Glucosylated free oligosaccharides are biomarkers of endoplasmic reticulum α-glucosidase inhibition

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The inhibition of ER (endoplasmic reticulum) α-glucosidases I and II by imino sugars, including NB-DNJ (N-butyl-deoxy- nojirimycin), causes the retention of glucose residues on N-linked oligosaccharides. Therefore, normal glycoprotein trafficking and processing through the glycosylation pathway is abrogated and misfolded glycoproteins are directed to undergo ERAD (ER-associated degradation), a consequence of which is the production of cytosolic FOS (free oligosaccharides). Following treatment with NB-DNJ, FOS were extracted from cells, murine tissues and human plasma and urine. Improved protocols for analysis were developed using ion-exchange chromatography followed by fluorescent labelling with 2-AA (2-aminobenzoic acid) and purification by lectin-affinity chromatography. Separation of 2-AA-labelled FOS by HPLC provided a rapid and sensitive method that enabled the detection of all FOS species resulting from the degradation of glycoproteins exported from the ER. The generation of oligosaccharides derived from glucosylated protein degradation was rapid, reversible, and time- and inhibitor concentration-dependent in cultured cells and in vivo. Long-term inhibition in cultured cells and in vivo indicated a slow rate of clearance of glucosylated FOS. In mouse and human urine, glucosylated FOS were detected as a result of transrenal excretion and provide unique and quantifiable biomarkers of ER- glucosidase inhibition.

Key words: deoxynojirimycin, glycoprotein, oligosaccharide degradation, renal excretion.

INTRODUCTION

The N-alkylated glucose analogue, NB-DNJ (N-butyl-deoxy- nojirimycin) [1–4], is an inhibitor of both N-glycan processing enzymes of the ER (endoplasmic reticulum), α-glucosidases I and II, and ceramide-specific glucosyltransferase, a key enzyme in the glycolipid biosynthetic pathway [5,6]. NB-DNJ has been developed as an effective treatment, known as SRT (substrate reduction therapy), for lysosomal storage diseases, in particular type I Gaucher disease, for which clinical trials have been undertaken [7]. NB-DNJ (miglustat, Zavesca®) is now an approved medicine in the U.S.A., Europe and Israel for this disorder. Clinically, a partial inhibition of the ceramide-specific glucosyltransferase by NB-DNJ is required to reduce glycolipid substrate synthesis at doses where side effects, such as glucosidase inhibition, are minimized. However, glucosidase inhibition also has therapeutic utility in reducing viral infectivity. Disruption of N-linked oligosaccharide processing by imino sugar-mediated ER glucosidase inhibition perturbs virus-sensitive protein-folding pathways in the cell [8,9].

Imino sugars quickly and efficiently cross the plasma membrane, such that the concentration of imino sugars in the cytosol is at equilibrium with the extracellular concentration [2]. In the cytosol, imino sugars interact directly with the ceramide-specific glucosyltransferase on the cytosolic side of the cis-Golgi, inhibiting glycolipid biosynthesis. However, to modulate N-linked oligosaccharide processing by glucosidase inhibition, imino sugars have to gain entry to the ER lumen. The rate of entry into the ER is unknown, but the concentration of imino sugar is assumed to be much lower in the ER lumen than is supplied exogenously to the cell. Evidence for this comes from cellular experiments where the concentration of N-alkylated DNJ-based imino sugars required to inhibit ER glucosidase I has been measured, often requiring 1000–10 000 times that which inhibits the purified enzyme in vitro [10].

Following access to the lumen of the ER, DNJ and its alkylated analogues inhibit the removal of glucose residues, mediated by α-glucosidases I and II. This results in a large increase in the amount of glucosylated high-mannose containing oligosaccharides present on glycoproteins that are unable to utilize the calnexin- and calreticulin-mediated folding pathways. These misfolded glycoproteins enter the ERAD (ER-associated degradation) pathway for subsequent removal of the oligosaccharide, and protein degradation. Therefore, an increase in glucosylated FOS (free oligosaccharides) in the presence of glucosidase inhibitors is observed in the cell [3]. Glucosylated FOS may also be produced as a result of the release of GlcManGlcNAc2 by ER-oligosaccharyltransferase-mediated hydrolysis of the dolichol precursor [11] which serves to control the size of the lipid-linked pool. Previous work has examined the relative level of FOS production by these separate pathways using virus infected, or non-infected, cells treated with glucosidase inhibitors and has shown that the FOS produced are almost exclusively protein derived [12] (D. S. Alonzi, D. C. A. Neville and T. D. Butters, unpublished work).

The removal of misfolded proteins from the ER and the production of FOS is a normal cellular process. Calnexin- or calreticulin-dependent, aberrantly-folded proteins and hyperglycosylated, aberrantly-folded proteins are ultimately translocated out of the ER into the cytosol via the Sec61p channel [13]. The N-linked oligosaccharide is subsequently released by a cytosolic PNGase (peptide:N-glycanase) (which may or may not be in

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Abbreviations used: 2-AA, 2-aminobenzoic acid; ConA, concanavalin A; ENGase, endo-β-N-acetylgalactosaminidase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FOS, free oligosaccharides; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; NB-DNJ, N-butyl-deoxy- nojirimycin; NP-HPLC, normal-phase HPLC; PGC, porous graphitized carbon; PNGase, peptide:N-glycanase; SRT, substrate reduction therapy; TFA, trifluoroacetic acid.
direct interaction with the Sec61p channel) [14,15], producing FOS. This process of selective protein export from the ER to the Golgi is termed ERAD. Cytosolic FOS produced are substrates for ENGase (endo-β-N-acetylglucosaminidase) [16] and cytosolic α-mannosidase [17], ultimately forming a Man₅GlcNAc₂ species that is transported to the lysosome. However, glucosylated FOS are unable to gain entry to the lysosome for degradation [18] and their fate remains to be determined. In addition to Man₅GlcNAc₂, other small amounts of FOS, including Glc₃Man₅GlcNAc, are produced by glycoproteins entering the ERAD pathway and represent the normal default pathway for degradation [3].

Previously, we have shown that N-alkylated DNJ imino sugars inhibit ER glucosidases, causing both an increase in the levels of cellular FOS and a change in the composition of the free oligosaccharide produced, i.e., an increase in the mono- and tri-glucosylated species was observed [3]. The present study optimizes the recovery of glucosylated FOS to allow full structural characterization of 2-AA (2-aminobenzoic acid)-fluorescently-labelled glycans using HPLC analysis, enzyme digestion and MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS. The FOS produced as a result of NB-DNJ treatment in vivo have been measured in different tissues, and the eventual fate of glucosylated FOS have been determined.

EXPERIMENTAL

Materials

Tissue culture media were from Gibco/Invitrogen or Sigma. AnalaR and HPLC grade solvents were from VWR International. All other reagents were from Sigma. Water was Milli-Q grade. NB-DNJ was provided by Celltech.

Cell culture

The human acute myeloid leukaemia cell line HL60 was cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen).

NB-DNJ animal dosing

All animal experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. FVB/N or C57Bl/6 mice were maintained on a diet of powdered chow mixed with NB-DNJ at a range of dose levels up to 2400 mg/kg/day for 5 weeks as described previously [19]. Body fluids and tissues were harvested and stored at −20 °C before extraction.

Clinical samples

Research ethics committee approval was given for the use of all clinical samples. Written informed consent was obtained from patients and/or their parents/legal guardians. Samples of plasma were obtained from an adult patient with Niemann-Pick disease type C treated with NB-DNJ (miglustat) for 16 months at various doses, until the last 3 months before analysis when 100 mg thrice daily was administered, as described in [20]. Urine samples were analysed from a juvenile Sandhoff disease patient treated with NB-DNJ for 6 months at a dose of 300 mg daily.

FOS isolation

HL60 cells were cultured to high density (1 × 10⁷ cells/ml) prior to growth in fresh medium containing NB-DNJ at various concentrations. The cells were seeded at a lower density to achieve a high density at the end of the incubation period. Following cell culture, the medium was removed and the cells were washed three times with PBS by centrifugation. Washed cells were stored at −20 °C for a short time before thawing and Dounce homogenization in water. An aliquot was taken for protein concentration determination using the Pierce BCA (bicinchoninic acid) protein assay reagent, following the manufacturer’s instructions. The maximum recovery of FOS was performed using the following conditions. The homogenate from 1–2 × 10⁶ cells (0.1–0.2 mg protein) was desalted and deproteinated by passage through a mixed-bed ion-exchange column [0.2 ml of AG50W-X12 (H⁺, 100-200 mesh) over 0.4 ml of AG3-X4 (OH−, 100-200 mesh)], pre-equilibrated with water (5 × 1 ml). The homogenate was added to the column which was washed with 4 × 1 ml of water, and the eluate collected. The extracted and purified FOS were then dried under vacuum or by lyophilizing.

Tissues (25 mg wet weight) were frozen and thawed in 1 ml of water before homogenization using a polytron. Murine serum and urine (100 µl), and human plasma (100 µl) and urine (1 ml) were used directly for FOS extraction as described above.

PGC (porous graphitized carbon) chromatography

Glucose contained in the tissue extract from the mixed-bed ion-exchange column was removed prior to labelling using a 1 ml (25 mg) PGC column (Thermo Electron). The column was pre-equilibrated with 1 ml of methanol, followed by 1 ml of water, 1 ml of acetonitrile containing 0.1% TFA (trifluoroacetic acid) and finally 2 × 0.5 ml of water. After sample loading, the column was washed with 2 × 0.5 ml of water before oligosaccharides were eluted with 2 ml of 50% acetonitrile containing 0.1% TFA.

Carbohydrate fluorescent labelling

The FOS were labelled with 2-AA and purified using DPA-6S columns as described previously [21]. Excess free, unconjugated 2-AA was removed following phase splitting using ethyl acetate. The 2-AA-labelled sample (1 ml in water) was added to 1.5 ml of ethyl acetate and vortexed before separating into two phases by centrifugation at 3000 g for 1 min at room temperature (25 °C). The upper phase was removed and a further 1.5 ml of ethyl acetate was added and separation was repeated. Following a further addition of ethyl acetate, the lower phase was removed and dried. The sample was then resuspended in 30 µl of water before a preparative run by HPLC to allow isolation of individual peaks for further analysis.

Purification of fluorescently labelled FOS

Labelled oligosaccharides in 50 mM Tris/HCl buffer, pH 7.2, were purified using a ConA (Concanavalin A)-Sepharose 4B column (100 µl packed resin). The column was pre-equilibrated with 2 × 1 ml of water followed by sequential 1 ml washes of 1 mM MgCl₂, 1 mM CaCl₂, 1 mM MnCl₂ and 2 × 1 ml of 50 mM Tris/HCl buffer, pH 7.2. The sample was added and allowed to pass through the column before washing with 2 × 1 ml of 50 mM Tris/HCl buffer, pH 7.2. The ConA-bound FOS were then eluted with 2 × 1 ml of hot (70 °C) 0.5 M methyl α-D-mannopyranoside in 50 mM Tris/HCl buffer, pH 7.2.

Methyl α-D-mannopyranoside was removed from ConA–Sepharose-purified 2-AA-labelled oligosaccharides in readiness for enzyme digestion using PGC chromatography as described above. No loss of FOS was observed following this procedure (results not shown).

Carbohydrate analysis by NP-HPLC (normal-phase HPLC)

ConA-Sepharose purified 2-AA-labelled oligosaccharides were separated by NP-HPLC (Waters) using a 4.6 × 250 mm TSKgel
Amide-80 column (Anachem) with slight modifications to the published method [21]. Glucose units were determined, following comparison with a 2-AA-labelled glucose oligomer ladder (derived from a partial hydrolysate of dextran) external standard using Peak Time software (developed in-house).

The peak area of each 2-AA-labelled species was measured using Waters Empower software and converted into molar amounts using an experimentally-derived conversion factor (i.e., using 2-AA labelling, comparing a standard oligosaccharide of known concentration and measurement of the peak area following HPLC separation).

**Enzyme digests**

Glycosidase digests were performed on the complete FOS populations and individually isolated peaks. Rat liver α-glucosidase I (0.1 M sodium phosphate buffer, pH 7, containing 0.8 % Lubrol-PX, 100 units/ml) and α-glucosidase II (80 mM triethylamine buffer, pH 7, containing 0.15 M NaCl and 10 % glycerol, 5 m-units/ml) were purified as previously described [22,23]. *Aspergillus saitoi* α,1,2-mannosidase (50 mM sodium acetate buffer, pH 5, 0.46 m-units/ml) and jack bean α-mannosidase (10 mM citric acid/sodium citrate buffer, pH 4.5, 0.46 m-units/ml) were purified in-house. Following enzyme treatment for 16 h at 37 °C, the reaction was stopped by addition of an equal volume of acetonitrile. Following enzyme treatment for 16 h at 37 °C, the reaction was stopped by addition of an equal volume of acetonitrile. The oligosaccharide reaction products were obtained after centrifugation through a 10,000 Da molecular weight cut-off filter (pre-washed with 150 µl of water) at 7000 g for 45 min at room temperature (25 °C), to remove proteins before HPLC analysis.

**In vitro glucosidase inhibition**

Free oligosaccharides were isolated from HL60 cells treated with 1 mM NB-DNJ, 2-AA-labelled and purified as substrates for either α-glucosidase I or II. Each fluorescently-labelled substrate was incubated with sufficient α-glucosidase I to generate 25 % hydrolysis of Glc3Man,GlcNAc or Glc3Man,GlcNAc in a 30 min reaction time. Similarly, α-glucosidase II was incubated for 2 h with Glc3Man,GlcNAc and for 20 min with Glc3Man,GlcNAc. In all cases, linear degradation of substrate occurred over the time of incubation. Reactions were performed in the presence of various concentrations of NB-DNJ, stopped by the addition of 30 µl acetonitrile and treated to remove protein as described above. Following HPLC separation of the reaction products, the amount of digestion was quantified using peak area analysis.

**MALDI-TOF MS of FOS**

Positive-ion MALDI-TOF MS were recorded with a Micromass TofSpec 2E reflectron-TOF mass spectrometer (Waters) fitted with delayed extraction and a nitrogen laser (337 nm). The acceleration voltage was 20 kV, the pulse voltage was 3000 V, with delayed extraction and a nitrogen laser (337 nm). The TofSpec 2E reflectron-TOF mass spectrometer (Waters) fitted in 100 µl of water. A 1 µl aliquot of each was mixed with 1 µl of a 50 mg/ml solution of 2,5-DHB (2,5-dihydroxybenzoic acid) in acetonitrile and allowed to air dry. The samples were re-crystallized from ethanol prior to analysis [24]. The m/z scale was calibrated using a 2-AA-labelled dextran hydrolysate ladder standard.

**NB-DNJ extraction and purification from tissue or plasma samples**

Plasma samples were centrifuged at 11,600 g for 5 min and the supernatant was made up to 400 µl with water and 1.0 µg of the imino sugar internal standard (N-propyl-DNJ) was added. Tissue samples were homogenized using an Ultra-Turrax T25 probe in 10 % methanol in water (v/v) at concentrations of 130 mg (wt weight/ml). After addition of an internal standard (as above), the homogenates were centrifuged at 100,000 g for 15 min at 2 °C. The supernatants were then used for imino sugar purification as described in [25].

**NB-DNJ quantification following cation-exchange chromatography**

A Dionex BioLC with an ED50 electrochemical detector was used in all experiments. A CS10 analytical column (4 x 250 mm) was isocratically eluted with a solvent containing 50 mM sodium sulphate, 2.5 mM sulphuric acid and 5 % (v/v) acetonitrile. The flow rate was 1 ml/min and the column was maintained at a temperature of 40 °C. A CMMS-II cation micro-membrane suppressor was used, with water as the regenerant, to convert the acidic eluant to basic conditions suitable for electrochemical detection. A triple-potential waveform was used for detection of carboxyanions. Imino sugar purified from plasma or tissue was analysed as described, in duplicate. The area under each peak, corresponding to the internal standard and NB-DNJ, was measured and, after application of a predetermined response factor for the two imino sugars, the amount of NB-DNJ was calculated [25].

**RESULTS**

**Characterization of FOS produced in HL60 cells in the presence/absence of 1 mM NB-DNJ**

Previously we have analysed the effect of NB-DNJ on the generation of FOS in HL60 cells to gain insights into important cellular processes that participate in the glycoprotein-folding pathway [3]. A simplified FOS isolation protocol, with accurate extraction and purification efficiency determination, has been developed that negates the use of organic solvents and phase separation, as was previously employed [3]. This new method gave a greater than 90 % recovery of total FOS as demonstrated by the recovery of known amounts of unlabelled oligosaccharides (see Supplementary Table 1, http://www.BiochemJ.org/bj/409/bj4090571add.htm). The use of hot (70 °C) methyl-α-D-mannopyranoside was necessary to maximize recovery of oligosaccharides bound to Con A-Sepharose. The FOS species were analysed by HPLC following 2-AA fluorescent labelling and the structures were characterized following a combination of enzyme digests (α-glucosidases I and II, *A. saitoi* α,1,2-mannosidase and jack bean α-mannosidase), MALDI-TOF MS analysis and by comparison of glucose unit (GU) values to known oligosaccharide standards. The combined results from these experiments enabled us to compile a library of FOS structures in cells, including the percentage of each structure in terms of the total FOS population, following a 24 h period in the absence or presence of NB-DNJ (Table 1). The control FOS (Figure 1A and Table 1) showed a range of structures, with the major component (peak 4, 50.81 %) being a Man,GlcNAc species with the structure Man2Man2Man2Man3-(Man6)Man4GlcNAc. There was also, interestingly, a major component that was monoglucosylated, Man,GlcNAc, (Glc3Man,GlcNAc, peak 5, 16.09 %) as well as further high-mannose structures similar to those found in previous studies [26,27]. The addition of the α-glucosidase inhibitor, NB-DNJ, induced cells to produce a nearly 3-fold increase in total FOS, from 544 pmol/mg to 1470 pmol/mg. This increase was due to the production of mono-, di- and tri-glucosylated FOS.
Table 1  FOS in HL60 cells following treatment with NB-DNJ

Structures, glucose unit values and proportions of FOS isolated peaks produced in HL60 cells in control cells (A) and following treatment with 1 mM NB-DNJ for 24 h (B). The numbers of the isolated peak correspond to the numbers in Figure 1. The nomenclature described in this paper refers to the structures below. The mean value from three experiments is shown +− S.D. * FOS structures observed in mouse tissue, serum and urine, and human plasma and urine, see Figures 4, 5 and 7.

<table>
<thead>
<tr>
<th>Isolated peak number</th>
<th>Glucose unit</th>
<th>Structure</th>
<th>Percentage in control cells</th>
<th>Percentage in treated cells</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.98</td>
<td>Man₅GlcNAc₅</td>
<td>1.07 ± 0.21</td>
<td>0.24 ± 0.01</td>
<td>core</td>
</tr>
<tr>
<td>1A</td>
<td>4.14</td>
<td>Contaminant</td>
<td>–</td>
<td>0.41 ± 0.02</td>
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</tr>
<tr>
<td>1B</td>
<td>4.56</td>
<td>Man₅GlcNAc₅</td>
<td>–</td>
<td>0.09 ± 0.01</td>
<td>linear</td>
</tr>
<tr>
<td>2</td>
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<td></td>
</tr>
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<td>2A</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>5.66</td>
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<td>0.59 ± 0.02</td>
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</tr>
<tr>
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<td>4C*</td>
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<td>Man₅GlcNAc₅</td>
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<td>–</td>
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<td>–</td>
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<td>–</td>
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</tr>
<tr>
<td>6A*</td>
<td>6.93</td>
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<td>7</td>
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<td>8</td>
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<td>–</td>
<td>/</td>
</tr>
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<td>0.3 ± 0.02</td>
<td>–</td>
<td></td>
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<tr>
<td>10</td>
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<td>0.92 ± 0.42</td>
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<td>–</td>
<td>4.15 ± 0.56</td>
<td>D2 missing</td>
</tr>
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<td>14</td>
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<tr>
<td>14A</td>
<td>10.29</td>
<td>Glc₃Man₅GlcNAc₅</td>
<td>–</td>
<td>0.9 ± 0.02</td>
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species (Figure 1B and Table 1), with the major FOS being a tri-glucosylated Man₅-based species (Glc₃Man₅GlcNAc₅, peak 10A, 34%). These results are qualitatively similar to those reported previously using 2-aminobenzamide-labelling of large FOS isolated from HL60 cells treated for 16 h with 1 mM NB-DNJ, but the improved method described here has increased recovery by 2.5–3.0-fold [3]. In our previous study [3], a triglucosylated high-mannose oligosaccharide
Glucosidase inhibition in vivo

Figure 1 HPLC analysis of 2-AA fluorescently-labelled FOS

HL60 cells were homogenized and FOS extracted as described in the Materials and methods section. Following 2-AA labelling, FOS were separated by HPLC. (A) Control cells and (B) cells treated with 1 mM NB-DNJ for 24 h. Peaks are numbered and structurally annotated as shown in Table 1.

containing a single GlcNAc reducing terminus (predicted to be Glc1Man5GlcNAc1), comprised 16.5 % of the total population. The increase in this triglucosylated species, using an increased time point (24 h) in the present study, is consistent with a greater effect on α-glucosidase I inhibition (see Figure 2A).

Kinetics of α-glucosidase inhibition

The results reported here support the idea that the measurement of FOS produced following imino sugar treatment may be used as a cellular–based assay for α-glucosidase I and/or II inhibition. The build up of both the mono-glucosylated and the tri-glucosylated Man5-based species were readily followed after 1 mM NB-DNJ treatment (Figure 2A). The initial step in the inhibition of N-linked oligosaccharide processing appears to be mediated by the reduction of α-glucosidase II activity, as indicated by the rapid increase in the amount of Glc1Man5GlcNAc1 observed (Figure 2A) before an equally rapid decline to control or below control levels. This increase, 153.5 ± 3.0 to 169.3 ± 5.0, was significant (95 % confidence limits, Student’s t test). A lag period before the effects of the inhibition of α-glucosidase I, resulting in the subsequent production of Glc1Man5GlcNAc1, was observed, supporting the preferential inhibition of α-glucosidase II seen in Figure 2(A). This inhibition of glucosidase II in cultured cells was in direct contrast to the in vitro inhibition by NB-DNJ (Table 2). The inhibition of glucosidase II in hydrolysing Glc1Man5GlcNAc1 was approximately 100-fold weaker than inhibition of glucosidase I using a triglucosylated substrate, irrespective of mannose structure or number of N-acetylglucosamine residues at the reducing terminus.

In cells, the amount of inhibition of α-glucosidase I activity in the presence of NB-DNJ, demonstrated by Glc1Man5GlcNAc1 as a FOS marker, reached a plateau by 24 h, indicating that the maximum effective ER concentration of inhibitor had been achieved. Therefore, the concentration dependence of the α-glucosidase inhibition was investigated. Various concentrations of NB-DNJ (0–1 mM NB-DNJ) were administered to HL60 cells for 24 h (Figure 2B). A concentration-dependent increase in Glc1Man5GlcNAc1 was observed, with a near maximal level of tri-glucosylated FOS being reached at 1 mM.

Fate of FOS in cells

Previously published experiments examining FOS biosynthesis and degradation have been carried out over short time periods using metabolically labelled substrate [12,27]. The method developed here enables the study of cells that have undergone...
longer-term treatment with imino sugars. This method also allowed the study of the cellular fate of the FOS following removal of inhibition (Figure 3). NB-DNJ treatment for 72 h (Figure 3C) showed not only the build up of Glc3Man4GlcNAc1, as seen after 24 h (Figure 3B), but also of Glc2Man3GlcNAc1. This indicated that Glc2Man3GlcNAc1 was further degraded in the cell and that cytosolic α-mannosidase was most probably, the enzyme responsible. However, the hydrolysis of Glc3Man3GlcNAc1 to Glc2Man4GlcNAc1 occurred at a much-reduced rate when compared to the hydrolysis of Glc3Man4GlcNAc1 to Glc2Man5GlcNAc1.

To investigate the origin of this Glc3Man3GlcNAc1 species, HL60 cells were treated for 24 h with 1 mM NB-DNJ to generate Glc3Man3GlcNAc1 in the cytosol before removing the imino sugar and hence the α-glucosidase blockage. The cells were allowed to recover for 72 h before purification and analysis of the FOS (Figure 3D). The disappearance of the majority of the hyperglucosylated species was observed. However, a significant, though reduced, amount of the Glc3Man3GlcNAc1 species was still present. These results indicate that the inhibition of ER-glucosidases was reversible but glucosylated FOS were retained in the cytosol before eventual clearance. The cytosolic α-mannosidase, or an as yet unidentified enzyme, is able to remove the accessible 4-Mano6 mannose residue to leave a linear Glc2Man5GlcNAc1 species. The analysis of FOS 120 h post-treatment revealed the continued presence of this tri-glucosylated FOS (results not shown). The eventual fate of glucosylated FOS was therefore examined in vivo.

Effect of NB-DNJ on mouse tissue FOS production

FVB/N mice were treated with 1200 mg/kg/day of NB-DNJ for 5 weeks to maintain a steady state of NB-DNJ in their serum. An overnight urine collection after 5 weeks treatment was performed using a metabolic cage. Heart, kidney, lung, liver, brain and spleen (25 mg wet weight tissue) were used for FOS analysis. These were compared to the same samples obtained from age-matched untreated FVB/N mice. The effect of NB-DNJ on total FOS production was pronounced in all samples, with a significant increase (at the 95% confidence level) in FOS amounts (Table 3, and Supplementary Table 2, http://www.BiochemJ.org/bj/409/bj4090571add.htm) and the appearance of glucosylated high-mannose FOS in the samples (Figure 4 and Table 1). In heart samples (Figure 4A), Glc3Man3GlcNAc1 was present in the largest amount (peak 6A, 37%) with the appearance of a lesser amount of Glc2Man4GlcNAc1 (peak 2A, 17.1%). The oligosaccharide Glc2Man3GlcNAc1 was the major glycan observed following treatment. This is believed to be a GlcNAc2-containing FOS, as demonstrated by MALDI-TOF MS analysis (results not shown), with the structure Glc2Man3GlcNAc1. The origin of this chitobiose-containing species is being explored further. In brain, the appearance of a small but significant amount of Glc2Man3GlcNAc1 (peak 5, 6.3%), Figure 4E) was seen, with a small increase in Glc2Man4GlcNAc1 also observed. However, in brain samples, the non-glucosylated species were still the majority of FOS observed. The liver had the largest amount of FOS observed prior to and following NB-DNJ treatment (29.4 and 86.2 pmol/mg, respectively). The heart differed from the other tissues examined, as Glc3Man3GlcNAc1 was the major glycan observed following treatment. This is probably due to a greater inhibition of α-glucosidase I rather than α-glucosidase II in this tissue. The occurrence of greater α-glucosidase I inhibition was also seen in the hind muscle and red blood cells of mice treated with NB-DNJ (results not shown). In spleen, greater inhibition of α-glucosidase II was again observed, consistent with the production of a Glc2Man4GlcNAc1 species.

Murine serum and urine were analysed for FOS (Figure 5 and Table 3) and sera were also used to determine NB-DNJ concentration. An average value of 26.4 μM NB-DNJ was measured.

| Table 3 FOS in murine tissues, serum and urine following treatment with NB-DNJ |
|-----------------------------------------------|-------|-----|
| Mouse tissue/sample                          | Total FOS |
|                                               | − NB-DNJ | + NB-DNJ |
| Heart                                         | 5.4 ± 0.1 pmol/mg | 12.6 ± 5.0 pmol/mg |
| Kidney                                        | 16.8 ± 1.9 pmol/mg | 52.9 ± 21.0 pmol/mg |
| Lung                                          | 12.0 ± 0.6 pmol/mg | 28.9 ± 3.6 pmol/mg |
| Liver                                         | 29.4 ± 5.8 pmol/mg | 86.2 ± 12.4 pmol/mg |
| Brain                                         | 3.2 ± 0.2 pmol/mg | 5.4 ± 1.0 pmol/mg |
| Spleen                                        | 4.1 ± 0.7 pmol/mg | 7.2 ± 2.3 pmol/mg |
| Serum                                         | 5.76 ± 0.8 nmol/ml | 12.4 ± 2.6 nmol/ml |
| Urine                                         | 10.6 ± 5.6 nmol/ml | 31.9 ± 7.2 nmol/ml |

Total amounts of FOS, pmol/mg (wet weight) in tissue samples and nmol/ml in serum and urine, were calculated from peak areas of all high mannose oligosaccharide species containing greater than three mannose residues having one or more core N-acetylglucosamine residues after analytical separation by NP-HPLC. The mean value from five animals per group is shown ± S.D. The increase in FOS in all tissues was significant at the 95% confidence level using Student’s t test.
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Figure 4 HPLC analysis of 2-AA fluorescently-labelled FOS from murine tissues

(A) Heart, (B) kidney, (C) lung, (D) liver, (E) brain and (F) spleen tissue from FVB/N control mice (dark line) and mice treated with 1200 mg/kg/day of NB-DNJ (light line) were homogenized and FOS extracted as described in the text. Following 2-AA labelling, FOS were separated using NP-HPLC. Peaks are numbered and structurally annotated as shown in Table 1.

found in treated mice. The FOS analysis closely matched the situation observed in tissues, with Glc1Man4GlcNAc1 (peak 2A, Figure 5 and Table 2) being the major glucosylated FOS produced following NB-DNJ treatment. Again, there was a statistically significant (95% confidence level, Student’s t test) increase in the total amount of FOS measured with an approximate 2-fold and 3-fold increase in serum and urine respectively. The Glc1Man3GlcNAc1 species, therefore, may represent the end-clearance product of glucosylated FOS from murine tissues. This analysis also demonstrated that the clearance of higher glucosylated FOS from tissues to urine, via serum, was also possible, as Glc1Man3GlcNAc1 and Glc1Man5GlcNAc2 FOS, observed in both tissues and serum, were also found in urine.

NB-DNJ production of glucosylated FOS in murine serum is time- and dose-dependent

Serum was obtained from mice (n = 3) treated with 2400 mg/kg/day of NB-DNJ for 2, 5, 9, 13 and 17 days. The serum was pooled and an aliquot was used for FOS analysis (Figure 6A). The level of Glc1Man3GlcNAc1 produced in serum, as a result of NB-DNJ treatment, reached a plateau after 5 days. A similar effect was seen on Glc1Man3GlcNAc1 levels in the serum of mice treated with escalating doses of NB-DNJ for 5 weeks, with a maximal level achieved at the highest dose, 2400 mg/kg/day (Figure 6B). This matches those results in cultured cells where there was a time- and concentration-dependent production of glucosylated FOS.

Reversal of α-glucosidase inhibition

Mice (C57Bl/6) were treated with 1200 mg/kg/day NB-DNJ for 5 weeks, prior to removal from the drug-treated diet, and placed in a metabolic cage for 0.5, 1, 2, 4, 6 and 8 h before being killed. The sera and urine of these animals were then used for FOS analysis and NB-DNJ concentration determination in the serum (Figure 6C). The amount of NB-DNJ declined rapidly in the serum following drug removal and was undetectable after 4 h, with an estimated half-life of 3.72 h. The FOS produced as a result of NB-DNJ-induced glucosidase inhibition had a similar serum half-life of 1.86 h and were finally cleared transrenally to urine with a half-life of 3.72 h. It was noticed that, following NB-DNJ treatment, the thymus was significantly reduced in size, as previously reported by this laboratory [19]. Thymi of mice killed following a 4 h removal from NB-DNJ were similar in appearance to control mice, suggesting that the effect on thymus acellularity is a direct effect of the drug. Following the clearance of NB-DNJ from serum, there were lag periods of approximately 4 h and 12 h until glucosylated FOS returned to control levels in serum and in urine respectively.

FOS in human samples following NB-DNJ treatment

To demonstrate that this technique was applicable to human samples, plasma and urine were obtained from human patients with lysosomal storage disorders that were undertaking SRT with NB-DNJ. Pre- and post-treatment samples were analysed for

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Figure 6 Glucosylated FOS in serum of mice treated with NB-DNJ is time- and dose-dependent, and reversible

(A) Monoglucosylated FOS (Glc\textsubscript{1}Man\textsubscript{4}GlcNAc\textsubscript{1}) in pooled serum (nmol/ml) of C57Bl/6 mice following treatment with 2400 mg/kg/day of NB-DNJ for 0–17 days. (B) Monoglucosylated FOS (Glc\textsubscript{1}Man\textsubscript{4}GlcNAc\textsubscript{1}) in serum (nmol/ml) of C57Bl/6 mice following treatment with 0–2400 mg/kg/day NB-DNJ for 5 weeks. (C) Monoglucosylated FOS (Glc\textsubscript{1}Man\textsubscript{4}GlcNAc\textsubscript{1}) in serum (■) and pooled urine (▲) expressed as nmol/ml, in C57Bl/6 mice following treatment with 1200 mg/kg/day of NB-DNJ for 5 weeks and subsequent removal for 0.5–16 h. NB-DNJ concentration in serum (µg/ml) over the same time period is shown (■). Where appropriate, the mean value for five animals per treatment group is shown, ±S.D.

Figure 7 HPLC analysis of 2-AA fluorescently-labelled FOS from human serum and urine

FOS were extracted from human serum and urine, pre-treatment (dark line) and post-treatment (light line) with NB-DNJ as described in the text. Following 2-AA labelling, FOS were separated by NP-HPLC. (A) Serum from a Niemann-Pick disease type C patient treated with 100–300 mg/day of NB-DNJ for 16 months and (B) urine from a juvenile Sandhoff patient treated with 300 mg/day of NB-DNJ for 6 months. Peaks are numbered and structurally annotated as shown in Table 1.

DISCUSSION

The aims of this study were to understand and characterise alterations in the biochemical pathways induced by iminosugar glucosidase inhibitors. Therefore, we have developed protocols that accurately measure FOS produced in cells as a functional consequence of ER-glucosidase inhibition. In the absence or presence of α-glucosidase inhibitors, misfolded glycoproteins are translocated to the lysosome where PNGase releases oligosaccharides and the protein is degraded by the proteosome. The FOS are substrates for cytosolic ENGase, which generates FOS containing a single reducing-terminal GlcNAc [28], and subsequently cytosolic α-mannosidase, which preferentially recognises these FOS that are trimmed to a Man\textsubscript{5}GlcNAc\textsubscript{1} structure [29–31]. This structure, Man\textsubscript{2}Man\textsubscript{2}Man\textsubscript{2}Man\textsubscript{2}(Man\textsubscript{6})Man\textsubscript{4}GlcNAc, is similar to the Man\textsubscript{5}GlcNAc\textsubscript{2} structure observed attached to dolichol on the extracellular leaflet of the ER. An ATP-dependent process subsequently translocates the non-glucosylated Man\textsubscript{5}GlcNAc\textsubscript{1} into the lysosome for degradation [30]. With a functional FOS catabolic pathway in cells it is no surprise that Man\textsubscript{5}GlcNAc\textsubscript{1} is the major species in control cells. This species may represent the normal flux of misfolded protein secreted via the calnexin/calreticulin-mediated ERAD pathway and is either resident in the cytoplasm or the lysosome.

The major species observed following NB-DNJ treatment in HL60 cells was a Glc\textsubscript{3}Man\textsubscript{5}GlcNAc\textsubscript{1} oligosaccharide derived subsequent to retrotranslocation of misfolded and hyperglycosylated proteins from the ER to the cytosol. This oligosaccharide may be unable to translocate into the lysosome for degradation since there is some evidence of the lack of an efficient glucosylated FOS lysosomal transporter [18,32]. Therefore, an increase in FOS is observed in agreement with previous studies [3].

When high concentrations of NB-DNJ (1 mM) were used to treat cells, α-glucosidase inhibition reached a plateau, indicative of a time-dependent NB-DNJ equilibration rate (cytosol–ER).
The initial build up of mono-glucosylated FOS at short time periods (0–2 h) before triglucosylated FOS are generated reveals the potent cellular inhibition of α-glucosidase II, compared to α-glucosidase I, and contrasts markedly with the inhibition of these enzymes using in vitro assays, where, on the basis of IC_{50} values, NB-DNJ is 100 times more efficient at inhibiting α-glucosidase I than II. The very small amount of diglucosylated FOS produced demonstrates that NB-DNJ is relatively poor at preventing the removal of the first α1,2-linked glucose. This reflects the kinetics of glucosidase I and II action in the ER, resulting in the efficient and rapid hydrolysis of tri- and di-glucosylated glycans to mono-glucosylated glycans to allow interaction with calnexin/calreticulin chaperones. The concentration-dependence of NB-DNJ inhibition suggests that a maximum level of DNJ-based imino sugar is achieved in the ER, beyond which some physiological aspect of the ER prevents further import or activates exit of the imino sugars from the ER.

The longer half-life of mono-glucosylated glycans, when compared to tri- and di-glucosylated species, is due to the slower hydrolysis rate of the proximal glucose residue by glucosidase II [33–35]. This contributes to a more favourable environment for NB-DNJ inhibition of this step. Removal of the first glucose residue by glucosidase I and the second glucose residue by glucosidase II in cultured cells probably occurs at close to their limiting rates (V_{max}), where addition of a competitive inhibitor has a limited effect on the observed rate. Removal of the third glucose residue by glucosidase II is much slower, suggesting that the rate is not close to V_{max}, and under these conditions a competitive inhibitor has a much greater effect on the rate. Since glucosidase II and calnexin both compete for the same substrate in the ER, Glc_{Man}GlcNAc_{2}–protein, if glucosidase II is unable to hydrolyse the substrate bound to calnexin, the presence of calnexin will significantly reduce the free substrate concentration, hence reducing the rate. Alternatively, if glucosidase II is able to hydrolyse the substrate bound to calnexin, the presence of calnexin is likely to change the K_{m}. An increase in K_{m} would also result in a reduced rate. Both possibilities have the same functional outcome: the rate of removal of the proximal glucose residue is reduced in cells and hence is more sensitive to the presence of a competitive inhibitor, resulting in a greater accumulation of mono-glycosylated glycans.

How do cells then deal with glucosylated FOS? Following treatment with NB-DNJ (24 and 72 h) the major proportion of FOS observed are Glc_{Man}GlcNAc_{1} structures. The presence of Glc_{Man}GlcNAc_{2} FOS demonstrates that cytosolic α-mannosidase, or an unknown α-mannosidase, may act on Man$_{n}$GlcNAc$_{1}$-containing FOS. The lack of lysosomal degradation, and increased cellular residence time of glucosylated Man$_{n}$GlcNAc$_{1}$-containing FOS observed, may explain the higher concentration of glucosylated Man$_{n}$GlcNAc$_{1}$-containing FOS observed following the 72 h treatment with NB-DNJ. Long-term (72 h) treatment with NB-DNJ, where a significant quantity of Glc$_{Man}$GlcNAc$_{1}$ is generated, followed by removal of inhibitor, reveals a slow decline in the amount of cellular Glc$_{Man}$GlcNAc$_{1}$. Analysis of the extracellular medium shows that Glc$_{Man}$GlcNAc$_{1}$ is present (results not shown) and suggests that some mechanism exists for cellular export of glucosylated FOS. The recovery experiments demonstrate that mono-glucosylated FOS, which occurs naturally, and also the tri-glucosylated FOS, produced in the presence of a glucosidase inhibitor, are removed from the cell. In Lec23 cells (glucosidase I-deficient cells), Glc$_{Man}$GlcNAc$_{1}$ is eventually cleared by an unknown mechanism at cell confluence [32].

The Man$_{n}$GlcNAc$_{1}$ species observed in control cells is presumably not formed in the lysosome following the action of lysosomal α-6-mannosidase, as this enzyme preferentially removes the α6Man$_{n}$Man$_{4}$GlcNAc$_{1}$ structure [36]. Although the cellular location of this species has yet to be determined, it is probable that this is produced as a result of a cytosolic α-mannosidase activity.

The methods developed here in tissue culture cells have been extended to characterize the pathway for FOS export from tissues in vivo to reveal cellular clearance and catabolic end-products. When administered to mice, NB-DNJ causes an increase in glucosylated FOS in tissues, sera and urine, similar to the effect previously demonstrated in cultured cells. The tissues with normally high concentrations of FOS prior to NB-DNJ treatment provided the greatest increase in glucosylated FOS, presumably due to greater glycoprotein synthesis/turover rates in the cells of these tissues that would contribute to the pool of ERAD substrates. The major species produced were Glc$_{Man}$GlcNAc$_{1}$ and Glc$_{Man}$GlcNAc$_{2}$, demonstrating quite clearly that once glucosylated FOS are produced in the cytosol, the cellular catabolic endpoint is a glucosylated Man$_{n}$GlcNAc$_{2}$ structure, not a glucosylated Man$_{n}$GlcNAc$_{1}$ structure as previously thought [32]. Analysis of tissue FOS supports the low dose results in cultured cells, with a monoglucosylated species being the major species produced following treatment in most tissues. At steady-state concentrations of NB-DNJ, there was sufficient access to the ER of cells to selectively inhibit α-glucosidase II-mediated hydrolysis of the Glc$_{2}$Man$_{3}$ linkage. As observed in cultured cells, this is the more inhibitor-sensitive step in the glycosylation pathway. However, the heart appears to show preferential α-glucosidase I inhibition, as Glc$_{Man}$GlcNAc$_{2}$ was the major species produced. This is not a result of increased NB-DNJ retention in the heart [37] but could result from a low level of N-linked glycoprotein biosynthesis in the heart.

The time-dependence of NB-DNJ administration on FOS production shows that a steady-state of α-glucosidase II inhibition, as evidenced by the detection of Glc$_{Man}$GlcNAc$_{1}$, is reached after 5 days. A similar effect of α-glucosidase inhibition was seen with 1200 mg/kg/day and 2400 mg/kg/day doses, indicating that a sufficient concentration of NB-DNJ had been reached to preferentially inhibit α-glucosidase II. A previous study of the effect of 14 days of treatment of C57BL/6 mice with 1200 mg/kg/day of NB-DNJ [19], examined changes to mature glycoproteins on a specific cell type (splenocytes), whereas the FOS assay developed here is more sensitive in revealing the effects of α-glucosidase inhibition on all tissue-resident cells. The relatively short serum half-life of NB-DNJ in mouse is approximately four times less than the half-life in man [7], in keeping with the higher metabolic rate per kilogram mass seen in mouse compared to man [38].

The present study has demonstrated that analysis of FOS in ER-α-glucosidase inhibitor-treated higher vertebrates reveals accurate and measurable biomarkers of the effects of NB-DNJ administration. The protocols developed here have shown that the FOS generation in tissue-cultured cells closely parallels that seen in both mouse and man. These protocols can be used non-invasively to analyse the effects on ER-glucosidase inhibition on protein folding by monitoring for the presence of glucosylated FOS in urine.

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