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Inhibition of glycogen breakdown by imino sugars *in vitro* and *in vivo*

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Abstract

The imino sugar *N*-butyldeoxynojirimycin (*N*B-DNJ) is a glucose analogue which inhibits the glycoprotein *N*-glycan processing enzymes α-glucosidases I and II and the ceramide glucosyltransferase that catalyses the first step of glycosphingolipid biosynthesis. This and other *N*-alkylated DNJ compounds have the potential to inhibit other glucosidase, including acid α-glucosidase and α-1,6-glucosidase, enzymes involved in glycogen breakdown. We have investigated the effect of *N*B-DNJ and *N*-nonyldeoxynojirimycin (*N*N-DNJ) on glycogen catabolism. Both *N*B-DNJ and *N*N-DNJ were potent inhibitors of acid α-glucosidase and α-1,6-glucosidase *in vitro*. *N*B-DNJ and *N*N-DNJ inhibited liver glycogen breakdown *in vivo* in fasting mice. Inhibition of glycogen catabolism occurred in the cytosol and lysosomes. The liver glycogen breakdown inhibition was only induced at high doses of *N*B-DNJ, whereas *N*N-DNJ caused glycogen accumulation at lower doses. The *in vivo* effect of *N*B-DNJ on liver glycogen was transient as there was no inhibition of breakdown after 90 days of treatment. The inhibition by *N*N-DNJ, was more pronounced, reached a plateau at 50 days and then remained unchanged. Increased glycogen was also observed in skeletal muscle in *N*B-DNJ- and *N*N-DNJ-treated mice. Since the effects on glycogen metabolism by *N*B-DNJ are transient and only occur at high concentrations, it is not predicted that glycogen breakdown will be impaired in patients receiving *N*B-DNJ therapy. *N*N-DNJ is the prototype of long alkyl chain derivatives of DNJ that are entering pre-clinical development as potential hepatitis B/hepatitis C (HBV/HCV) therapeutics. Depending on the dose of these compounds used, there is the potential for glycogen catabolism to be partially impaired in experimental animals and man.

Keywords: Imino sugar; Deoxynojirimycins; Glycogen; Glycosphingolipid; Enzyme inhibition; Lysosome

1. Introduction

N-Alkylated derivatives of the imino sugar DNJ were originally developed for their antiviral activity, which was believed to result from inhibition of the *N*-glycan processing enzymes α-glucosidases I and II [1]. It was later found that *N*-alkylation of DNJ with a three-carbon chain or longer resulted in inhibition of the ceramide-specific glucosyltransferase involved in glycosphingolipid (GSL) synthesis [2]. Based upon the latter activity, *N*B-DNJ is undergoing clinical evaluation as a treatment for GSL storage diseases with brain involvement, and is approved for use in type 1 Gaucher's disease in Europe, Israel and the USA [3]. By inhibiting the glucosyltransferase and thereby limiting the synthesis of GSLs, a balance between the reduced activity of GSL breakdown in the affected cells and the concentration of GSL substrate is achieved. A few adverse effects of *N*B-DNJ treatment have been reported both in clinical trials and in mice [3,4]. The possibility of these effects being due to GSL depletion has been ruled out in mice where the more specific ceramide glucosyltransferase inhibitor *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) inhibited GSL synthesis but did not show any detectable adverse effects [5]. Since *N*B-DGJ is a galactose analogue, whereas *N*B-DNJ is a glucose analogue and a known glucosidase inhibitor [6], it is predicted that at least

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Abbreviations: *N*B-DNJ, *N*-butyldeoxynojirimycin; *N*N-DNJ, *N*-nonyldeoxynojirimycin; *N*B-DGJ, *N*-butyldeoxygalactonojirimycin; GSL, glycosphingolipid; GAA, acid α-glucosidase; ER, endoplasmic reticulum; EM, electron microscopy; GI, gastrointestinal; ww, wet weight; HBV, hepatitis B virus; HCV, hepatitis C virus.

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some adverse effects are due to inhibition of one or more glucosidases. In the present report, one of the activities observed in mice treated with *N*B-DNJ (but not with *N*B-DGJ), the increase in basal liver glycogen [5], has been investigated *in vivo* and *in vitro* to establish the basis, tissue and cellular location, and long-term development of the elevated glycogen concentration.

Glycogen exists both as a cytosolic and a lysosomal form which are broken down by different pathways, the cytosolic form by the traditional glycogenolytic pathway, including the phosphorylase and the debranching enzymes, and the lysosomal form by a single acidic glucosidase (acid α-glucosidase (GAA) or acid maltase). The non-alkylated DNJ and the derivatives miglitol (*N*-hydroxyethyl-DNJ) and emiglitate (*N*-[β-(4-ethoxycarbonylphenoxy)-ethyl]-DNJ) have previously been reported to inhibit GAA and the α-1,6-glucosidase activity of the glycogen debranching enzyme [7–9]. Other enzymes involved in glycogen breakdown, such as phosphorylase, glucose-6-phospatase and the glucosyltransferase, of the debranching enzyme were all unaffected by these compounds [7–9]. Although the *N*-alkyl derivatives of DNJ have different properties (such as hydrophobicity and stereochemistry) compared to DNJ, miglitol and emiglitate, it is likely (although it remains to be proven) that high glycogen levels previously found in livers of *N*B-DNJ-treated mice after a 12-hr fast [5] can be explained by α-1,6-glucosidase and/or GAA inhibition. The differential activities thus far described for alkylated imino sugars depend both on the sugar structure and the length of the alkyl chain [10]. *N*N-DNJ has previously been evaluated primarily on the basis of its antiviral activity [11,12] and has been included in this study for a comparison of how elongation of the alkyl chain, and thereby an increase in hydrophobicity [13], affects the inhibition of glycogen breakdown. A lower dose of *N*N-DNJ compared to *N*B-DNJ was selected because of the former compounds higher tissue uptake and because previous studies had shown that at these doses (250 mg/kg/day for *N*N-DNJ and 2400 mg/kg/day for *N*B-DNJ) a similar level of GSL depletion was achieved in mice.[1] The present study shows that although similar IC_{50} values of *N*B-DNJ and *N*N-DNJ were obtained for the *in vitro* inhibition of glycogen breakdown enzymes, treatment *in vivo* with a 10-fold lower dose of *N*N-DNJ compared to *N*B-DNJ still caused a much higher concentration of tissue glycogen. The results showed that at a high concentration of *N*B-DNJ, glycogen breakdown was inhibited in liver and muscle, that both lysosomal and cytosolic glycogen was affected, but that the effect was transient. For *N*N-DNJ, however, the inhibition was more pronounced with lower concentrations needed to induce the effect, and the effect persisted over time after glycogen concentrations reached a plateau level at about 50 days of treatment. The implications of these findings, in the context of the clinical use of members of this family of compounds, are discussed.

[1] F.M. Platt, unpublished observation.

2. Materials and methods

2.1. Compounds

*N*B-DNJ was a gift from the Monsanto/Searle and Oxford GlycoSciences, and *N*N-DNJ was a gift from Synergy Pharmaceutical Inc. DNJ and *N*B-DGJ were purchased from Toronto Research Biochemicals. *N*-Octyl-DNJ was synthesised as described [13]. Human recombinant GAA (EC 3.2.1.20) and glycogen debranching enzyme (4-α-glucosyltransferase, EC 2.4.1.25, and α-1,6-glucosidase, EC 3.2.1.33) [14] were generously provided by Professor Y.-T. Chen, Duke University Medical Center.

3. Animals

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

3.1. GAA assay

The activity of the lysosomal enzyme GAA was measured using the artificial substrate 4-methylumbelliferryl-α-D-glucoside (4-MUαG). The reaction mixture contained inhibitor (DNJ, *N*B-DNJ, *N*N-DNJ or *N*B-DGJ at different concentrations), 0.8 μg/mL GAA (specific activity at 100 nmol/min/mg protein) and 0.55 mM 4-MUαG in 50 mM sodium acetate buffer (pH 4). After incubation for 20 min at 37°, the reaction was stopped by adding 1 mL of NaOH–glycine (0.3 M, pH 10.5), and the released 4-MU was measured at 350ex/460em nm (Perkin Elmer fluorimeter).

3.2. α-1,6-Glucosidase assay

The α-1,6-glucosidase activity of the glycogen debranching enzyme was assayed in the reverse direction by measuring the incorporation of [^{14}C]glucose into glycogen [15]. Glycogen debranching enzyme (0.22 mg/mL at specific activity 6 nmol/min/mg protein) was incubated in a total volume of 20 μL containing 2.6 mM [^{14}C]glucose (12 μCi/μM), 12.5% rabbit liver glycogen, 2.1 mM EDTA, 0.02% gelatine, 4.1 mM mercaptoethanol, and inhibitor (DNJ, *N*B-DNJ, *N*N-DNJ or *N*B-DGJ at different concentrations) in 45 mM glycylglycine, pH 6.5. After 1-hr incubation at 30° the reaction was stopped with 20 μL of 0.2 M HCl, and 30 μL aliquots were spotted onto glass microfibre discs (GF/C). The discs were washed in 66% ethanol three times for 20 min whilst stirring, dipped into acetone for 10 min, dried under a heating lamp and transferred to vials containing 4 mL of scintillation fluid.

Glycogen-incorporated glucose was determined by counting [^{14}C] and subtracting counts obtained for blank samples (with no debranching enzyme).

3.3. Treatment of mice with NB-DNJ and NN-DNJ

Mice (6 weeks old) were fed a diet of powdered chow (expended Rat and Mouse Chow 3, ground, SDS Ltd), with or without *N*B-DNJ or *N*N-DNJ. 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 at 2400 mg/kg/day for 3–180 days or 1200 mg/kg/day for 3–14 days, or on *N*N-DNJ at 250 mg/kg/day for 1–120 days or 10–250 mg/kg/day for 30 days. When fasted, the mice were deprived of food but not water for 12 hr overnight.

3.4. Determination of glycogen concentration in liver and muscle

Glycogen concentrations in mouse liver and gastrocnemius muscle were determined as described [16]. Briefly, the tissue was homogenised in ice-cold 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 2 M KOH for 20 min at 70°. The solution was centrifuged (10 min, 13,000 rpm) 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). The concentration of amyloglucosidase was sufficiently high to avoid any potential inhibitory effects by the glucosidase inhibitors *N*B-DNJ and *N*N-DNJ (confirmed in assays containing up to 1 mM added imino sugar). 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 U/mL HK, 0.85 U/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, 13,000 *g*), and the absorbance of the supernatant at 340 nm measured.

3.5. Electron microscopy (EM) of mouse liver

Mice were anaesthetised (0.3 mL Hypnorm:Hypnoval:H_20, 1:1:2, i.p.), perfused through the left cardiac ventricle with heparinized saline followed by 2% paraformaldehyde/2% glutaraldehyde in PBS. Perfused tissue was dissected and fixed further at 4° for 2–3 days. Small pieces of the fixed tissue were washed three times in 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer containing 1.5% potassium ferrocyanide for 2 hr. The tissue was rinsed three times in 70% ethanol, incubated in a mixture of 70% ethanol and 1% uranyl acetate for 2 hr, dehydrated through an ethanol series and propylene oxide, and embedded in Araldite. Ultra-thin sections (60 nm) were cut and examined using Joel JEM 100 CX electron microscope.

3.6. Determination of serum and liver NN-DNJ concentration

The concentration of *N*N-DNJ in mouse serum and liver was determined according to Mellor *et al*. [17]. Briefly, serum or liver homogenates had an internal standard (*N*-octyl-DNJ) added, were filtered, purified on a cation exchange column and a C18 column, and finally quantified by HPLC.

3.7. Serum metabolite measurements

Commercially available kits (Sigma) were used to measure glucose, free fatty acids (NEFAs) and triglycerides in mouse serum. Measurements were performed according to manufacturer's instructions.

3.8. 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 two-tailed Student's *t* test for unpaired observations. Results in the text and tables are presented as means ± SEM.

4. Results

4.1. In vitro inhibition of GAA and α-1,6-glucosidase

The compounds DNJ, *N*B-DNJ, *N*N-DNJ and *N*B-DGJ were tested for their inhibitory action against two enzymes involved in glycogen breakdown, the lysosomal GAA and the α-1,6-glucosidase activity of the debranching enzyme (Fig. 1 and Table 1). All three DNJ compounds were shown to be potent inhibitors of both enzymes with similar *in vitro* IC_{50} values (Table 1), whereas the galactose analogue

Table 1
IC_{50} values of imino sugars for *in vitro* inhibition of glycogen breakdown enzymes acid α-glucosidase and α-1,6-glucosidase

	IC_{50} (μM)	
	Acid α-glucosidase (lysosomal enzyme)	α-1,6-Glucosidase (debranching enzyme)
DNJ	0.38	1.8
*N*B-DNJ	1.9	4.5
*N*N-DNJ	0.42	8.4
*N*B-DGJ	NI	NI

NI: not inhibitory at 2 mM. Data are derived from three independent experiments.

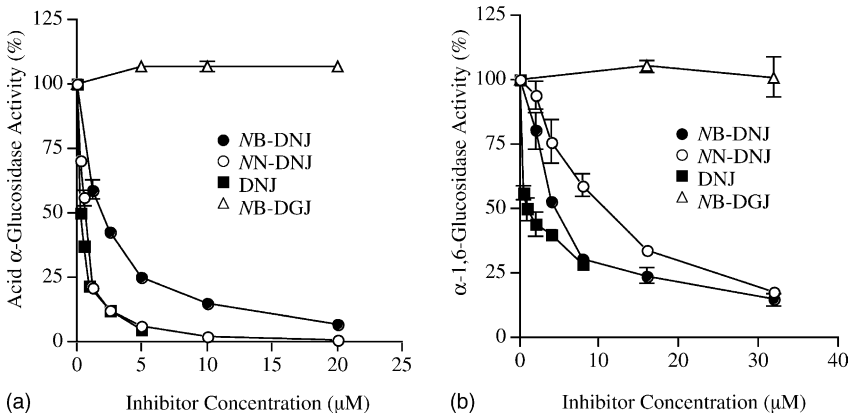

Fig. 1. *In vitro* inhibition of glycogen breakdown enzymes by imino sugars. (a) Acid α-glucosidase activity and (b) α-1,6-glucosidase activity expressed as % of control activity (no inhibitor present) at different concentrations of *N*B-DNJ (●), *N*N-DNJ (○), DNJ (■) and *N*B-DGJ (△). Data are derived from three independent experiments (means ± SEM).

*N*B-DGJ did not inhibit either enzyme at the concentrations tested (up to 2 mM).

4.2. Glycogen levels in liver, muscle and heart in NB-DNJ- and NN-DNJ-treated mice

Mice were treated with *N*B-DNJ or *N*N-DNJ for up to 180 and 120 days, respectively, after which the glycogen levels in liver and muscle were determined at different time points (either in the fed state or after 12 hr of fasting). After 30 days treatment, the liver glycogen concentration was 14 ± 1 μmol/g wet weight (ww) for control livers, 31 ±3 μmol/g wet for *N*B-DNJ treated and 52± 6 μmol/g wet for *N*N-DNJ ($P < 0.0001$ and $P < 0.001$ for differences compared to controls for *N*B-DNJ and *N*N-DNJ, respectively, Fig. 2a). Longer *N*B-DNJ treatment did not increase the glycogen concentration further, but rather cause a slight reduction back to normal levels. Livers from mice treated with *N*B-DNJ for longer than 30 days had a glycogen concentration not significantly different to control mice ($P > 0.43$). The glycogen concentration in livers of *N*N-DNJ-treated mice stayed high throughout the treatment period of 120 days (liver glycogen concentrations of 67–81 μmol/g, $P < 0.03$ when compared to control livers). The glycogen concentrations in livers of fed mice were not significantly different between the groups. The values were 318 ± 28 μmol/g ww, 268 ± 39 μmol/g ww and 246± 26 μmol/g ww for control, *N*B-DNJ- and *N*N-DNJ-treated mice, respectively ($P \geq 0.14$, Fig. 2b). All the biochemical data were supported by histochemical analysis (data not shown).

The dose of *N*B-DNJ and *N*N-DNJ required to cause glycogen breakdown inhibition in the liver was determined (Fig. 2c and d). The results showed that 1200 mg/kg/day of *N*B-DNJ was not sufficient to inhibit glycogen breakdown ($P = 0.78$), this was only observed at the higher concentration 2400 mg/kg/day (Fig. 2c). For *N*N-DNJ, dose-dependent inhibition was observed (after 30 days treatment), with significant differences compared to control mice from an *N*N-DNJ concentration of 50 mg/kg/day ($P = 0.008$, Fig 2d).

In muscle, the differences between imino sugar-treated and control mice were detectable both in the fed and in the fasted mice (Fig. 3a and b), which was also confirmed histochemically (not shown). Signs of accumulated glycogen were also occasionally observed in heart sections from *N*N-DNJ-treated mice. This was not observed in control or *N*B-DNJ-treated mice (data not shown).

4.3. EM analysis of liver glycogen in imino sugar-treated mice

Mice were treated with *N*B-DNJ (2400 mg/kg/day) or *N*N-DNJ (250 mg/kg/day) for 5 weeks and then fasted for 12 hr prior to dissection and preparation for EM. Glycogen was observed in both the lysosomes and cytosol of livers from imino sugar-treated mice after starvation (Fig. 4). The organelles that looked to store glycogen in *N*N-DNJ-treated mice appeared larger than normal lysosomes (Fig. 4j), whereas in *N*B-DNJ they were small but dense (Fig. 4h). Minimal or no glycogen was observed in livers of starved control mice (Fig. 4f).

4.4. NN-DNJ concentration in mouse serum and liver

C57Bl/6 mice were fed *N*N-DNJ at a dose of 250 mg/kg/day for 1–21 days. After dissection and homogenising the concentration of *N*N-DNJ in the serum and livers was determined by HPLC. The results showed a stable serum concentration of approximately 5–7 μM from day 3 (Fig. 5). In liver, the *N*N-DNJ accumulated over the 21 days, reaching 22 nmol/g ww liver.

4.5. Mouse body weights and serum concentrations of glucose, NEFAs and triglycerides

As observed in previous studies [5], mice on *N*B-DNJ lost body weight rapidly following initiation of treatment

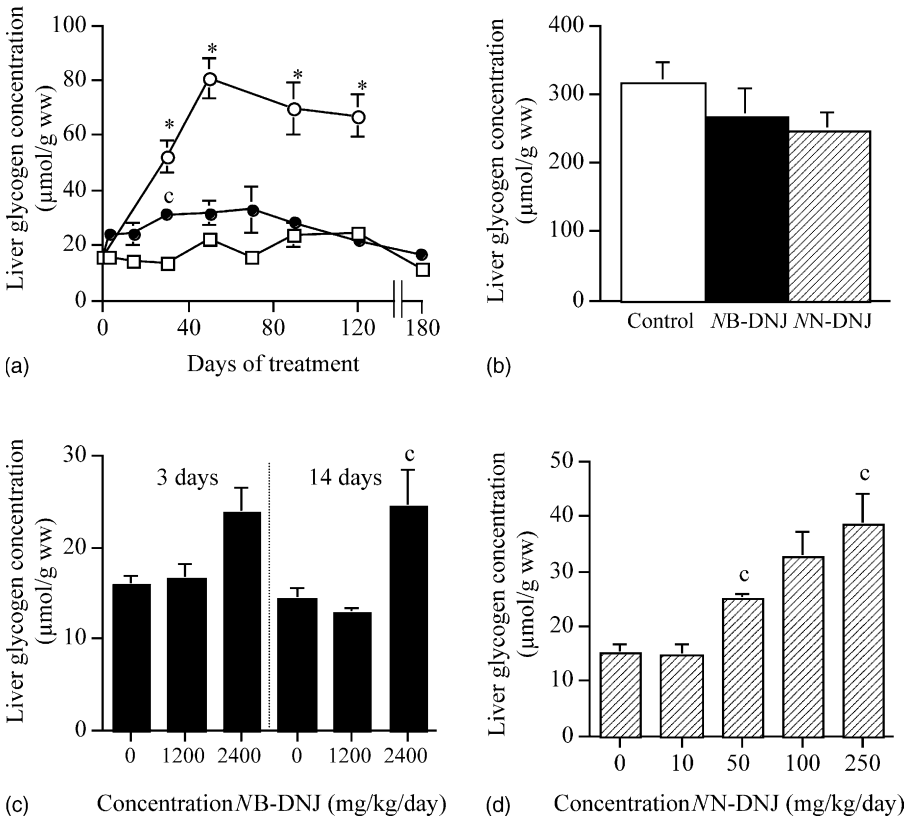

Fig. 2. Glycogen concentration in mouse liver after imino sugar treatment. The glycogen concentration was determined in mouse liver after imino sugar treatment up to 180 days with or without 12 hr of fasting before sacrificing. The concentrations are expressed as μmol glucose units per g wet weight tissue. (a) Liver glycogen concentration in 12-hr fasted mice treated with control diet (□), 2400 mg/kg/day *N*B-DNJ (●) or 250 mg/kg/day *N*N-DNJ (○). The N numbers varied from 3 to 8 at different time points. (b) Liver glycogen concentration in fed mice treated with control diet, 2400 mg/kg/day *N*B-DNJ or 250 mg/kg/day *N*N-DNJ. The values are averages over the treatment period (N = 5). (c) Liver glycogen at different doses of *N*B-DNJ. The mice were fasted after 3 or 14 days of treatment with 0–2400 mg/kg/day *N*B-DNJ (N = 3). (d) Liver glycogen at different doses of *N*N-DNJ. The mice were fasted after 30 days of treatment with 0–250 mg/kg/day *N*N-DNJ (N = 3).

but then paralleled the growth curve of control mice (Fig. 6a). However, they never reached the same weight as control mice and always looked lean with almost no subcutaneous fat observed on dissection. The body weights of *N*N-DNJ-treated mice were generally unaffected at the dose evaluated (250 mg/kg/day).

Serum collected from mice sacrificed at different time points following drug treatment were assayed for glucose, NEFAs and triglycerides. The results for 12-hr fasted (after 3–125 days treatment) and fed mice (after 30 days treatment) are shown in Fig. 6b–d. In fed mice, treatment with *N*B-DNJ and *N*N-DNJ resulted in lower glucose concentration ($P < 0.02$). Under conditions of starvation, however, when the control mice lowered their serum glucose levels, the *N*B-DNJ- and *N*N-DNJ-treated mice retained a similar level as before starvation and therefore showed

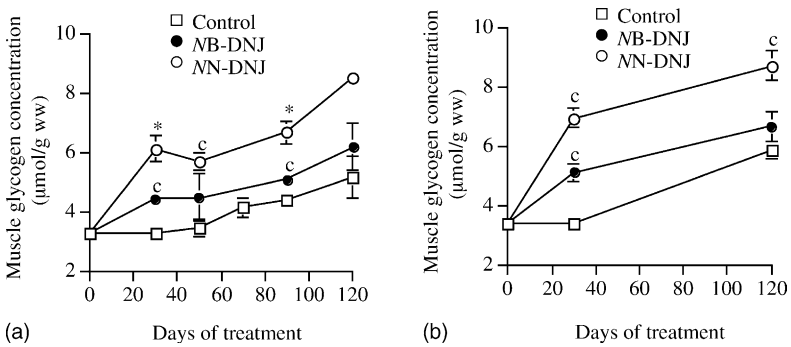

Fig. 3. Glycogen concentration in gastrocnemius muscle after imino sugar treatment. Mice were treated as in Fig. 2 and muscle glycogen determined. (a) Muscle glycogen concentration in 12-hr fasted mice treated with control diet (□), 2400 mg/kg/day *N*B-DNJ (●) or 250 mg/kg/day *N*N-DNJ (○) ($3 \leq N \leq 8$). (b) Muscle glycogen concentration in fed mice at 30 and 120 days of treatments as in panel a. [c]Significant difference compared to control mice; [*]significant difference compared to *N*B-DNJ-treated and control mice ($P < 0.05$). Group sizes ranged from four to eight animals per group.

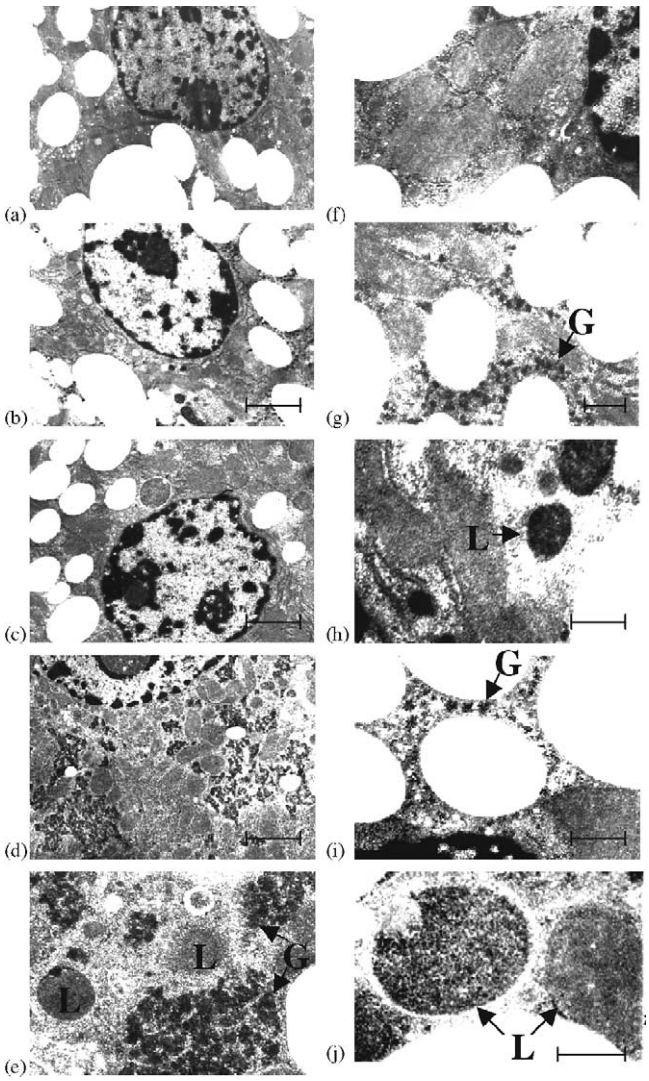

Fig. 4. Electron microscopy of liver from *N*B-DNJ- and *N*N-DNJ-treated mice. Mice were fed *N*B-DNJ (2400 mg/kg/day) or *N*N-DNJ (250 mg/kg/day) for 30 days, fasted or fed, and whole body perfusion fixed. Sections of liver were stained with potassium ferrocyanide to stain glycogen. The bar in panels a–d represent 2 μm, and in the higher magnification photos (e–j) 500 nm. Representative (N = 3 mice per treatment, 20 fields examined per sample) electron microscopy images show (a) fasted control liver, low magnification, (b) fasted liver from *N*B-DNJ-treated mouse, low magnification, (c) fasted liver from *N*N-DNJ-treated mouse, low magnification, (d) fed control liver, low magnification, (e) fed control liver showing glycogen (G) and lysosome (L), (f) fasted control liver empty of glycogen, (g) cytosolic glycogen (G) of *N*B-DNJ-treated mouse, (h) lysosomal glycogen (L) of *N*B-DNJ-treated mouse, (i) cytosolic glycogen (G) of *N*N-DNJ-treated mouse, (j) lysosomal glycogen (L) of *N*N-DNJ-treated mouse.

higher glucose levels than the control mice ($P < 0.02$, Fig. 6b). Both NEFA and triglyceride concentrations in the fed state were lower in *N*B-DNJ-treated mice compared to control and *N*N-DNJ-treated mice (although the differences were not significant, $P > 0.58$). Control mice increased NEFA and triglyceride levels in the serum on starvation. This was also observed in the drug-treated mice, although their levels stayed slightly lower than in the control mice throughout the treatment period. The mobilisation of fat during starvation was also visualised by

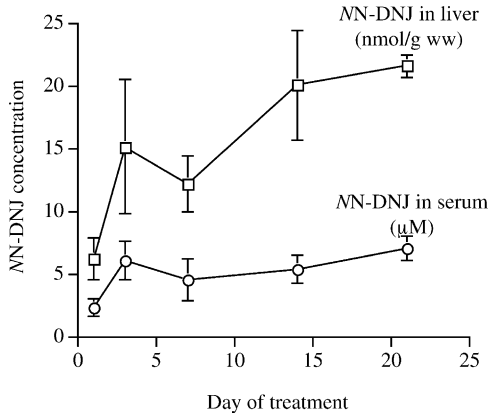

Fig. 5. Concentration of *N*N-DNJ in serum and liver. C57Bl/6 mice were treated with *N*N-DNJ for 1–21 days at a dose of 250 mg/kg/day. After sacrifice, serum was collected and livers were dissected, homogenised and its content of *N*N-DNJ was purified and analysed by HPLC. The *N*N-DNJ concentration in serum (○) is expressed in μM, and in liver (□) as nmol/g wet weight of tissue. The data are based upon a group size of five mice per compound per time point.

numerous large fat droplets in the liver EM images (Fig. 4a–c). There was no obvious difference in size or frequency of lipid droplets with different treatments.

5. Discussion

Over the past decade there has been increasing interest in the development of imino sugar compounds for human therapy (for reviews, see Refs. [10,18]). The glucose analogue *N*B-DNJ is a differential inhibitor of several cellular enzymes in different intracellular compartments [10]. The consequences of its administration *in vivo* do not always correlate with *in vitro* potency against target enzymes, due to differential accessibility of the drug to different compartments of the cell. For instance, *N*B-DNJ is a potent α-glucosidase inhibitor *in vitro* (K_i 0.2 μM) and a modest inhibitor of the ceramide glucosyltransferase (K_i 7 μM) [9]. However, *in vivo* the dominant effect of *N*B-DNJ administration is on the latter enzyme due to its greater accessibility to the drug (the catalytic domain of the transferase is facing the cytosol, whereas processing α-glucosidases reside in the lumen of the ER). *In vivo* evaluation is therefore essential in order to establish how significant potential enzyme inhibitory activities are, to study their consequences, and to relate these findings to doses of the drug being used in man.

An observation made in a previous study was that increased levels of glycogen were detected in livers of fasted mice receiving *N*B-DNJ treatment compared to fasted control mice [5]. The aim of the present study was therefore to identify the cause of the increased glycogen, investigate the extent of the accumulation, determine whether other organs, such as muscle, is affected and to determine whether the glycogen accumulates progressively with time (i.e. does glycogen storage occur?). The two

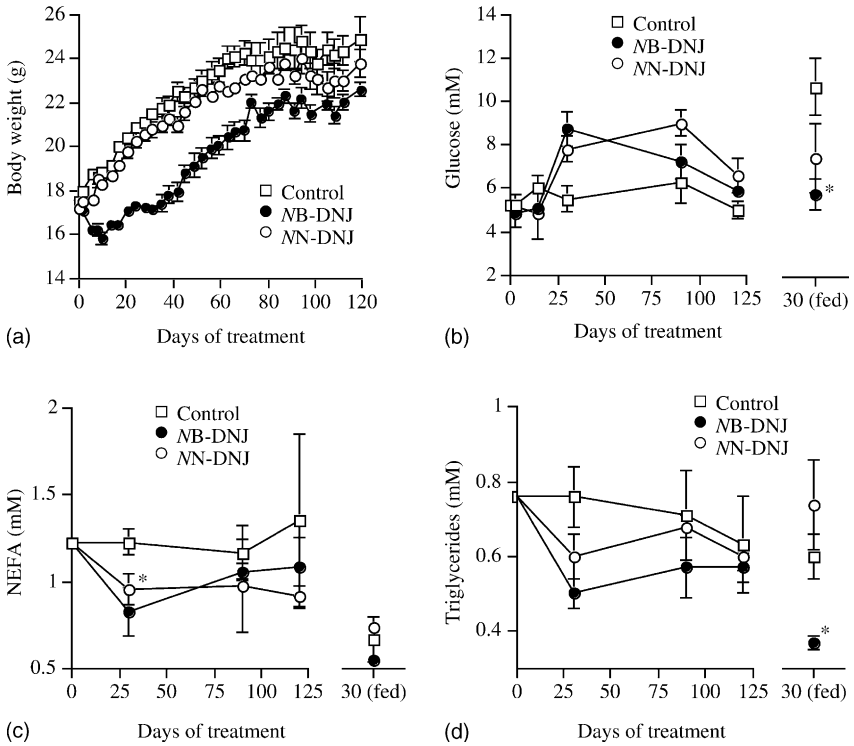

Fig. 6. Body weights and serum metabolite concentrations of imino sugar-treated mice. Mice were given a control diet (□), 2400 mg/kg/day *N*B-DNJ (●) or 250 mg/kg/day *N*N-DNJ (○). At dissection they were either kept fed or fasted for 12 hr. The results from drug treatment plus 12-hr fasting are shown for time points 0–120 days of treatment. The results from mice kept fed until dissection are shown for 30 days treatment only. (a) Mouse body weights during treatment. N = 17 between days 0 and 90, N = 6 between days 90 and 120. The difference between control and *N*B-DNJ-treated mice was significant from day 2 until the end of the treatment period ($P < 0.01$). (b) Serum glucose concentrations in fed and fasted mice. (c) Serum-free fatty acid (NEFA) concentration in fed and fasted mice. (d) Serum triglyceride concentration in fed and fasted mice. The N values in panels b–d varied from 3 to 6 depending on time point. *Significant difference compared to control mice ($P < 0.02$).

compounds used for the *in vivo* studies were *N*B-DNJ and *N*N-DNJ. *N*N-DNJ has been of interest because of its potential use as an antiviral drug [11,12], and is here used for biochemical comparison of imino sugars with varying *N*-alkyl chain length and therefore differing hydrophobicities (*N*B-DNJ is hydrophilic, *N*N-DNJ hydrophobic).

The liver glycogen levels in fed mice were the same or slightly lower in *N*B-DNJ- or *N*N-DNJ-treated mice compared to controls, whereas during fasting when glycogen breakdown is promoted, their levels were significantly higher than in control mice. This implied that the observed effects of the imino sugars are caused by inhibition in the breakdown of glycogen. There are two pathways of glycogen breakdown, the lysosomal hydrolytic pathway and cytosolic glycogenolysis. Both these pathways have metabolic disorders associated with them [19]. It is clearly important that using imino sugars for treatment of other diseases does not cause pathological symptoms similar to the inherited glycogenoses, i.e. glycogen storage. The lysosomal glycogen is hydrolysed by a single enzyme, the GAA (acid maltase), and genetic defects in the expression or activity of this enzyme cause the severe lysosomal storage disorder Pompe's disease (type II glycogenosis). The active site of GAA is very similar to that of sucrase-isomaltase [19,20] which is an enzyme already shown to be potently inhibited by *N*B-DNJ and other DNJ derivatives [5]. Inhibition of GAA is therefore one potential mechanism leading to elevated glycogen in fasted *N*B-DNJ- and *N*N-DNJ-treated mice. The glycogen stored in the cytosol is broken down by the enzymes glycogen phosphorylase and glycogen debranching enzyme, which in turn has two different activities (glucosyltransferase and α-1,6-glucosidase). The α-1,6-glucosidase activity of the debranching enzyme has previously been shown to be inhibited by DNJ (whereas the transferase activity and the phosphorylase enzyme were unaffected) [8,9], which makes inhibition of this enzyme another potential explanation for the glycogen breakdown inhibition resulting from *N*B-DNJ and *N*N-DNJ treatment. Deficiency of glycogen debranching enzyme is the cause of type III glycogenosis (Cori's disease), which is a relatively mild disorder that often can be treated by dietary management [19]. *In vitro* experiments in the present study showed potent inhibitory activity of both *N*B-DNJ and *N*N-DNJ towards both the GAA and the glycogen debranching enzyme. EM of livers from *N*B-DNJ-treated mice show the presence of glycogen granules in the cytosol and dense glycogen filled lysosomes. In livers from starved *N*N-DNJ-treated mice, EM showed cytosolic glycogen and numerous large lysosomes storing glycogen, with similar structure to that seen in storage lysosomes in Pompe's disease patients [19]. This indicates that both GAA and the debranching enzyme are inhibited

by *N*B-DNJ or *N*N-DNJ administration, albeit to differing degrees. The galactose analogue *N*B-DGJ, however, did not inhibit either of these activities *in vitro*. *N*B-DGJ has previously not been found to inhibit glucosidases, and after *N*B-DGJ treatment in mice the liver glycogen levels were normal as predicted [5].

When feeding mice almost 10 times less *N*N-DNJ compared to *N*B-DNJ, the effect on glycogen breakdown was still more pronounced in *N*N-DNJ mice. Since IC_{50} values for both the GAA and the debranching enzyme were similar for both compounds, this suggests a difference in uptake and/or retention of the two compounds in tissue, in different cells, or in different organelles. Slower but increased *in vivo* uptake over time of *N*N-DNJ compared to *N*B-DNJ has been reported [13] with greater tissue retention of *N*N-DNJ, in agreement with findings in the present study. The *N*N-DNJ concentration determination in this study indicated a continuous accumulation over at least the first 3 weeks in liver, whereas the serum concentration had already levelled out after 3 days of treatment. An important observation in the comparison between the two analogues is that *N*B-DNJ treatment causes weight loss, whereas *N*N-DNJ does not at the concentrations evaluated in this study. In a previous study, we suggested that inhibition of glycogen breakdown could be a factor involved in weight loss seen following *N*B-DNJ treatment [5]. The explanation was that mice would utilise fat rather than the glycogen they could not use because of breakdown inhibition. However, in this report it is shown that *N*N-DNJ causes a higher degree of glycogen breakdown inhibition but does not cause weight loss at the concentration tested. This implies that lack of glucose derived from glycogen is not a major contributor to the weight loss seen in mice treated with high doses of *N*B-DNJ. During treatment with *N*B-DNJ and *N*N-DNJ, there was a tendency in the fed mice towards lower glycogen levels in the imino sugar-treated mice. This is consistent with studies of other glucosidase inhibitors, such as emiglitol, miglitol and acarbose [7], and could be due to a decreased glucose uptake from the GI tract as a result of intestinal glucosidase inhibition and/or lower food intake. After fasting, however, the basal liver glycogen levels were 2-fold higher in *N*B-DNJ-treated mice and almost 4-fold higher in *N*N-DNJ-treated at their highest levels compared to control mice. With *N*B-DNJ treatment this effect was transient and after about 90 days of treatment the basal glycogen levels returned to normal. The mechanism for this could either be a down-regulation of glycogen synthesis or an up-regulation of the glycogen breakdown enzymes. Further investigation will be needed to resolve this matter. The high liver glycogen seen in fasted *N*N-DNJ mice is not decreased over the treatment period but seems to plateau at about 50 days of treatment. The reason for the different time courses of glycogen breakdown inhibition *in vivo* seen with *N*B-DNJ and *N*N-DNJ has not been established. However, as discussed above, recent *in vivo* compound distribution data suggest that the more hydrophobic imino sugar analogues, such as *N*N-DNJ, are slower but more effectively taken up by tissue and are also retained longer. So even if there is a similar change in glycogen metabolism (synthesis down-regulation/degradation up-regulation) with both inhibitors, the long retention and possibly accumulation of *N*N-DNJ in tissue could explain a more persistent *in vivo* glycogen breakdown inhibition by *N*N-DNJ.

The rate of clearance of *N*B-DNJ in mice is two orders of magnitude faster than in humans [4], thus high dose therapy is required in mice to recapitulate human therapeutic levels of the drug. Feeding mice 2400 mg/kg/day of *N*B-DNJ gives a serum level of approximately 50 μM as compared to 2–5 μM achieved in Gaucher patients when given a therapeutic dose of 300 mg/day [3]. It was shown in the present study that feeding mice 1200 mg/kg/day of *N*B-DNJ (serum concentration of 31 μM in mouse) [4] had no detectable effect on glycogen metabolism, which means that this adverse effect can only be observed at concentrations far higher than current therapeutic levels (5–10 μM). For *N*N-DNJ, the glycogen effect in liver was seen from 50 mg/kg/day and increased with dose. The serum levels achieved by feeding 250 mg/kg/day *N*N-DNJ stabilised at approximately 5–7 μM. This means that in the murine system, low serum levels of *N*N-DNJ is sufficient to cause glycogen breakdown inhibition.

In addition to liver, the other organs storing energy in the form of glycogen are muscle and heart. In both Pompe's and Cori's diseases, these organs accumulate abnormal amounts of glycogen which, especially in the case of Pompe's disease, leads to severe pathology, such as muscle weakness and cardio-respiratory failure [19]. It was therefore important in this study to investigate glycogen levels in muscle and heart during treatment with the imino sugars. The glycogen levels of muscle are not as greatly affected by fasting in contrast to liver glycogen, so differences between treated and control mice were detected both in fed and fasted mice. Both *N*B-DNJ- and *N*N-DNJ-treated mice showed increased glycogen, with the latter compound giving the larger increase. The difference in muscle glycogen between control and imino sugar-treated mice does not change over the treatment period. In heart, no signs of glycogen accumulation were observed in *N*B-DNJ-treated mice, whereas hearts from *N*N-DNJ mice frequently showed regions of glycogen staining within the fibres, which was not seen in control or *N*B-DNJ-treated mice (data not shown).

In addition to slightly lower liver glycogen concentrations in fed mice treated with *N*B-DNJ and *N*N-DNJ, a lower serum glucose concentration was observed, which again could be explained by decreased glucose uptake from the intestine. When fasted, these mice maintained the same glucose level at almost the same concentration as when fed, whereas control mice as expected lowered their glucose concentration. This could be explained by the fact that the

glycogen breakdown has been inhibited and the liver depot of glycogen is still quite high. The mice therefore do not seem to switch to a fasted metabolic state but keep the same energy regulation as in the fed state. This is consistent with the fatty acid and triglyceride results where the imino sugar-treated mice do not mobilise fatty acids to the same extent as the controls, and release triglycerides and NEFAs from the adipose tissue into the serum during fasting. The NEFAs are then used by the liver either for fatty acid oxidation or ketone body production in order to conserve glucose. The seemingly low concentrations of NEFAs and triglycerides in the fed *N*B-DNJ mice can be explained by the lack of adipose tissue in these mice. *N*B-DNJ-treated mice lose weight rapidly and on dissection it can be noted that they are very lean with little subcutaneous fat.

Taken together, these results suggest that *N*B-DNJ therapy should cause no long-term effects on glycogen metabolism, since *N*B-DNJ serum concentrations above 10 times that achieved in clinical trials is needed for any effect to be observed. The effect seen in liver at these high *N*B-DNJ concentrations is transient and in muscle is stable over time, and no glycogen accumulation in the heart could be observed. However, *N*N-DNJ treatment did lead to potential adverse levels of glycogen accumulation at the doses tested. This may prove to be a factor when evaluating the therapeutic range of imino sugar with long alkyl chains and glucosidase inhibitory properties, depending on the therapeutic dose required. In addition to the clinical implication of these studies, imino sugars may prove to be useful tools for generating models of glycogenoses.

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