Biochemical Pharmacology, Vol. 59, pp. 821–829, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00384-6

N-Butyldeoxygalactonojirimycin: A More Selective Inhibitor of Glycosphingolipid Biosynthesis than *N*-Butyldeoxynojirimycin, *In Vitro* and *In Vivo*

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ABSTRACT. *N*-Butyldeoxynojirimycin (*N*B-DNJ) inhibits the ceramide glucosyltransferase which catalyses the first step in glycosphingolipid (GSL) biosynthesis. It has the potential to be used for the treatment of the GSL lysosomal storage diseases and is currently in clinical trials for the treatment of type 1 Gaucher's disease. However, *N*B-DNJ is also a potent inhibitor of other enzymes, including α-glucosidase I and II, which could potentially cause side effects in patients receiving life-long therapy. We therefore evaluated a potentially more selective GSL biosynthesis inhibitor, *N*-butyldeoxygalactonojirimycin (*N*B-DGJ), *in vitro* and *in vivo*. The distribution and degree of GSL depletion in the liver of mice treated with *N*B-DGJ or *N*B-DNJ were equivalent. Mice treated with *N*B-DGJ had normal body weights and lymphoid organ sizes, whereas *N*B-DNJ-treated mice showed weight loss and partial lymphoid organ shrinkage. *N*B-DNJ inhibited glycogen catabolism in the liver, whereas *N*B-DGJ did not. *N*B-DNJ was also a potent inhibitor of sucrase and maltase *in vitro* but not of lactase, while *N*B-DGJ inhibited lactase but not sucrase or maltase. *N*B-DGJ is therefore more selective than *N*B-DNJ, and deserves to be evaluated for human therapy. BIOCHEM PHARMACOL **59**;7:821–829, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. imino sugars; *N*-butyldeoxynojirimycin; *N*-butyldeoxygalactonojirimycin; glycosphingolipid; inhibitors; glycosphingolipid lysosomal storage diseases

The GSL† lysosomal storage diseases are a group of severe disorders for which the therapeutic options are currently limited [1, 2]. The diseases result from the inheritance of defects in the genes encoding the enzymes required for catabolism of GSLs within lysosomes [1]. Pathology is caused by the storage of incompletely degraded GSLs in the lysosomes of affected cells. Potential therapeutic strategies include enzyme replacement, gene therapy, allogeneic bone marrow transplantation (BMT), and substrate deprivation [2, 3]. Gene therapy is still experimental [4] and the results of BMT have been mixed [5, 6]. To date, only type 1 Gaucher's disease, which is characterised by glucocerebrosidase deficiency in the absence of neuropathology, has been treated successfully with enzyme replacement therapy [7–9]. However, skeletal abnormalities associated with the disease respond slowly to treatment [10], and the neuoronopathic forms of the disease (types 2 and 3) are refractory to therapy. The cost of this treatment is prohibitively high, precluding many patients from access to therapy [11].

Substrate deprivation is an approach which could offer a general strategy for the treatment of the GSL storage disorders, irrespective of the specific genetic defect involved [2, 3]. A partial reduction in GSL biosynthesis would allow the residual enzyme activity present in the juvenile and adult onset variants to fully catabolize the GSLs entering the lysosome. Inhibition of the first step in GSL biosynthesis, where glucose is transferred to ceramide forming glucosylceramide (GlcCer), could potentially offer a therapeutic approach for treating all disorders involving the storage of GlcCer-derived GSLs (Gaucher's disease types 1, 2 and 3, Fabry, Tay-Sachs, Sandhoff, and G_{M1} gangliosidosis) [2]. The addition of glucose to ceramide is catalysed by a ceramide-specific glucosyltransferase, and several inhibitors of this enzyme have been identified [2, 12], including the *N*-alkylated imino sugar *N*B-DNJ [2, 13]. It has been shown both *in vitro* and *in vivo* (in mice) that treatment with *N*B-DNJ is well tolerated and significantly reduces GSL biosynthesis [13, 14]. In an asymptomatic mouse model of Tay-Sachs disease, *N*B-DNJ treatment reduced pathological storage in the brain [15], while in a neurodegenerative mouse model of Sandhoff's disease, life expectancy was significantly extended [16]. Currently, *N*B-DNJ is being evaluated in type I Gaucher patients in Europe and Israel. It would be predicted that for peripheral GSL storage diseases (type I Gaucher and Fabry's disease), only low-level dosing with *N*B-DNJ will be required. This

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† *Abbreviations:* *N*B-DNJ, *N*-butyldeoxynojirimycin; *N*B-DGJ, *N*-butyldeoxygalactonojirimycin; GSL, glycosphingolipid; GM2, monoganglioside GalNAcβ4(NeuAcα3)Galβ4Glcβ3Ceramide; ER, endoplasmic reticulum; and GI, gastrointestinal.

Received 22 May 1999; accepted 15 September 1999.

is because the storage cells are readily accessible to the drug (macrophages and endothelial cells, respectively). However, in order to achieve therapeutic levels of *N*B-DNJ in the CNS for treatment of neuropathological forms of storage disorders, high systemic doses will have to be administered, as only approximately 10% of the serum level of *N*B-DNJ is present in the cerebrospinal fluid [15]. For life-long therapy for the diseases with CNS pathology, a more selective inhibitor would therefore be preferable. In addition to its inhibitory activity against the glucosyltransferase, *N*B-DNJ is also a potent inhibitor of the ER lumen *N*-glycan-processing enzymes α-glucosidase I and II, β-glucocerebrosidase, and possibly other glucosidases [17]. This could potentially lead to unwanted side effects. For example, *N*B-DNJ at high concentrations causes diarrhoea in humans [18], which is thought to be the result of inhibition of disaccharidases in the intestine. In previous reports, it has also been observed that *N*B-DNJ treatment decreases body weight and reduces the size of lymphoid organs in mice, although the mechanisms underlying these changes are unclear [14].

A potentially more selective glucosyltransferase inhibitor is the galactose analogue *N*B-DGJ. This compound has been shown to inhibit GSL biosynthesis *in vitro* as effectively as *N*B-DNJ, but does not inhibit α-glucosidase I and II or β-glucocerebrosidase [19]. In the present study, *N*B-DGJ was evaluated in healthy mice. The distribution of *N*B-DGJ *in vivo* was found to be equivalent or superior to that of *N*B-DNJ and both inhibited GSL biosynthesis, but most significantly it was shown that *N*B-DGJ treatment does not cause the side effects associated with *N*B-DNJ. The biochemical basis for this increased selectivity of *N*B-DGJ was evaluated.

MATERIALS AND METHODS

Compounds

*N*B-DNJ was a gift from the Monsanto/Searle Company and Oxford GlycoSciences, [^{14}C] *N*B-DNJ was a gift from Monsanto/Searle, and *N*B-DGJ was purchased from Toronto Research Biochemicals. *N*-3-methyl-butyl-DNJ was synthesised according to previously published methods [13, 19].

Animals

Female C57BL/6 mice were housed under standard non-sterile conditions. The mice were provided with water *ad lib*. and prior to drug administration were fed pelleted chow (expanded Rat and Mouse Chow 1, SDS Ltd.). All experiments were performed on age-matched animals.

Treatment of Mice with NB-DNJ and NB-DGJ

The mice (6 weeks old) were fed a diet of powdered chow (expanded Rat and Mouse Chow 3, ground, SDS Ltd.) or diet containing *N*B-DNJ or *N*B-DGJ. The diet and compound (both as dry solids) were mixed thoroughly, stored at room temperature, and used within 7 days of mixing. The mice were maintained on *N*B-DNJ or *N*B-DGJ at doses of 300–4800 mg/kg/day for 10 days, or 2400 mg/kg/day for 5 weeks.

Radiolabelling of NB-DGJ

A galactose oxidase/Na[^{3}H]$_4$B method was used to radiolabel the C6 carbon of *N*B-DGJ. A solution of *N*B-DGJ (1.3 mg), galactose oxidase (80 units), and catalase (37,000 units) in 200 μL 10 mM sodium phosphate buffer was incubated for 24 hr at room temperature whilst stirring. The reaction was stopped by heating the solution to 95° for 5 min. After centrifuging (10 min, 14,000 *g*), 1M NaOH was added to the supernatant until pH 10–12 was achieved. Na[^{3}H]$_4$B (4.3 mCi) was added and the solution incubated for 2 hr at 30°, after which $NaBD_4$ (1 mg) was added and the solution incubated for 1 hr at 30°. The solution was neutralised with 1 M acetic acid, and then dried down. After removing borate by washing with acidified methanol (0.6% glacial acetic acid in methanol) 5–10 times, the [^{3}H]*N*B-DGJ mixture was resuspended in water, added to an AG50 column (equilibrated with water), and eluted with 1–4 M NH_3. [^{3}H]*N*B-DGJ was further purified on HPLC (Dionex CS10 hpcec chromatography, isocratic elution with 50 mM Na_2SO_4, 2.5 mM H_2SO_4, and 5% acetonitrile, pulsed amperometric detector), and the AG50 column step was repeated.

Short-term Distribution of [^{14}C]NB-DNJ and [^{3}H]NB-DGJ in Mice

Mice were orally gavaged with 100 μL water containing 25 μg (10^6 cpm) [^{14}C]*N*B-DNJ or [^{3}H]*N*B-DGJ and 1 mg non-radiolabelled *N*B-DNJ or *N*B-DGJ, respectively. Urine and faeces were collected over 90 min. After 90 min, the mice were killed and the serum, organs, and any additional urine and faeces collected. Organs were homogenised (Ultra-Turrax, speed five, 1 min) in a 4-fold volume of water, and faeces in a 10-fold volume. Aliquots of 500 μL homogenate, 100 μL urine, or 50 μL serum were mixed with 4 mL scintillation fluid and [^{14}C] or [^{3}H] counts measured. The quenching by the different tissues of both isotopes was determined by measuring the counts of known amounts of radiolabelled compound added to tissue homogenates, and the results were corrected accordingly.

GSL Analysis of Mouse Liver

Liver samples were homogenised (Ultra-Turrax, speed five, 1 min) in water and lyophilized. Dried homogenates were extracted twice in chloroform:methanol (2:1, v/v), first overnight at 4° and then for 3 hr at room temperature, pooled, and dried under nitrogen. The extracts were resuspended in 500 μL chloroform:methanol (1:1, v/v), base-treated by adding 83 μL of 0.35 M NaOH in methanol and

digested for 90 min at room temperature, and partitioned by adding 83 μL water:methanol (9:1, v/v), 166.5 μL water, and 416 μL chloroform. The upper phase containing the gangliosides was separated from the lower phase after mixing and low-speed centrifugation, and the lower phase was washed twice with Folch (chloroform:methanol:0.74% KCl, 3:48:47, v/v). Upper phases were combined, dried down to half-volume under nitrogen, dialysed against water, lyophilized, and resuspended in chloroform:methanol (2:1, v/v). An equivalent of 5 mg dry weight of tissue was separated by TLC (chloroform:methanol:0.22% $CaCl_2$, 60:35:8, v/v). The TLC plate was air-dried, sprayed with orcinol:sulphuric acid (0.2% (w/v):2N), and heat-treated (90° for 10 min). The intensity of bands was quantified by scanning densitometry (NIH Image 1·49 Software).

Determination of NB-DNJ *and* NB-DGJ *Concentrations in Serum and Liver*

Serum and supernatant of liver homogenate (130 mg/mL in 10% methanol) were centrifuged three times through a Millipore Ultrafree filter, after an internal standard (*N*-3-methylbutyl-DNJ) had been added to the samples. The pooled filtrates were purified on an HCl-preconditioned SCX column, eluted with 1% NH_3 in MeOH, dried down, resuspended in water, further purified on a C18 column (MeOH preconditioning, H_2O wash, and MeOH elution), and finally quantified by HPLC (Dionex CS10 hpcec chromatography, isocratic elution with 50 mM Na_2SO_4, 2.5 mM H_2SO_4, and 5% acetonitrile, pulsed amperometric detector).

Purification of Disaccharidases and Measurement of Sucrase, Maltase, and Lactase Activity

The enzymes sucrase–isomaltase (EC 3.2.1.10/48) and lactase–phlorizin hydrolase (EC 3.2.1.62/108) were purified from porcine intestine at 4° as follows. The intestine (100 g) was cut into small pieces, washed by stirring in 250 mL of 150 mM NaCl/ 10 mM KCl for 30 min, and extracted twice with 125 mL of 2M urea, 50 mM EDTA, and 50 mM KCl at pH 7. The urea extracts were combined and homogenised (Waring blender), the homogenate was centrifuged at 60,000 *g* for 75 min, and the pellet was resuspended in 50 mL of a solution containing 10 mM EDTA and 10 mM l-cysteine HCl in 50 mM potassium phosphate buffer at pH 7.5 (pre-equilibrated to 37°). After addition of papain (15 units/mL), the mixture was incubated for 30 min at 37°, and centrifuged at 105,000 *g* for 60 min. The supernatant was removed and precipitated in 75 mL of ethanol at −20° for 1 hr. The precipitate was recovered by centrifugation at 5000 *g* for 10 min, dissolved in 5–10 mL of 10 mM potassium phosphate buffer at pH 7.5, and the solution was centrifuged at 30,000 *g* for 60 min. The supernatant was removed and stored at 4° in the presence of 0.02% sodium azide. Sucrase, maltase, and lactase activity were determined in the enzyme preparation (diluted to a suitable concentration) by incubating 50 μL enzyme, 125 μL sodium citrate buffer (60 mM, pH 6), and 125 μL disaccharide substrate at 37° for 30 min, heating to 100° for 3 min to inactivate the enzyme, centrifuging the mixture at 13,000 *g* for 10 min, and determining the glucose concentration by adding 50 μL of the supernatant to 1 mL Trinder reagent (Sigma) and reading the absorbance at 505 nm after 18 min.

Determination of Glycogen Concentration in Liver

Glycogen concentrations in mouse livers were determined as described [20]. Liver was homogenised (Ultra-Turrax, speed five, 1 min) in extraction buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, and 1 mM EDTA at pH 8.2), and 100 μL homogenate was incubated with 100 μL 2M KOH for 20 min at 70°. The solution was centrifuged (10 min, 14,000 *g*) and 40 μL of the supernatant was added to 200 μL of 0.75% amyloglucosidase (w/v) and 2.5% glacial acetic acid (v/v) in 0.3 M acetate buffer (pH 4.8). After incubation at room temperature overnight, the glucose released into the solution was quantitated spectrophotometrically by measuring the NADPH production in the hexokinase / glucose-6-phosphate dehydrogenase (HK/G-6-PDH) reaction. A mixture of 50 μL of the solution and 1 mL of glucose reagent (1.7 units/mL HK, 0.85 units/mL G-6-PDH, 3 mM ATP, 0.56 mM $NADP^+$, and 3 mM $MgSO_4$ in 0.3 M triethanolamine buffer, pH 7.5) was incubated for 40 min at room temperature, centrifuged (2 min, 14,000 *g*), and the absorbance at 340 nm measured.

Statistical Analysis

Conventional statistical methods were employed to calculate mean values and standard errors of the mean (SEM). Differences between groups of mice were tested for significance using Student's *t*-test for unpaired observations. Results in the text and tables are presented as means ± SEM.

RESULTS

Short-term Distribution of [^{3}H]NB-DGJ and [^{14}C]NB-DNJ in Mice

The short-term distribution of *N*B-DGJ and *N*B-DNJ in mice was determined by giving the compounds to mice by oral gavage. The radioactive counts in organs, serum, faeces, and urine were measured after 90 min. The concentration of *N*B-DNJ was 28% higher than that of *N*B-DGJ in the total urine collected, while in the intestine there was 77% more *N*B-DGJ than *N*B-DNJ (Fig. 1A). This suggests that *N*B-DGJ passed more slowly out of the GI tract relative to *N*B-DNJ. There appeared to be no difference in distribution of the two compounds in other tissue (Fig. 1B). The serum concentration, however, differed significantly, with a lower level of *N*B-DGJ relative to *N*B-DNJ (Fig. 1C), possibly reflecting the slower uptake of *N*B-DGJ from the GI tract. When adjusted for differential serum levels,

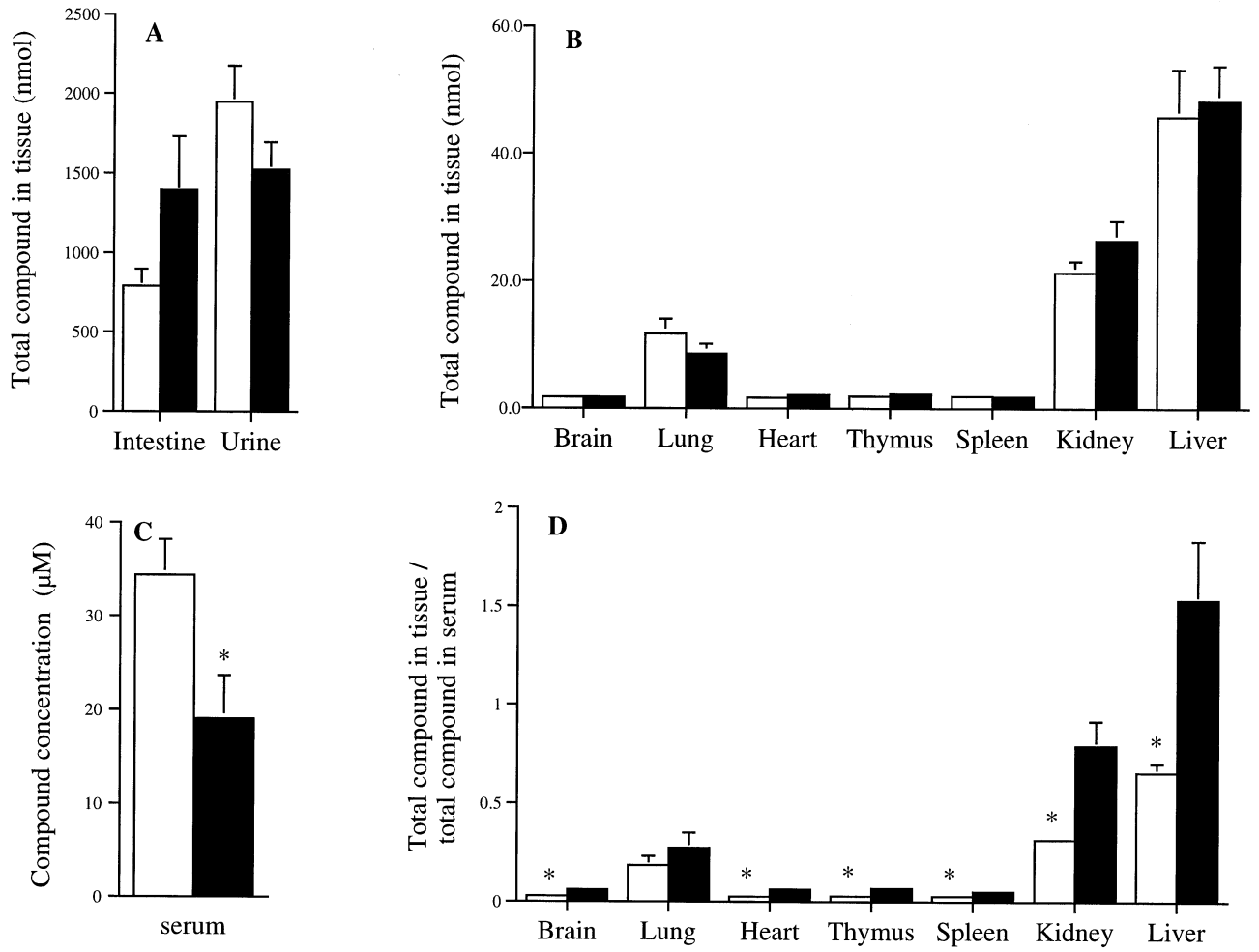

FIG. 1. Short-term distribution of radiolabelled NB-DNJ and NB-DGJ in mouse. Mice were dissected 90 min after oral administration of [^{14}C]NB-DNJ (open bars) or [^{3}H]NB-DGJ (filled bars). (A) Total compound in intestine and urine. (B) Total compound in organs. (C) Compound concentration in serum. (D) Compound in organs expressed as a ratio to compound in serum. The data are presented as the mean values ± SEM, N = 5. * denotes a significant difference between the NB-DNJ and NB-DGJ-treated mice ($P < 0.05$).

*N*B-DGJ was distributed to the tissue more efficiently than *N*B-DNJ (Fig. 1D).

Long-term Distribution of NB-DGJ and NB-DNJ in Mouse Serum and Liver

To assay the steady-state levels of the compounds when administered long-term via the oral route, the concentrations of *N*B-DGJ and *N*B-DNJ in serum and liver were determined by HPLC after treating mice with 2400 mg/kg/day of *N*B-DNJ or *N*B-DGJ (non-radiolabelled) for 5 weeks (Table 1). Both serum and liver concentration of drug were higher in *N*B-DGJ-treated mice compared to *N*B-DNJ-treated mice (66 ± 3.1 μM compared to 51 ± 13.3 μM for serum, and 207 ± 30.6 μM compared to 103 ± 21.2 for liver).

TABLE 1. Concentration of NB-DGJ and NB-DNJ in serum and liver

	Compound concentration (μM)	
	Serum	Liver
*N*B-DGJ	66 ± 3.1	207 ± 30.6
*N*B-DNJ	51 ± 13.3	103 ± 21.2

Mice were treated with 2400 mg/kg/day of *N*B-DGJ or *N*B-DNJ for 5 weeks ($n = 2$), and the compound concentration in serum and liver was then determined by duplicate runs on HPLC.

Depletion of GSL by NB-DGJ and NB-DNJ

The degree of GSL depletion in liver after 10 days or 5 weeks of treatment was compared in mice administered *N*B-DGJ or *N*B-DNJ. The livers were chloroform:methanol-extracted, gangliosides were analysed by TLC, and the G_{M2} band intensity was quantitated by densitometry. The relative G_{M2} concentrations (compared to control mice) in livers of mice treated with a range of *N*B-DGJ or *N*B-DNJ doses (300–4800 mg/kg/day) for 10 days showed a dose-dependent response to both compounds (Fig. 2A). There was no significant difference between the G_{M2} depletion achieved by the two compounds at any of the concentra-

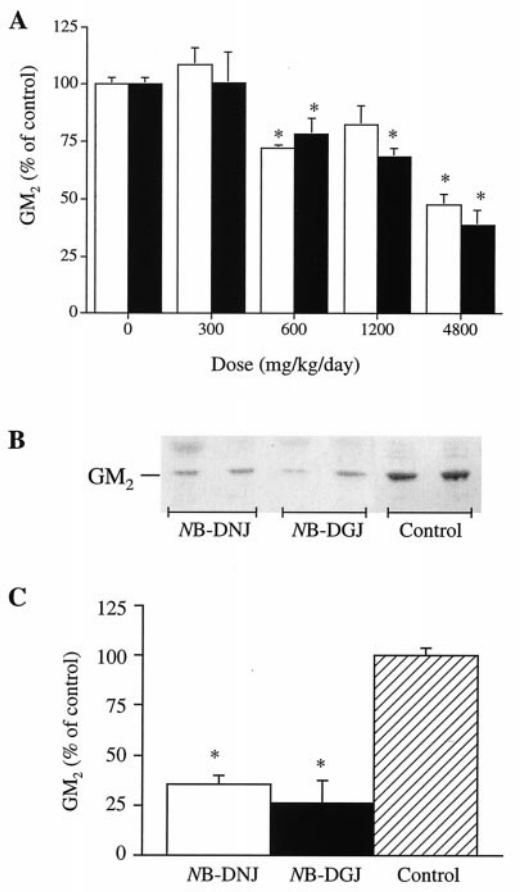

FIG. 2. Glycosphingolipid depletion in mouse liver after feeding NB-DNJ or NB-DGJ. Gangliosides were purified from liver and separated by TLC. G_{M2} concentration was measured by densitometry of the scanned TLC chromatograms. (A) G_{M2} concentration in livers of mice fed 300–4800 mg/kg/day NB-DNJ (open bars) or NB-DGJ (filled bars) for 10 days. The data are presented as the mean values ± SEM, N = 5. (B) TLC-separated G_{M2} band of livers from mice treated for 5 weeks with 2400 mg/kg/day. (C) Densitometry of TLC in B. * denotes significantly lower concentration that the control concentration ($P < 0.05$).

tions tested. After longer treatment (2400 mg/kg/day for 5 weeks), the G_{M2} concentrations in livers of mice treated with *N*B-DNJ or *N*B-DGJ were reduced to 35 ± 4% and 26 ± 11%, respectively, in relation to the concentration in control livers (Fig. 2, B and C).

Effects of NB-DGJ *and* NB-DNJ *on Growth and Lymphoid Organ Size*

To examine the overall well-being of the mice treated with *N*B-DGJ or *N*B-DNJ (2400 mg/kg/day for 5 weeks), the mice were monitored 2–3 times per week, body weights recorded, and the effects of *N*B-DGJ and *N*B-DNJ on growth rates determined (Fig. 3). The *N*B-DNJ-treated mice grew slower than untreated control mice, while *N*B-DGJ-treated mice showed no difference in growth rates relative to the untreated controls. After 5 weeks of treatment, the *N*B-DNJ mice weighed 25% less than control

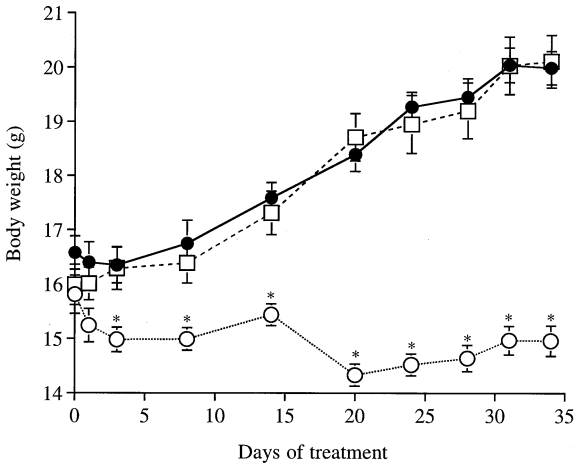

FIG. 3. Growth of mice fed NB-DNJ or NB-DGJ. Mice were fed 2400 mg/kg/day of NB-DNJ (○), NB-DGJ (●), or a control diet (□). The data are presented as the mean values ± SEM, N = 10. * denotes a significant difference compared to control weights ($P < 0.01$).

and *N*B-DGJ mice. Thymuses and spleens removed from *N*B-DNJ mice were smaller than those of control or *N*B-DGJ-treated mice (Fig. 4), *N*B-DNJ reduced the thymus weight by 61 ± 2% and spleen weight by 62 ± 3% compared to organs from control mice. In contrast, *N*B-DNJ had no effect on lymphoid organ weight. The loss of body weight in NB-DNJ treated mice did not account for the large reduction in lymphoid organ size. If expressed as a ratio to body weight, the organ weights were still reduced significantly (the thymus to body weight ratio was reduced by 45 ± 5% and the spleen to body weight ratio by 48 ± 4% in *N*B-DNJ mice compared to controls). It was observed that *N*B-DNJ-treated mice had less fat associated with their organs (kidney, spleen, etc.) and lacked subcutaneous fat compared to control or *N*B-DGJ-treated mice (data not shown).

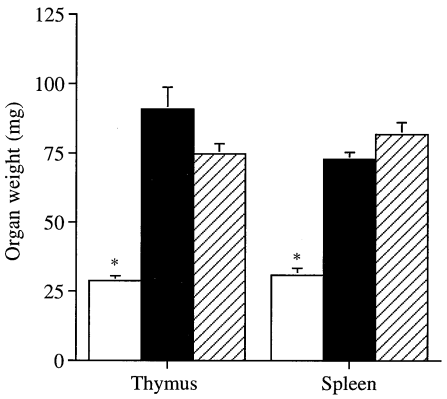

FIG. 4. Lymphoid organ size in mice after NB-DNJ or NB-DGJ treatment. Wet weight of thymus and spleen was determined at dissection after 5 weeks of treatment with 2400 mg/kg/day of NB-DNJ (open bars), NB-DGJ (filled bars), or a control diet (dashed bars). The data are presented as the mean values ± SEM, N = 4. * denotes a significant difference compared to control weights ($P < 0.001$).

TABLE 2. K_is for the inhibition of sucrase and maltase

	K_i (μM)	
	Sucrase	Maltase
DNJ	0.03	0.07
*N*B-DNJ	0.26	0.37
*N*B-DGJ	2 mM	NI

NI, non-inhibitory at 2 mM.

TABLE 3. K_is for the inhibition of lactase

	K_i (mM)
DNJ	0.013
*N*B-DNJ	4
DGJ	0.030
*N*B-DGJ	0.085

Inhibition of Disaccharidases In Vitro

*N*B-DGJ, *N*B-DNJ, and the parental non-alkylated compound DNJ were assessed for their capacities to inhibit the sucrase and maltase activities of the enzyme sucrase–isomaltase (which has disaccharidase activities for the breakdown of sucrose, maltose, and isomaltose). Inhibition of this enzyme by DNJ has previously been reported [21]. Both substrate and inhibitor concentrations were varied and the K_i calculated (Table 2). *N*B-DNJ and DNJ were found to be potent inhibitors of both sucrase and maltase (K_i [sucrase] = 0.03 μM and K_i [maltase] = 0.07 μM for DNJ, and *K*i [sucrase] = 0.26 μM and K_i [maltase] = 0.37 μM for *N*B-DNJ, while *N*B-DGJ was less potent (K_i [sucrase] = 2 mM, with maltase non-inhibitory at 2 mM). *N*B-DNJ, DNJ, *N*B-DGJ, and DGJ were also tested for their capacity to inhibit lactase (Fig. 5 and Table 3). DNJ, *N*B-DGJ, and DGJ all inhibited lactase (K_i *of* 13, 30, and 85 μM for DNJ, DGJ, and *N*B-DGJ, respectively). Lactase inhibition by *N*B-DNJ was very weak (K_i = 4 mM).

Effect of NB-DGJ *and* NB-DNJ *on Glycogen Metabolism*

Mice treated with *N*B-DGJ or *N*B-DNJ (2400 mg/kg/day for 5 weeks) were starved for 12 hr to promote glycogen breakdown. After starvation, the mice were killed and basal liver glycogen concentrations were measured (Fig. 6). In control and *N*B-DGJ-treated starved mice, the glycogen concentrations were 14 ± 0.9 μmol/g wet weight and 13 ± 0.9 μmol/g wet weight, respectively. *N*B-DNJ-treated mice, however, did not show as extensive a degree of glycogen depletion and had a significantly higher basal liver glycogen concentration (32 ± 1.6 μmol/g wet weight).

DISCUSSION

Substrate deprivation using the GSL biosynthesis inhibitor *N*B-DNJ has been shown to be effective in animal models of Tay Sachs and Sandhoff's disease [15, 16], and is currently undergoing clinical evaluation in type 1 Gaucher's disease patients. The major clinical study with *N*B-DNJ prior to the ongoing study in Gaucher's disease was in HIV-infected patients, evaluating this compound's antiviral efficacy [18]. It was not possible to achieve high enough *N*B-DNJ concentrations in the lumen of the ER of these patients to achieve the desired inhibition of *N*-linked oligosaccharide processing (through the inhibition of α-glucosidase I and II). This drug, therefore, had a minimal impact on viral parameters in these patients [18]. Although the drug was relatively well tolerated in man, side effects were reported and primarily involve GI tract distress, presumably due to disaccharidase inhibition [18]. One of the concerns when considering potential life-long therapy for patients with GSL storage diseases is tolerability. It is therefore important to determine whether or not other compounds can be identified with greater selectivity for the GSL biosynthetic pathway. This may also be a very impor-

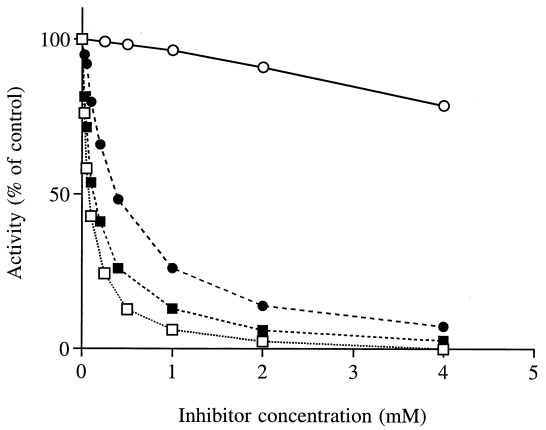

FIG. 5. Inhibition of lactase activity by NB-DNJ, NB-DGJ, DNJ and DGJ. Lactase activity expressed as % of control activity at different concentrations of NB-DNJ (○), NB-DGJ (□), DNJ (□), and DGJ (■).

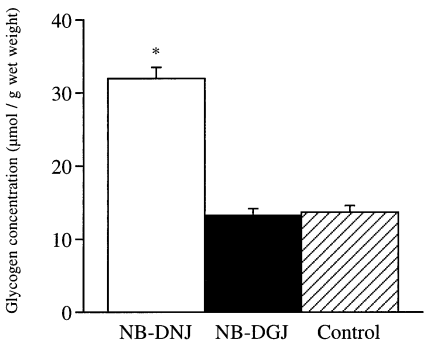

FIG. 6. Basal glycogen levels in the liver of NB-DNJ- and NB-DGJ-treated mice. Drug-treated mice (2400 mg/kg/day for 5 weeks) were starved for 12 hr before dissection, and the liver glycogen concentration determined. The concentration is calculated as μmol glucosyl units per gram liver (wet weight). The data are presented as the mean values ± SEM, N = 5. * denotes a significant difference compared to control concentration ($P < 0.001$).

tant issue when considering this class of drugs in the treatment of paediatric patients. In this study, we have therefore evaluated a second compound, the imino sugar *N*B-DGJ, to determine whether it has improved selectivity and whether it is a potential second generation analogue for clinical evaluation.

The tissue distribution of *N*B-DGJ was compared to *N*B-DNJ *in vivo* in normal mice. From the results in the short-term distribution experiment with orally administered radiolabelled compounds, *N*B-DGJ left the GI tract more slowly than *N*B-DNJ, but the organ concentrations in relation to the serum concentration was higher for *N*B-DGJ. In the liver, the levels of *N*B-DGJ (relative to serum) were 2-fold higher than the levels of *N*B-DNJ. This suggests selective uptake of the galactose analogue by cells of the liver, when compared to the glucose analogue. The basis of this selective uptake is not currently known. This is also the case after long-term feeding (5 weeks) of unlabelled compounds, where the serum levels for the two analogues were comparable, but the concentration in liver was twice as high for *N*B-DGJ compared to *N*B-DNJ. *N*B-DGJ may therefore enter tissues more efficiently and persist longer than the glucose analogue *N*B-DNJ. Both analogues (*N*B-DNJ and *N*B-DGJ) were shown to be potent inhibitors of GSL biosynthesis *in vivo*. After 10 days of treatment, dose-dependent GSL depletion was seen in livers of mice fed either *N*B-DNJ or *N*B-DGJ. The lowest dose causing GSL depletion was 600 mg/kg/day (25% reduction). The highest dose evaluated (4800 mg/kg/day) caused 60–70% depletion. Similar data were obtained with both compounds. Although there was a 2-fold higher concentration of *N*B-DGJ in liver, this was not observed when GSL depletion was measured, where both compounds gave comparable inhibition of G_{M2} biosynthesis. This may reflect differential cellular uptake of the compounds into hepatocytes, endothelial cells, and Kupffer cells, as G_{M2} may be primarily the product of one cell type, where as the compound could be sequestered in non-G_{M2}-synthesising cells. This will require further experimentation to resolve. GSL depletion after longer treatment at a dosage of 2400 mg/kg/day was also determined. After 5 weeks of feeding, the G_{M2} concentration was reduced by 74% by *N*B-DGJ and 65% by *N*B-DNJ. The drug distribution and G_{M2} depletion imply that treatment of GSL storage disorders should be as effective with *N*B-DGJ, since it has been shown that *N*B-DNJ reduces storage in mouse models of these diseases and *N*B-DGJ is equivalent to *N*B-DNJ in inhibiting GSL biosynthesis *in vivo*.

The fact that loss of body weight and reduction of lymphoid organ size is caused by *N*B-DNJ but not by *N*B-DGJ suggests that these effects are a function of glucosidase inhibition (or an as yet unidentified activity) by *N*B-DNJ, not GSL biosynthesis inhibition (an activity shared by both compounds). The effect of *N*B-DNJ in the present study on the inhibition of glycogen breakdown could provide a possible explanation for at least part of the weight loss observed in *N*B-DNJ-treated mice. It was shown that after 12 hr of starvation, when the control and *N*B-DGJ-treated mice had depleted most of their glycogen, *N*B-DNJ-treated mice still had a significant amount of glycogen in their livers. Both following starvation and between episodes of feeding, the mouse would normally break down glycogen to provide the brain, muscles, and other tissues of the body with glucose. However, if the glycogenolysis was partially inhibited, as in the *N*B-DNJ-treated mice, it would have to use other fuel sources, such as fat, to meet its energy demand. The store of adipose tissue would decrease with time, resulting in reduced body weight. This hypothesis fits with the observation that the *N*B-DNJ-treated mice (both fed and starved) had very little subcutaneous fat compared to normal or *N*B-DGJ-treated mice. The inhibition of glycogenolysis by *N*B-DNJ is probably due to inhibition of the glycogen debranching enzyme (4-α-glucanotransferase, EC 2.4.1.25 and α-1,6-glucosidase, EC 3.2.1.33). Although never reported for *N*B-DNJ, inhibition of the α-1,6-glucosidase activity of this enzyme has previously been observed for other DNJ derivatives [22, 23]. If this is also the case for *N*B-DNJ, over prolonged treatment periods this could cause (pathological) glycogen storage. If this does occur, however, it is exceedingly slow storage, as animals on drug for prolonged periods in excess of six months show no overt signs of pathology (data not shown). What may be occurring is that the basal level of glycogen is increased due to partial enzyme inhibition, but that this remains relatively constant over time at the doses of inhibitor used in this study. The direct inhibition of α-1,6-glucosidase by *N*B-DNJ is currently under investigation. It also remains to be determined whether or not the effect of *N*B-DNJ on glycogen breakdown is the only cause of weight loss or one of several underlying metabolic changes occurring in response to this glucose analogue.

*N*B-DNJ-treated mice had consistently smaller lymphoid organs, which is in agreement with previous studies [14]. However, *N*B-DGJ did not show this effect, implying that this effect on lymphoid tissues does not result from GSL biosynthesis inhibition in animals treated with *N*B-DNJ. Reduction in the size of lymphoid organs in *N*B-DNJ-treated mice has not yet presented any problems in terms of increased disease susceptibility, but long-term effects and the combination of *N*B-DNJ treatment with disease states or infection have not been evaluated. Spleen size reduction in response to *N*B-DNJ treatment has practical implications for GSL storage disease therapy, since hepatosplenomegaly is a hallmark of Gaucher's disease, and spleen size is used as one of a number of diagnostic markers when evaluating drug efficacy in this disease. Other clinical criteria, therefore, have to be considered when evaluating *N*B-DNJ in Gaucher patients. However, this does not constitute a major problem, as several other disease parameters can be reliably measured. For example, the surrogate marker chitotriosidase is highly elevated in Gaucher patients, and levels of this macrophage-derived enzyme are reduced in response to enzyme replacement therapy [24]. As

*N*B-DGJ does not affect spleen weight, evaluation of the efficacy of this compound in type 1 Gaucher patients will be more straightforward and can include spleen volume as a reliable parameter for therapeutic monitoring.

In the HIV clinical trial, it was noticed that the primary side effect of the drug was osmotic diarrhoea [18]. The diarrhoea is thought to be caused by inhibition of disaccharidases in the intestine, which means that sugars such as sucrose and maltose cannot be catabolised and absorbed from the digestive system. Sucrose consists of one glucose and one fructose residue, and maltose of two glucose residues. It is therefore not surprising that the results in this study show that the glucose analogues *N*B-DNJ and DNJ are very potent inhibitors of sucrase and maltase activity, while the galactose analogue *N*B-DGJ is not inhibitory. The study was extended to look at the catabolism of the galactose-containing disaccharide lactose (galactose–glucose disaccharide). It was found that DNJ, *N*B-DGJ, and DGJ all inhibited lactase, but the K_is were at least 10^2 times higher than for sucrase and maltase inhibition by the glucose analogues. *N*B-DNJ, however, was not a good inhibitor of lactase (K_i 4mM). In practical terms, this could mean that *N*B-DGJ might be best tolerated on a lactose-free diet, but will not interfere with the digestion of other carbohydrates. It is also possible that enzymes such as lactosylceramidase may be inhibited by *N*B-DGJ. However, even if potent inhibition of this enzyme occurred, storage would not take place due to this compound's ability to inhibit the GSL biosynthetic pathway (substrate deprivation). Inhibition of galactosidases with non-GSL substrates could potentially lead to storage of glycoconjugates. Additional enzyme inhibitory properties of *N*B-DGJ are therefore currently under investigation. We would predict that the lack of side effects associated with *N*B-DGJ *in vivo* may have important implications for the potential treatment of infants and young children, where these side effects could reduce tolerability to a greater extent than those experienced in adults [18].

In this study, *N*B-DGJ has been shown to deplete GSLs *in vivo* and to exhibit far fewer *in vitro* and *in vivo* enzyme inhibitory properties, making this a more selective compound. *N*B-DGJ should therefore be considered for use, either alone or in combination with enzyme-augmenting therapies, in GSL storage disorders. Of the six potential side effect activities listed (Table 4), lactase inhibition is the only one associated with *N*B-DGJ and is probably the simplest to overcome by restricting dietary intake of lactose. The findings in this study would support full preclinical evaluation of this compound with a view to its development as a therapeutic agent for the treatment of the glycosphingolipidoses.

We are indebted to the Research Trust for Metabolic Diseases in Children (RTMDC) for raising the funds for the purchase of NB-DGJ. *We would like to thank Julia McAvoy and David Smith for excellent technical assistance. F. P. is a Lister Institute Research Fellow. U. A. is supported by BBSRC and the British Council.*

TABLE 4. Summary of activities associated with NB-DNJ and NB-DGJ

	NB-DNJ	NB-DGJ
GSL biosynthesis inhibition	+	+
Weight loss	+	−
Lymphoid organ size reduction	+	−
ER α-glucosidase I and II inhibition*	+	−
Glycogen breakdown inhibition	+	−
Sucrase and maltase inhibition†	+	−
Lactase inhibition‡	−	+

*See Reference [19].
†K_i (sucrase) = 0.26 μM, K_i (maltase) = 0.37 μM for *N*B-DNJ.
‡K_i (lactase) = 85 μM for *N*B-DGJ.

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