_2O ratio of 30 : 60 : 8 (solvent A) by adding 48 mL $CHCl_3$: CH_3OH (1 : 2 by vol). The lower phase was evaporated under a stream of nitrogen, re-suspended in 5 mL $CHCl_3$: CH_3OH (2 : 1 by vol), and stored at 4°C for neutral lipid isolation and purification.

Gangliosides were purified using DEAE-Sephadex (A-25, Pharmacia Biotech, Upsala, Sweden) column chromatography. The ganglioside fraction suspended in solvent A was applied to a DEAE-Sephadex column with a bed volume of 1.2 mL that had been equilibrated prior with solvent A. The column was washed with 20 mL solvent A. Gangliosides were then eluted from the column with 30 mL of solvent B ($CHCl_3$: CH_3OH : 0.8 M Na acetate, 30 : 60 : 8 by vol).

An aliquot of the ganglioside fraction was evaporated under a stream of nitrogen and analyzed for sialic acid content using a modified resorcinol method (Svennerholm 1957; Miettinen and Takki-Luukkainen 1959; Suzuki 1964). *N*-acetylneuraminic acid (Sigma, St Louis, MO, USA) was used as an external standard. Samples were dissolved in 1 mL of resorcinol reagent : dH_2O (1 : 1 by vol), boiled for 15 min, and then cooled in an ice bath. Butyl acetate : 1-butanol (1.5 mL of 85 : 15 by vol) was then added and the samples were vortexed and centrifuged at 1200 *g*. The violet supernatant was removed and analyzed at 580 nm in the Shimadzu UV-1601 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

Base treatment and desalting

After removing aliquots for the resorcinol assay, the ganglioside fraction was dried by rotary evaporation and treated with mild base (1 mL of 0.15 M NaOH) in a shaking water bath at 37°C for 1.5 h. Base and salts were separated from the gangliosides using a modification of a previously described method (Williams and McCluer 1980). The sample was applied to a C18 reverse-phase Bond Elute column (Varian, Harbor City, CA, USA) that was equilibrated with 5 mL each of $CHCl_3$: CH_3OH (1 : 1 by vol), CH_3OH, and 0.1 M NaCl. The column was washed with 25 mL of dH_2O to remove salts. Gangliosides were eluted from the column with 2 mL CH_3OH followed by 4 mL $CHCl_3$: CH_3OH (1 : 1 by vol). Samples were evaporated under a stream of nitrogen, re-suspended in 5 mL $CHCl_3$: CH_3OH (1 : 1 by vol), and stored at 4°C.

Neutral lipid isolation and purification

GA1 was isolated from the lower phase after the Folch partitioning procedure described above. A 2 mL aliquot of the lower phase was evaporated under a stream of nitrogen and treated with mild base (1 mL of 0.15 M NaOH) in a shaking water bath at 37°C for 1.5 h. The solution was converted to $CHCl_3$: CH_3OH : dH_2O (8 : 4 : 3 by vol) by addition of 4 mL $CHCl_3$: CH_3OH (2 : 1 by vol) and the Folch partitioning procedure was repeated to separate GA1 from salts and saponified phospholipids. The upper aqueous phase was discarded and the lower organic phase was washed once with 1.7 mL of the Folch 'pure solvent upper phase.' The upper phase was again discarded and the lower phase containing GA1 was evaporated under a stream of nitrogen, re-suspended in 5 mL $CHCl_3$: CH_3OH (2 : 1 by vol), and stored at 4°C.

Ceramide, sphingomyelin, and other non-polar neutral lipids were also isolated from the lower phase after the Folch partitioning procedure described above. The remaining 3 mL of the lower phase suspended in $CHCl_3$: CH_3OH (2 : 1 by vol) was converted to solvent A by addition of 1 mL $CHCl_3$, 5 mL CH_3OH, and 0.8 mL dH_2O. Neutral lipids were then purified using DEAE-Sephadex column chromatography as described previously. This neutral lipid fraction was dried by rotary evaporation, re-suspended in 5 mL $CHCl_3$: CH_3OH (2 : 1 by vol), and stored at 4°C under nitrogen.

High-performance thin-layer chromatography

Gangliosides, GA1, and non-polar neutral lipids were analyzed qualitatively by high-performance thin-layer chromatography (HPTLC) following modifications of previously described methods (Ando *et al*. 1978; Seyfried *et al*. 1978b, 1984). Lipids were spotted on 10 × 20 cm Silica gel 60 HPTLC plates (E. Merck, Darmstadt, Germany) using a Camag Linomat III auto-TLC spotter (Camag Scientific Inc., Wilmington, NC, USA). The amount of lipid per lane was equivalent to 1.5 μg total sialic acid for gangliosides and either 0.4 mg or 0.3 mg brain dry weight for GA1 or neutral lipids, respectively. Purified lipid standards were either purchased from Matreya, Inc. (Pleasant Gap, PA, USA) or were a gift from Dr Robert Yu (Medical College of Georgia, Augusta, GA, USA).

For gangliosides and GA1, the HPTLC plate was developed by a single ascending run with $CHCl_3$: CH_3OH : dH_2O (55 : 45 : 10, by vol for gangliosides and 65 : 35 : 8, by vol for GA1) containing 0.02% $CaCl_2$ $2H_2O$. Plates were sprayed with either resorcinol-HCl or orcinol-H_2SO_4 reagent and heated on an aluminum block heater at 95°C for approximately 30 min to visualize gangliosides or GA1, respectively (Svennerholm 1957). For neutral lipids, the plate was developed to a height of 4.5 cm with chloroform : methanol : acetic

acid : formic acid : water (35 : 15 : 6 : 2 : 1 by vol), then developed to the top with hexanes : diisopropyl ether : acetic acid (65 : 35 : 2 by vol). Neutral lipids were visualized by charring with 3% cupric acetate in 8% phosphoric acid solution, followed by heating the plate at 140°C as previously described (Seyfried *et al*. 1984).

The percentage distribution of the individual bands was determined by scanning the plate on a Personal Densitometer SI with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA) for gangliosides and neutral lipids or on a ScanMaker 4800 with ScanWizard5 V7.00 software (Microtek, Carson, CA, USA) for GA1. The total brain ganglioside distribution was normalized to 100% and the percentage distribution values were used to calculate sialic acid concentration of individual gangliosides as we previously described (Seyfried *et al*. 1982). The density values for GA1, ceramide, and sphingomyelin were fit to a standard curve of the respective lipid and used to calculate individual concentrations. All brain lipid concentrations are expressed as μg/100 mg dry weight.

Ceramide analysis

Ceramide was further characterized following preparative HPTLC where 5.4 mg tissue dry weight (corresponding to approximately 16 μg ceramide) was developed according to methods described above. Ceramide was eluted from the Silica gel by sonication in 25 mL $CHCl_3$: CH_3OH (2 : 1 by vol) for 30 min followed by centrifugation at 1200 *g* for 10 min. The supernatant was collected and the pellet was washed with 10 mL $CHCl_3$: CH_3OH (2 : 1 by vol) and centrifuged. The pooled supernatants were filtered through a Buchner funnel using medium-porosity quantitative filter paper (Fisher Scientific, Pittsburgh, PA, USA) to remove residual Silica gel. The eluant was dried under a stream of nitrogen, re-suspended in 2 mL $CHCl_3$: CH_3OH (2 : 1 by vol), and stored at 4°C for gas-liquid chromatography (GLC).

Following methanolysis (Gaver and Sweeley 1965), the purified ceramide was fractionated into fatty acid and long-chain base constituents according to previous methods (Carter and Gaver 1967; Ando and Yu 1979). The fractionated ceramide was analyzed by GLC (6890 Series, Agilent Technologies, Colorado Springs, CO, USA) using a capillary column (HP-1, 10 m length, 0.53 mm internal diameter, 2.65 μm film, Agilent Technologies) and an OV-1 column (3% OV-1 on 100/120 ChromosorbWHP, 6 feet × 1/4 inch glass, 4 mm internal diameter, Supelco, Bellefonte, PA, USA) for fatty acid and long chain base analysis, respectively.

DNA extraction and fragmentation analysis

Genomic DNA was isolated from cerebral cortex of neonatal B6 and β-gal (–/–) mice using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). DNA was electrophoresed and examined for fragmentation according to previously described methods (Huang *et al*. 1997). In brief, 2 μg of genomic DNA was electrophoresed on a 1.8% agarose gel containing ethidium bromide (0.04 μg/mL) at 50 V. The DNA bands were visualized under UV light and compared to a 1 kb ladder (Gibco-BRL, Division of Life Technologies Inc., Gaithersburg, MD, USA).

Results

Our objective was to determine if the imino sugar, *N*B-DGJ, could reduce brain ganglioside and GM1 content in normal B6 mice and in B6/129Sv β-gal (–/–) mice at neonatal ages. Our results show that four daily *N*B-DGJ injections of either 600 mg or 1200 mg/kg/day from p-2 to p-5 caused significant reductions in whole brain ganglioside content in these mice (Table 1). These drug concentrations reduced brain ganglioside content by 25% and 28% in the B6 mice, and by 19% and 32% in the β-gal (–/–) mice. In fact, the brain ganglioside concentrations in the drug-treated β-gal (–/–) mice (332 μg and 280 μg/100 mg dry weight, respectively) were lower than those in the untreated control B6 mice (354 μg/100 mg dry weight). The drug treatments did not reduce viability and had no significant effect on body weight, brain weight, or brain water content in the neonatal B6 and β-gal (–/–) mice (Table 1).

The influence of *N*B-DGJ on the qualitative and quantitative distribution of individual gangliosides in the B6 and the β-gal (–/–) mice is shown in Fig. 2 and Table 2. Certain gangliosides that were absent or comprised less than 1% of the total distribution (GM4, GM3, and GM2) were not included in the computations. A fourfold elevation in GM1

Table 1 Brain ganglioslide content in neonatal B6 and β-gal mice treated with NB-DGJ[a]

Strain	NB-DGJ (mg/kg)	N[b]	Body Weight (g)	Brain Weight (mg)	Water Content (%)	Ganglioside Sialic Acid (μg/whole brain)	Ganglioside Sialic Acid (μg/100 mg dry wt)
B6	0	13	2.3 ± 0.01	165.8 ± 3.7	87.07 ± 0.03	76 ± 1	354 ± 3
	600	7	2.3 ± 0.01	165.4 ± 6.4	87.04 ± 0.10	57 ± 2*	267 ± 6*
	1200	6	2.2 ± 0.01	154.6 ± 3.3	87.07 ± 0.06	51 ± 2*	253 ± 8*
β-gal (-/-)	0	6	2.3 ± 0.01	165.6 ± 7.1	86.93 ± 0.05	89 ± 3	412 ± 7
	600	4	2.2 ± 0.02	151.7 ± 8.1	86.97 ± 0.07	66 ± 4*	332 ± 5*
	1200	4	2.3 ± 0.01	159.1 ± 0.7	86.76 ± 0.05	60 ± 4*	280 ± 18*

[a]Values represent the mean ± SEM.

[b]N, the number of independent mice analyzed.

*Indicates that the value is significantly different from that of the control mice at $p < 0.01$ as determined from the two-tailed *t*-test.

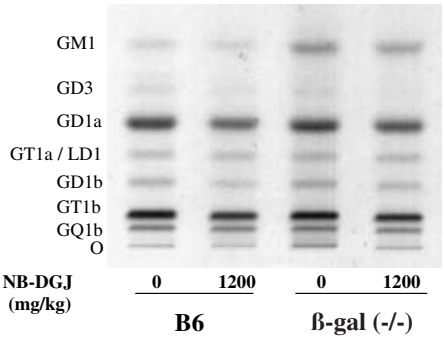

Fig. 2 HPTLC of brain gangliosides in neonatal (p-5) B6 and β-gal (–/–) mice treated with *N*B-DGJ from p-2 to p-5. The amount of ganglioside sialic acid spotted per lane was equivalent to approximately 0.75 mg brain dry weight. The plate was developed by a single ascending run with $CHCl_3$: CH_3OH : dH_2O (55 : 45 : 10, by vol) containing 0.02% $CaCl_2$ $2H_2O$. The bands were visualized with the resorcinol-HCl spray.

content was the most noticeable difference in ganglioside distribution between the untreated B6 and β-gal (–/–) mice, indicating that GM1 ganglioside accumulation occurs during early neonatal ages in these β-gal (–/–) mice. No major changes in brain ganglioside distribution were found in either the B6 or the β-gal (–/–) mice following treatment with *N*B-DGJ (Fig. 2).

*N*B-DGJ treatment with either 600 mg or 1200 mg/kg generally reduced the concentration of GM1 and most other gangliosides in the B6 and the β-gal (–/–) mice Table 2. These reductions were seen for gangliosides in both the 'a' (GM1 and GD1a) and 'b' (GD3, GD1b, GT1b, GQ1b) metabolic pathways. At the 1200 mg/kg dosage, *N*B-DGJ reduced GM1 concentration in the B6 mice by about 4.9 μg/100 mg dry wt, corresponding to a 34% reduction (Table 2). This same dosage reduced GM1 concentration in the β-gal (–/–) mice by about 21.5 μg/100 mg dry wt, corresponding to a 37% reduction. The inhibitory effects of *N*B-DGJ were

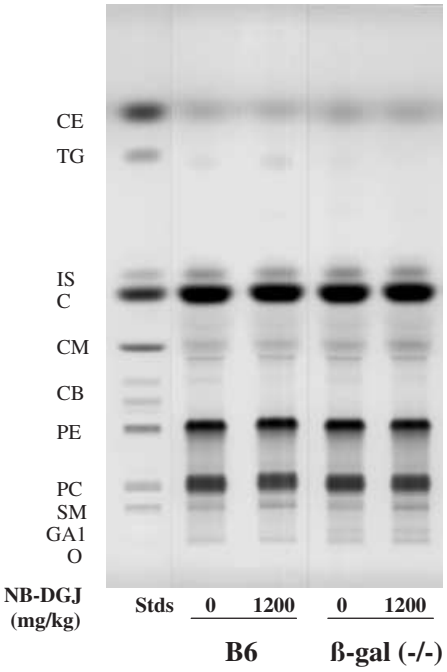

Fig. 3 HPTLC of brain neutral lipids in neonatal (p-5) B6 and β-gal (–/–) mice treated with *N*B-DGJ from p-2 to p-5. The amount of neutral lipids spotted per lane was equivalent to approximately 0.3 mg brain dry weight. The plate was developed to a height of 4.5 cm with chloroform : methanol : acetic acid : formic acid : water (35 : 15 : 6 : 2 : 1 by vol), then developed to the top with hexanes : diisopropyl ether : acetic acid (65 : 35 : 2 by vol). The bands were visualized by charring with 3% cupric acetate in 8% phosphoric acid solution. CE, cholesterol esters; TG, triglycerides; IS, internal standard; C, cholesterol; CM, ceramide; CB, cerebrosides (doublet); PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; GA1, asialo-GM1, O, origin.

generally less for gangliosides GT1a/LD1 than for other ganglioside species in both mouse groups and for GQ1b in the B6 mice. These findings indicate that *N*B-DGJ reduces

Table 2 Brain ganglioslide distribution in neonatal B6 and β-gal (-/-) mice treated with NB-DGJ[a]

Strain	NB-DGJ (mg/kg)	N[b]	Concentration (μg sialic acid/100 mg dry wt)[c]						
			GM1	GD3	GD1a	GT1a/LD1	GD1b	GT1b	GQ1b
B6	0	4	14.2 ± 0.7	9.3 ± 0.4	119.0 ± 3.4	19.4 ± 1.0	31.8 ± 1.0	126.7 ± 2.0	35.8 ± 2.5
	600	3	11.1 ± 0.9	7.2 ± 0.4	91.3 ± 5.1*	17.8 ± 1.0	19.8 ± 1.4*	96.1 ± 5.5*	29.3 ± 1.9
	1200	4	9.3 ± 1.0*	4.1 ± 0.5*	71.6 ± 1.6*	14.8 ± 0.3*	17.6 ± 0.4*	97.4 ± 2.5*	35.5 ± 1.4
β-gal (-/-)	0	6	58.8 ± 1.4	7.6 ± 1.7	133.9 ± 1.5	18.9 ± 1.4	28.0 ± 2.2	127.5 ± 6.0	37.7 ± 1.1
	600	4	36.6 ± 1.4*	4.8 ± 0.6	113.5 ± 3.0*	16.3 ± 0.9	19.7 ± 1.1	108.9 ± 1.6	32.7 ± 0.6*
	1200	3	37.3 ± 2.5*	2.6 ± 0.2	100.0 ± 2.6*	14.2 ± 0.4	14.4 ± 0.8*	75.0 ± 2.9*	19.7 ± 0.5*

[a]Values represent the mean ± SEM.

[b]N, the number of independent whole brain samples analyzed.

[c]Determined from densitometric scanning of HPTLC as shown in Fig. 2.

*Indicates that the value is significantly different from that of the control mice at $p < 0.01$ as determined from the two-tailed *t*-test.

Table 3 Brain neutral lipids in neonatal B6 and β-gal (-/-) mice treated with NB-DGJ[a]

Strain	NB-DGJ (mg/kg)	ceramide[b] (μg/100 mg dry wt)	sphingomyelin[b] (μg/100 mg dry wt)	GA1[c] (μg/100 mg dry wt)
B6	0	263 ± 7	546 ± 24	–
	1200	279 ± 13	728 ± 66	–
β-gal (-/-)	0	305 ± 6	558 ± 28	186 ± 1
	1200	445 ± 36*	765 ± 56*	207 ± 7*

[a]Values represent the mean ± SEM of 3–4 independent whole brain samples.
[b]Determined from densitometric scanning of HPTLC as shown in Fig. 3.
[c]Determined from HPTLC analysis as described in Materials and Methods.
*Indicates that the value is significantly different from that of the control mice at $p < 0.05$ as determined from the two-tailed *t*-test.

the concentration of GM1 and other neonatal brain gangliosides without producing major changes in ganglioside distribution.

The influence of *N*B-DGJ on the qualitative and quantitative distribution of non-polar neutral brain lipids in the B6 and the β-gal (–/–) mice is shown in Fig. 3 and Table 3. The objective of these studies was to determine if *N*B-DGJ treatment altered the distribution of upstream ganglioside metabolites, i.e. ceramide, sphingomyelin, and galactocerebroside (Fig. 1). Galactocerebroside was undetectable in either the control or *N*B-DGJ-treated B6 or β-gal (–/–) mice (Fig. 3). This finding is consistent with the absence of mature myelin at this age (Seyfried an Yu 1980). While ceramide and sphingomyelin content was elevated in the *N*B-DGJ-treated B6 and the β-gal (–/–) mice, these elevations were statistically significant only in the β-gal (–/–) mice. As ceramide elevation has been implicated in apoptosis (Wiesner and Dawson 1996), we evaluated apoptosis in the brains of the control and *N*B-DGJ-treated mice using DNA fragmentation analysis. No DNA fragmentation was detected in either the B6 or the β-gal (–/–) mice treated with *N*B-DGJ at 1200 mg/kg (data not shown). GA1 was undetectable in the brains of either the control or the *N*B-DGJ-treated B6 mice (Fig. 3 and Table 3). In contrast, noticeable amounts of GA1 were found in the control β-gal (–/–) mice and *N*B-DGJ treatment increased these levels by about 11% (Table 3).

To determine if *N*B-DGJ treatment at neonatal ages had noticeable effects at juvenile ages (p-21), we analyzed brain ganglioside content in juvenile B6 and β-gal (–/–) mice that received either saline or *N*B-DGJ *(*600 mg/kg) daily from p-2 to p-5. We chose this drug dosage because at p-5 it produced total brain ganglioside reductions similar to that of the higher dosage in the B6 mice (Table 1). No significant differences were found at p-21 between the control and *N*B-DGJ-treated mice for cerebral cortex or cerebellar ganglioside content (Table 4). Also, no differences were found between the mice for body weight, brain weight, or brain water content. Righting reflex at p-14 (occurring within 1 s) and eye opening at p-14 to p-15 were also similar for all control and *N*B-DGJ-treated mice. These findings indicate that *N*B-DGJ treatment (600 mg/kg/day) at neonatal ages had no noticeable effects on brain development or ganglioside content at juvenile ages. We do not exclude the possibility that effects could occur using higher drug dosages.

Discussion

The gene and enzymatic defects responsible for the GM1 gangliosidoses have been known for more than 30 years, but no effective therapies are available (Suzuki *et al*. 1995). The failure to access glycolipid storage products in CNS cells at critical developmental periods has hindered therapeutic progress. As ganglioside accumulation occurs during embryonic and early neonatal periods in the infantile- and juvenile-onset forms of the GM1 gangliosidoses, early intervention may be necessary for the effective management of these diseases (Takaura *et al*. 2003). Recent studies suggest that SRT with oral administration of the imino sugar, *N*B-DNJ, is effective in reducing brain ganglioside storage in adult mouse models of Tay-Sachs disease and Sandhoff disease (Platt *et al*. 1997, 2003; Jeyakumar *et al*. 1999). *N*B-DNJ, however, induces body and organ weight loss that would be

Table 4 Brain ganglioslide content in juvenile B6 and β-gal (-/-) mice treated with NB-DGJ at neonatal ages[a]

Strain	NB-DGJ (mg/kg)	Body Weight (g)	Brain Weight (mg)		Water Content (%)		Ganglioside Sialic Acid (μg/100 mg dry wt)	
			Cerebral cortex	Cerebellum	Cerebral Cortex	Cerebellum	Cerebral Cortex	Cerebellum
B6	0	9.0 ± 0.3	280.6 ± 0.6	50.4 ± 0.07	80.60 ± 0.01	79.10 ± 0.02	571 ± 5	360 ± 3
	600	9.0 ± 0.1	282.6 ± 2.1	51.6 ± 0.08	80.67 ± 0.07	79.07 ± 0.01	556 ± 1	352 ± 10
β-gal (-/-)	0	6.3, 8.5	283.3, 291.8	47.5, 47.6	80.66, 80.23	79.58, 79.20	752, 720	488, 493
	600	7.1, 8.2	277.2, 270.9	47.5, 51.6	80.48, 80.88	78.74, 79.07	768, 753	473, 514

[a]Values represent the mean ± SEM of 3 independent samples for the B6 mice and the values for the β-gal (-/-) mice are from two independent samples.

more of a concern in younger than older mice. *N*B-DGJ, the galactose analogue of *N*B-DNJ, may be better suited for SRT in younger mice, since prior studies show that adverse effects are absent or minimal with this compound (Brigande *et al*. 1998; Andersson *et al*. 2000).

We found that *N*B-DGJ significantly reduced total brain and GM1 ganglioside content when administered from p-2 to p-5 in B6 and β-gal (–/–) mice. These neonatal ages in mice would be comparable to pre-natal ages in humans as they precede the period of active myelinogenesis and cerebellar development (Verbitskaya 1969; Seyfried and Yu 1980; Seyfried *et al.* 1983; Morell and Quarles 1999). Although we found that brain GM1 content was significantly greater in the β-gal (–/–) mice than in the B6 mice, previous studies showed that clear histopathological changes do not appear in β-gal (–/–) mice until about p-14 (Itoh *et al*. 2001). Our studies at p-5 precede the onset of observable histopathological storage in the brains of these mice. Hence, further studies will be necessary in older β-gal (–/–) mice to determine if *N*B-DGJ can reduce the histopathology of GM1 storage.

No observable adverse effects on brain or body weights were found in the neonatal mice following *N*B-DGJ treatment. Although brain ceramide levels were elevated in the treated B6 and β-gal (–/–) mice, no evidence was found for apoptosis. These observations concur with previous findings in normal embryonic mice treated with *N*B-DGJ (Brigande *et al*. 1998). Also, no residual effects on brain development or ganglioside distribution were found in juvenile B6 mice and β-gal (–/–) mice that were treated transiently with *N*B-DGJ at neonatal ages. Thus, *N*B-DGJ-induced reduction of brain ganglioside content at neonatal ages does not persist until juvenile ages.

Brain GA1 content is generally greater in the β-gal (–/–) mouse models than in the human disease possibly due to a more active mouse sialidase (Sango *et al*. 1995; Hahn *et al*. 1997; Matsuda *et al*. 1997). We found that brain GA1 content was greater in the *N*B-DGJ-treated β-gal (–/–) mice than in the untreated control mice. These findings suggest that *N*B-DGJ may enhance mouse sialidase activity. Recent studies showed that *N*B-DGJ could enhance the activity of mutant forms of human β-gal through chaperone effects (Tominaga *et al*. 2001; Matsuda *et al*. 2003). Further studies will be needed to evaluate the influence of *N*B-DGJ on normal and mutant forms of lysosomal enzyme activities under *in vivo* conditions.

Ganglioside biosynthesis in neonatal brain is thought to occur through a multiglycosyltransferase system (Roseman 1970; Caputto *et al*. 1974; Giraudo and Maccioni 2003). According to this hypothesis, each ganglioside species is synthesized on its own microsomal assembly line under a specific complex of glycosyltransferases. Metabolic intermediates neither leave the synthetic site nor enter a common pool of identical intermediates (Caputto *et al*. 1974; Giraudo *et al*. 2001). Our results in the neonatal B6 and β-gal (–/–) mice support this hypothesis as *N*B-DGJ reduced to a similar degree the concentration of several major gangliosides in both the 'a' and 'b' metabolic pathways. The reduction of GM1 content in the *N*B-DGJ-treated β-gal (–/–) mice may therefore result from the inhibition of GM1 synthesis itself together with the inhibited synthesis of other more complex gangliosides that would be catabolized to GM1. This might explain the 4.9 μg and 21.5 μg/100 mg dry wt reductions in brain GM1 content between the *N*B-DGJ-treated B6 and β-gal (–/–) mice, respectively. These findings suggest that the effects of *N*B-DGJ as a GM1 gangliosidosis therapy would be greater at younger ages when ganglioside synthesis and turnover rates are fast than at older ages when these rates are much slower.

Our previous results in embryonic mice (E-11) and in mouse brain tumors showed that *N*B-DGJ and *N*B-DNJ respectively, reduced ganglioside content through reduction of ganglioside precursors, i.e. GM3 and GD3, according to the substrate availability hypothesis of ganglioside biosynthesis (Nakamura *et al*. 1990; Chou *et al*. 1991; Brigande *et al*. 1998; Ranes *et al*. 2001). Although *N*B-DGJ reduced GD3 in the neonatal mice, GD3 was a minor ganglioside and the reduction was not markedly different from that of other more complex gangliosides. In contrast to the p-5 neonatal mice where most brain cells are differentiating, most cells in embryonic mice and in brain tumors are proliferating. Recent studies suggest that GSL biosynthesis is influenced by physiological state (Giraudo and Maccioni 2003). Hence, the stage of brain differentiation and degree of brain cell proliferation may influence the therapeutic efficacy of *N*B-DGJ for GM1 gangliosidosis.

In summary, our results show that *N*B-DGJ is effective in reducing total brain ganglioside content in normal B6 mice and in β-gal (–/–) mice at early neonatal ages. Furthermore, this reduction was observed for GM1 and several other gangliosides and was not associated with observable adverse effects. These findings suggest that *N*B-DGJ may be an effective early intervention therapy for GM1 gangliosidosis and possibly other GSL lysosomal storage diseases.

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