Cellular effects of deoxynojirimycin analogues: inhibition of N-linked oligosaccharide processing and generation of free glucosylated oligosaccharides

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In the accompanying paper [Mellor, Neville, Harvey, Platt, Dwek and Butters (2004) *Biochem. J.* 381, 861–866] we treated HL60 cells with N-alk(en)yl-deoxynojirimycin (DNJ) compounds to inhibit glucosphingolipid (GSL) biosynthesis and identified a number of non-GSL-derived, small, free oligosaccharides (FOS) most likely produced due to inhibition of the oligosaccharide-processing enzymes α -glucosidases I and II. When HL60 cells were treated with concentrations of N-alk(en)ylated DNJ analogues that inhibited GSL biosynthesis completely, *N*-butyl- and *N*-nonyl-DNJ inhibited endoplasmic reticulum (ER) glucosidases I and II, but octadecyl-DNJ did not, probably due to the lack of ER lumen access for this novel, long-chain derivative. Glucosidase inhibition resulted in the appearance of free Glc_{1–3}Man structures, which is evidence of Golgi glycoprotein endomannosidase processing of oligosaccharides with retained glucose residues. Additional large FOS was also detected in cells following a 16 h treatment with *N*-butyl- and *N*-nonyl-DNJ. When these FOS structures (> 30, including > 20 species not present in

control cells) were characterized by enzyme digests and MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS, all were found to be polymannose-type oligosaccharides, of which the majority were glucosylated and had only one reducing terminal GlcNAc (*N*-acetylglucosamine) residue (FOS-GlcNAc₁), demonstrating a cytosolic location. These results support the proposal that the increase in glucosylated FOS results from enzyme-mediated cytosolic cleavage of oligosaccharides from glycoproteins exported from the ER because of misfolding or excessive retention. Importantly, the present study characterizes the cellular properties of DNJs further and demonstrates that side-chain modifications allow selective inhibition of protein and lipid glycosylation pathways. This represents the most detailed characterization of the FOS structures arising from ER α -glucosidase inhibition to date.

Key words: alkyl chain, deoxynojirimycin, endomannosidase, glucosidase, HL60 cell, imino sugar.

INTRODUCTION

N-Alkyl-deoxynojirimycin (DNJ) derivatives inhibit the ceramide glucosyltransferase (CGT, EC 2.4.1.80) involved in the first step of glucosphingolipid (GSL) biosynthesis [1,2]. In cells, these compounds rapidly inhibit the enzyme at the cytosolic face of the Golgi at concentrations consistent with their inhibition constants (see the accompanying paper [2a]). In our accompanying study [2a], where we analysed the depletion of GSL by N-alk(en)ylated DNJs using endoglycosidase release and fluorescence labelling of the oligosaccharides, it was apparent that several non-GSL-derived free oligosaccharide (FOS) species were generated in response to inhibitor treatment. Since these compounds are also inhibitors of the ER (endoplasmic reticulum) oligosaccharide-processing enzymes α -glucosidases I and II, it is likely that the FOS observed were the result of aberrant N-linked glycosylation. Using structural analysis, the present study aimed to determine if the generation of FOS is the result of inhibition of this pathway.

N-linked glycosylation of proteins commences in the ER lumen with the direct transfer of Glc₃Man₉GlcNAc₂ from dolichol-PP to the nascent polypeptide [3]. α -Glucosidases I and II then remove the glucose residues on the oligosaccharide. To facilitate

protein folding, misfolded proteins can be acted on by uridine diphosphate glucose:glycoprotein glucosyltransferase and α -glucosidase II. The re-glucosylation/de-glucosylation reactions performed by the concerted action of these two enzymes allow proper interaction of the protein with the chaperones calnexin and calreticulin, which facilitate protein folding [4,5]. It is the failure of certain viral envelope proteins to fold correctly when α -glucosidases are inhibited that is responsible for the antiviral effects of *N*-butyl-DNJ (NB-DNJ) [6]. However, a second antiviral activity of N-alkylated imino sugars, which is independent of glucosidase and GSL inhibition, has been described, whose mode of action has yet to be elucidated [7].

Our continued interest in the biological effects of N-alkylated imino sugars, in particular the structure–function relationships of these small molecules, has led to the generation of a series of N-alkylated DNJ derivatives with side chains ranging in length from C₄ to C₁₈ [8]. In the present study we have examined the effect of these analogues on N-linked oligosaccharide-processing pathways and have demonstrated a chain-length-dependent selectivity of DNJ analogues for the inhibition of α -glucosidases I and II in HL60 cells. We have also characterized in detail compound-induced changes in free cellular oligosaccharides that have not been previously observed with these imino sugar analogues.

Abbreviations used: 2-AB, 2-aminobenzamide; CGT, ceramide glucosyltransferase; NB-DGJ, *N*-butyl-deoxygalactonojirimycin; DNJ, deoxynojirimycin; C₁₈-DNJ, *N*-cis-13-octadecenyl-DNJ; NB-DNJ, *N*-butyl-DNJ; NN-DNJ, *N*-nonyl-DNJ; Endo H, endoglycosidase H; ER, endoplasmic reticulum; FOS, free oligosaccharide(s); GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GSL, glucosphingolipid(s); GU, glucose unit; HexNAc, *N*-acetylhexosamine; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; Man, mannose; NeuAc, *N*-acetylneuraminic acid; PNGase, peptide:N-glycanase.

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EXPERIMENTAL

Compounds

N-alk(en)ylated imino sugars were synthesised as reported previously [8].

Isolation of small FOS from imino-sugar-treated HL60 cells

HL60 cells were cultured to high density before the medium was replaced with fresh medium containing NB-DNJ (1 mM), N-nonyl-DNJ (NN-DNJ; 100 μ M), N-cis-13-octadecenyl-DNJ (C₁₈-DNJ; 10 μ M), DNJ (2 mM) or N-butyl-deoxygalactonojirimycin (NB-DGJ; 1 mM). The cells were cultured overnight (16 h), then the medium was removed and the cells were washed with PBS by centrifugation. Washed cells were stored at -20°C for 1 week before thawing and thorough homogenization. Protein assays were performed on the cell homogenates and a fraction was taken, containing 500 μ g of protein, for extraction with chloroform/methanol/water (4:8:3, by vol.), as described in the accompanying paper [2a].

Optimized isolation of FOS from imino-sugar-treated HL60 cells

Samples of cell homogenate (standardized to 500 μ g of protein) from HL60 cells treated with imino sugar analogues, as described above, were prepared in 200 μ l of 4 mM MgCl₂, to which 400 μ l of methanol was added followed by 600 μ l of chloroform, with mixing at each step. The samples were centrifuged at 600 g for 5 min to separate the phases, the upper aqueous phase was removed and the lower phase was washed twice with 250 μ l of Folch theoretical upper-phase solvents (chloroform/methanol/water, 1:10:10, by vol.). The three upper phases were combined and dried under nitrogen and then vacuum. The samples were resuspended in 200 μ l of Milli-QTM water and added to an OasisTM HLB cartridge (Waters), which had been pre-equilibrated with 1 ml of methanol and 1 ml of Milli-QTM water. The eluates, a Milli-QTM water wash (100 μ l) and a 5 % methanol-in-water wash (200 μ l), were collected. The pooled oligosaccharides were dried under vacuum, resuspended in 20 μ l of DMSO/acetic acid (7:3, v/v) and de-salted using a GlycoClean S cleanup cartridge (Oxford GlycoSciences, Oxford, U.K.) according to the manufacturer's instructions.

2-Aminobenzamide (2-AB) labelling

Samples were resuspended in 5 μ l of 2-AB-labelling mixture (Ludger Ltd., Oxford, U.K.) by vortex-mixing and were incubated at 65°C for 2 h. Underivatized 2-AB was removed by using GlycoClean S cleanup cartridges or by ascending paper chromatography with acetonitrile. The labelled carbohydrates were eluted from the paper strips with Milli-QTM water.

HPLC analysis of 2-AB-labelled carbohydrates

The 2-AB-labelled sugars were analysed by normal-phase HPLC, as described previously [9,10]. Briefly, the equipment comprised a Waters Alliance 2695XE separations module and Waters 474 fluorescence detector set at wavelengths of 330 nm and 420 nm for excitation and emission respectively. Labelled sugars were separated on a 4.6 mm $\times$ 250 mm TSK-Gel Amide-80 column (Anachem) at 30°C (see accompanying paper [2a]). After initial HPLC analysis of the samples, further preparative HPLC runs were used to isolate the FOS from other 2-AB-labelled or auto-fluorescent material in the samples. The amount of each carbohydrate species present in the imino-sugar-treated HL60 cells

(pmol/mg of cellular protein) was calculated by measuring individual peak areas using Waters MilleniumTM software and then by comparing these values with those obtained from a standard curve of 2-AB.

MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS

Positive-ion MALDI-TOF mass spectra were recorded with a Micromass ToFSpec 2E reflectron-TOF mass spectrometer [Waters-Micromass (UK) Ltd., Manchester, U.K.] fitted with delayed extraction and a nitrogen laser (337 nm) [11]. For further details, see the accompanying paper [2a].

Glycosidase digestions

To characterize the 2-AB-labelled carbohydrates in more detail, a series of digestions was performed using linkage-specific glycosidases. Preparative HPLC runs were performed, as described, to isolate the FOS from other 2-AB-labelled or autofluorescent material in the samples. The appropriate fractions were collected, concentrated under vacuum and repeatedly freeze-dried to remove ammonium formate derived from the HPLC buffer.

α -Glucosidase I and II digestions

2-AB-labelled carbohydrates were dried under vacuum, and α -glucosidase I and/or α -glucosidase II were added. The enzymes were purified as described previously [12,13]. The samples were mixed and incubated for 16 h at 37°C .

α -Mannosidase digestions

2-AB-labelled carbohydrates were resuspended in 100 mM sodium acetate buffer, pH 5.0, and jack bean α -mannosidase (Glyko, San Leandro, CA, U.S.A.) was added to give a final concentration of 25 units/ml. Samples were mixed and incubated at 37°C for 24 h.

Endo H (endoglycosidase H) digestions

2-AB-labelled carbohydrates were resuspended in 10 μ l of water containing 1 μ l of $10\times$ buffer (0.1 M sodium citrate, pH 5.5), which was supplied by the manufacturer. To this, 2 μ l of Endo H (500 000 units/ml; New England Biolabs) was added. The samples were mixed and incubated for 24 h at 37°C .

After the incubation period, the enzymes were denatured at 100°C for 10 min and the digestion products were dissolved in 100 μ l of water/acetonitrile (1:1, v/v) for HPLC analysis as described above.

RESULTS

HPLC characterization of non-GSL small FOS generated following imino sugar treatment of HL60 cells

After treatment with the DNJ analogues, three peaks, which were not present in the control cells, were found in significant quantities in the chromatograms of the chloroform/methanol/water extracts from the NB-DNJ- and NN-DNJ-treated HL60 cells (marked X, Y and Z in Figures 1A–1C). The increase in the amount of these structures was concurrent with a decrease in GSL levels, which were reduced by an equivalent amount in the presence of all three DNJ analogues (see accompanying paper [2a]). We determined that these structures were free carbohydrates, not GSL-derived, by the absence of these structures in chromatograms obtained

Figure 1 HPLC chromatograms illustrating the identification of $\text{Glc}\alpha 3\text{Man}$, $\text{Glc}\alpha 3\text{Glc}\alpha 3\text{Man}$ and $\text{Glc}\alpha 2\text{Glc}\alpha 3\text{Glc}\alpha 3\text{Man}$ structures (small FOS) by enzyme digestion with α -glucosidases I and II

The oligosaccharides marked X, Y and Z, which were found in the extracts of HL60 cells treated with NB-DNJ and NN-DNJ, were identified by incubating the 2-AB-labelled carbohydrates with purified ER carbohydrate-processing enzymes α -glucosidases I and II. The samples were subsequently analysed by normal-phase HPLC, as described in the text, and the peaks were found to be $\text{Glc}\alpha 3\text{Man}$ -2AB (X), $\text{Glc}\alpha 3\text{Glc}\alpha 3\text{Man}$ -2AB (Y) and $\text{Glc}\alpha 2\text{Glc}\alpha 3\text{Glc}\alpha 3\text{Man}$ -2AB (Z). The structures of the relevant peaks and the positions of each digestion are shown. The other peaks in the chromatogram are GSL-derived oligosaccharides as described in the accompanying paper [2a]. (A) NB-DNJ (1 mM), no enzyme; (B) NB-DNJ (1 mM), α -glucosidase I; (C) NB-DNJ (1 mM), α -glucosidases I and II. ○, mannose; □, glucose.

following flow dialysis of the cell homogenates prior to solvent extraction, enzyme release and labelling (results not shown). HL60 cells treated with the imino sugar NB-DGJ, which inhibits GSL biosynthesis but not the ER α -glucosidases [14], did not result in the generation of these structures (results not shown). We postulated that the appearance of these carbohydrates may result from interference with the N-linked oligosaccharide processing pathway due to the inhibitory activities of DNJ analogues on ER α -glucosidases I and II.

As these carbohydrates had glucose unit (GU) values consistent with two hexoses (X), three hexoses (Y) and four hexoses (Z) (see Figure 1), we hypothesised that they were the products of Golgi endomannosidase activity on mono-, di- and tri-glucosylated polymannose-type N-linked oligosaccharides to yield $\text{Glc}\alpha 3\text{Man}$, $\text{Glc}\alpha 3\text{Glc}\alpha 3\text{Man}$ and $\text{Glc}\alpha 2\text{Glc}\alpha 3\text{Glc}\alpha 3\text{Man}$ respectively. To test this hypothesis, α -glucosidase I and II digestions were performed. When α -glucosidase I (which is specific for $\text{Glc}\alpha 2\text{Glc}$ linkages) was used, peak Z (Figure 1A) collapsed to peak Y (Figure 1B). When α -glucosidase I was used in combination with α -glucosidase II (specific for $\text{Glc}\alpha 3\text{Glc}$ -Man linkages), both Z and Y collapsed to peak X (Figure 1C), thus confirming the presence of $\text{Glc}\alpha 2\text{Glc}$ and $\text{Glc}\alpha 2\text{Glc}\alpha 3\text{Glc}$ structures. The reason for peak X not digesting into Man-2-AB in the presence of α -glucosidase II is most likely due to the inability of the enzyme to recognize the open-ring form of the mannose residue generated by reductive amination during fluorescence labelling. However, it

Figure 2 HPLC chromatograms showing optimized recovery of large FOS derived from HL60 cells treated with N-alkylated DNJs

FOS were extracted from HL60 cells treated for 16 h with or without imino sugar analogues, as described in the text, using optimized methods for recovery. Samples were prepared in 4 mM MgCl_2 to which methanol and chloroform were added to obtain upper and lower phases. Carbohydrates were purified from the upper aqueous phase using an OasisTM cartridge and de-salted using a GlycoClean S cleanup cartridge. The sugars were 2-AB labelled and analysed by normal-phase HPLC as described in the text. Chromatograms shown are representative of those obtained from three HL60 cell extracts obtained after treatment with or without imino sugar analogues. (A) DNJ (2 mM); (B) NB-DNJ (1 mM); (C) NN-DNJ (100 μM); (D) C_{18} -DNJ (10 μM); (E) NB-DGJ (1 mM); (F) control, no imino sugar. The relationships between peak number, GU values, structure and abundance are given in Table 2.

is also possible that α -glucosidase II does not correctly recognize this structure, as the 'usual' substrate is the extended arm of a polymannose-type N-linked oligosaccharide. The specificity of α -glucosidases I and II was confirmed using a 2-AB-labelled $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ oligosaccharide [15].

Optimized extraction and HPLC characterization of FOS in imino-sugar-treated HL60 cells

In order to study additional changes in free carbohydrates induced by treatment of HL60 cells with N-alk(en)ylated DNJs, FOS were isolated from cells using the optimized conditions for extraction of hydrophilic components as described above. The FOS was 2-AB labelled and analysed by HPLC. Differences in the FOS profiles were seen not only between control and drug treated cells, but relative differences were observed between the profiles produced by DNJ compounds with different chain lengths (Figure 2). Treatment with glucosidase inhibitors DNJ, NB-DNJ and NN-DNJ (Figures 2A–2C), but not C_{18} -DNJ (Figure 2D), led to the generation of more than 20 large FOS structures not present in control cells (Figure 2F) that are additional to peaks X, Y and Z. There

Table 1 FOS estimates following treatment of HL60 cells with imino sugar inhibitors

Cells were treated with 1 mM NB-DNJ or 100 μ M NN-DNJ, or untreated (control) as described in the text. FOS were extracted using optimized conditions for both small and large FOS, and following 2-AB labelling and HPLC separation the peak area of individual components was measured and converted into pmol/mg of cell protein. For composition and structure of small and large FOS see Figures 1 and 2 respectively. The values of FOS shown for each treatment are the average of 3 experiments where FOS were extracted from cells, labelled with 2-AB and analysed (means $\pm$ S.D.). ND, not detected.

Treatment	FOS (pmol/mg of protein)				Large (GU 4–12)	Total (small + large FOS)
	Small (GU 1–4)					
	X	Y	Z			
Control	ND	ND	ND	ND	178 ± 50	178 ± 50
NB-DNJ	67 ± 2	21 ± 2	238 ± 3	326 ± 4	595 ± 6	921 ± 7
NN-DNJ	128 ± 3	45 ± 1	124 ± 3	297 ± 4	592 ± 8	889 ± 9

were no significant changes in the profile or overall quantity of large FOS between control cells (Figure 2F, 178 $\pm$ 50 pmol/mg of protein, and Table 1) and those treated with the non-glucosidase inhibitor NB-DGJ (Figure 2E, 201 $\pm$ 52 pmol/mg of protein). Estimation of the amounts of both small FOS (GU, 1–4; Figure 1) and large FOS (GU, 4–12; Figure 2) in response to NB-DNJ- and NN-DNJ-mediated glucosidase inhibition are shown in Table 1. NB-DNJ and NN-DNJ treatment produced a more than 5-fold increase in total FOS levels over the 16-h treatment period to 921 $\pm$ 7 and 889 $\pm$ 9 pmol/mg of protein respectively.

Large FOS analysis following α -glucosidase digestion

α -Glucosidase I and II digestions were performed to determine if retention of glucose residues could account for some or all of the new structures generated by treatment with the imino sugars. Most (85 % of total) of the large FOS structures in the NB-DNJ-treated cells were susceptible to digestion with glucosidases I and II (Figure 3D and Table 2) and 53 % of total large FOS were glucosidase I-susceptible triglycosylated structures (Figure 3B and Table 2). The profile seen after double digestion (Figure 3D) is qualitatively similar to the control (Figure 3H), indicating that similar core structures were present. Two glucosylated FOS, 7 % of total large FOS, were found in the control cells (peak numbers 9 and 37, Figure 3E). These were identified as being a mono-glucosylated structure [GU, 6.61 (A)] and a triglycosylated structure (GU, 11.71), both of which were present in the imino-sugar-treated cells and in similar relative amounts (see Table 2).

Large FOS analysis following α -mannosidase digestion

The NB-DNJ-treated and control FOS samples were digested with α -glucosidases I and II followed by jack bean α -mannosidase to determine if the oligosaccharides present were exclusively glucosylated polymannose structures. As shown in Figures 4(A) and 4(B), in both the NB-DNJ-treated and control cells, the oligosaccharides all collapsed to structures containing a β -mannose and either a single terminal GlcNAc or a chitobiose core. Structures with a single reducing terminal GlcNAc accounted for > 90 % of total in both cases. Thus all of the original structures were polymannose-type oligosaccharides with or without glucosylation (Table 2). The Glc₃Man₇GlcNAc₂ standard digested to a Man-GlcNAc₂-2-AB core as expected (Figure 4C).

Large FOS analysis of the reducing GlcNAc terminus

To determine how many of the structures in the FOS profiles had a GlcNAc₂ core, the samples were treated with Endo H, which

Figure 3 HPLC chromatograms showing digestion of large FOS from NB-DNJ-treated and control HL60 cells with α -glucosidases I and II

FOS were extracted from HL60 cells and fluorescently labelled as described in the text. 2-AB-labelled carbohydrates were dried under vacuum and treated with α -glucosidase I and/or α -glucosidase II for 16 h at 37 °C before HPLC analysis, as described in the text. Note the α -glucosidase susceptible peaks 9 and 37 in the undigested control chromatogram (E). (A) NB-DNJ (1 mM), no enzyme; (B) NB-DNJ (1 mM), α -glucosidase I; (C) NB-DNJ (1 mM), α -glucosidase II; (D) NB-DNJ (1 mM), α -glucosidases I and II; (E) control, no enzyme; (F) control, α -glucosidase I; (G) control, α -glucosidase II; (H) control, α -glucosidases I and II. In (B), peaks labelled 'n' show new species generated following α -glucosidase I digestion. The relationships between peak number, GU values, structure and abundance are given in Table 2.

Table 2 Composition and relative abundance of large free high-mannose oligosaccharides from NB-DNJ-treated HL60 cells

All structures were high-mannose-type oligosaccharides as confirmed by jack-bean α -mannosidase digestion (see the text). The GU values were determined by comparison of the high-mannose-FOS HPLC column retention times with those of a 2-AB-labelled dextran series. The number of glucose residues (if present) and the number of core GlcNAc residues (1 or 2) are indicated for each structure as determined by glucosidase I and II and EndoH digestions respectively. The proportion of each FOS structure present is expressed as a percentage of total FOS for each treatment (average of 3 experiments where FOS were extracted from cells, labelled with 2-AB and expressed as the means $\pm$ S.D.).

Peak no.	GU	NB-DNJ-treated cells			Control cells		
		No. of glucose residues	No. of core GlcNAc residues	Percentage of total oligo-mannose FOS	No. of glucose residues	No. of core GlcNAc residues	Percentage of total oligo-mannose FOS
1	4.61	—	—	—	0	1	2.07 $\pm$ 0.78
2	4.65	0	1	1.54 $\pm$ 0.07	—	—	—
3	4.94	0	1	3.82 $\pm$ 0.15	0	1	18.53 $\pm$ 0.18
4	5.38	1	1	7.22 $\pm$ 0.02	—	—	—
5	5.76	0	1	1.19 $\pm$ 0.02	0	1	2.30 $\pm$ 0.08
6	5.85	0	1	5.05 $\pm$ 0.06	0	1	37.10 $\pm$ 0.94
7	6.05	2	1	1.38 $\pm$ 0.01	—	—	—
8	6.48	0	1	0.74 $\pm$ 0.01	—	—	—
9	6.61 (A)	1	1	5.61 $\pm$ 0.04	1	1	6.36 $\pm$ 0.19
10	6.61 (B)	0	1	1.86 $\pm$ 0.07	0	1	3.66 $\pm$ 0.15
11	6.91	—	—	—	0	1	2.20 $\pm$ 0.06
12	7.00 (A)	0	1	0.77 $\pm$ 0.05	—	—	—
13	7.00 (B)	3	1	6.16 $\pm$ 0.07	—	—	—
14	7.06	—	—	—	0	2	1.87 $\pm$ 0.03
15	7.29	2	1	2.83 $\pm$ 0.05	—	—	—
16	7.37	—	—	—	0	1	5.41 $\pm$ 0.12
17	7.42	1/2	1	2.19 $\pm$ 0.03	—	—	—
18	7.81	0	1	1.95 $\pm$ 0.09	0	1	1.79 $\pm$ 0.05
19	7.93	0	2	2.06 $\pm$ 0.04	—	—	—
20	7.95	—	—	—	0	1	3.19 $\pm$ 0.22
21	8.11	2	1	1.06 $\pm$ 0.03	—	—	—
22	8.29	3	1	16.55 $\pm$ 0.15	—	—	—
23	8.49	—	—	—	0	1	1.86 $\pm$ 0.09
24	8.59 (A)	1	1	3.09 $\pm$ 0.06	—	—	—
25	8.59 (B)	1	2	2.59 $\pm$ 0.08	—	—	—
26	8.84	0	1	1.98 $\pm$ 0.01	0	1	4.30 $\pm$ 0.12
27	9.12	3	1	5.75 $\pm$ 0.03	—	—	—
28	9.40	3	1	1.31 $\pm$ 0.04	—	—	—
29	9.53	—	—	—	0	1	8.93 $\pm$ 0.35
30	9.54	3	1	1.72 $\pm$ 0.04	—	—	—
31	9.81	3	1	3.68 $\pm$ 0.02	—	—	—
32	10.25 (A)	3	1	5.16 $\pm$ 0.07	—	—	—
33	10.25 (B)	3	1	1.68 $\pm$ 0.02	—	—	—
34	10.92	3	1	1.87 $\pm$ 0.03	—	—	—
35	11.07	3	2	0.68 $\pm$ 0.01	—	—	—
36	11.25	3	1	3.74 $\pm$ 0.08	—	—	—
37	11.71	3	2	0.85 $\pm$ 0.04	3	2	0.74 $\pm$ 0.20
38	11.92	3	1	3.91 $\pm$ 0.14	—	—	—
Total				99.99			100.31

cleaves between the two core GlcNAc residues, removing the terminal GlcNAc residue linked to 2-AB and hence any FOS with a GlcNAc₂ core from the HPLC profile. The results of the Endo H digests are shown in Figure 5. Four structures in the NB-DNJ-treated samples and two in the control had GlcNAc₂ cores (peak numbers 19, 25, 35 and 37 in Figure 5A and peak numbers 14 and 37 in Figure 5C). The assignment of the reducing terminus for each FOS structure is summarized in Table 2.

The structural information obtained from the results of the enzyme digestions was supported by MALDI-TOF MS analysis of the large FOS in NB-DNJ-treated cells, where the predominant series was Hex₅₋₁₂HexNAc₁ and a minor series comprising Hex₇₋₁₂HexNAc₂ (results not shown). Two major high-mannose FOS were found in the control at GU values of 4.94 and 5.85 and accounted for over 50 % of the oligosaccharides analysed (Table 2). In support of these results, MALDI-TOF MS detected the presence of two abundant ions with mass values consistent with Hex₅HexNAc₁ and Hex₆HexNAc₁ (results not shown).

DISCUSSION

N-Alkylation of DNJ modifies the selectivity of this compound for inhibition of CGT and α -glucosidase in cultured HL60 cells. We have previously shown that by increasing the N-alkyl chain-length of DNJ, the biological properties of the resulting compounds were significantly altered [8]. Using *in vitro* assays, it was shown that increasing N-alkyl chain length resulted in improvements to inhibitory potency for CGT, but not for α -glucosidase. For example, we found C₁₈-DNJ to be 10-fold better at inhibiting CGT than NB-DNJ, and a 3-fold worse at inhibiting α -glucosidase I [8]. In the accompanying paper [2a] we showed that the presence of a long side chain conferred increased cellular retention. However, the differential in cellular retention displayed by C₄ and C₁₈ compounds did not necessarily result in increased inhibition of all cellular enzymes. When used at concentrations that resulted in an equivalent degree of CGT inhibition, a significant increase in glucosylated FOS was found following treatment with NB-DNJ

Figure 4 HPLC chromatograms showing glucosidase-I- and II-digested large FOS from NB-DNJ-treated and control HL60 cells following treatment with jack-bean α -mannosidase

2-AB-labelled carbohydrates were prepared in 100 mM sodium acetate buffer, pH 5.0, to which jack-bean α -mannosidase was added at a final enzyme concentration of 25 units/ml. Samples were mixed and incubated at 37 °C for 24 h. The products of the enzyme digestions are shown after incubation with α -mannosidase and the peaks are labelled with their structures. (A) NB-DNJ (1 mM); (B) control, no imino sugar; (C) Glc₃Man₇GlcNAc₂. *, contaminant present prior to enzyme digest; ■, GlcNAc; ○, mannose.

and VN-DNJ, but not with C₁₈-DNJ. This further demonstrates the selectivity of C₁₈-DNJ for inhibiting the GSL biosynthetic pathway in comparison with the shorter-chain DNJ analogues. CGT is located on the cytosolic side of the *cis*-Golgi [16–18] and is therefore more accessible to inhibitors than the ER luminal-localized α -glucosidases. We propose that the structure of the very-long-chain imino sugars limits accessibility to the ER lumen.

Although treatment of HL60 cells with DNJ, NB-DNJ or VN-DNJ resulted in increases in FOS levels, differences were observed in the relative FOS profiles. These differences reflect variation in the degree of glucosylation of the immature glucosylated glycans, and probably indicate that there is some difference in the selectivity of the DNJ analogues for inhibiting α -glucosidase I and II in the ER. A differential selectivity of NB-DNJ and DNJ for inhibition of α -glucosidases has previously been observed in cultured mouse lymphoma cells [19]. Glycopeptides contained equivalent molar amounts of glucosylated oligosaccharides, but significantly more Glc₃ structures were found than Glc₂ after NB-DNJ treatment, with the reverse being the case for DNJ-treated cells.

Endomannosidase trims oligosaccharides by specifically cleaving the α 2 linkage between the glucose-substituted mannose and the rest of the core α 3-mannose branch of an N-linked oligosaccharide [20]. Endomannosidase isolated from rat liver was found to process Glc₂- and Glc₃-containing oligosaccharides much less efficiently than Glc₁-containing structures (approx. 10-fold) [21]. The enzyme is not inhibited by any of the agents that inhibit glucosidases [21]. The endomannosidase pathway has been shown to provide a bypass route that allows the formation of complex

Figure 5 HPLC chromatograms showing digestion of large FOS from NB-DNJ-treated and control HL60 cells with Endo H

2-AB-labelled carbohydrates were prepared in 10 μ l of sodium citrate buffer, pH 5.0, as described in the text and incubated with 1000 units of Endo H for 24 h at 37 °C. (A) NB-DNJ (1 mM), no enzyme; (B) NB-DNJ (1 mM) + Endo H; (C) control, no imino sugar, no enzyme; (D) control, no imino sugar + Endo H. Peak numbers 19, 25, 35 and 37 in (A) and 14 and 37 in (C) represent oligosaccharide structures which were susceptible to Endo H digestion and, therefore, contained a chitobiose core. The relationships between peak number, GU values, structure and abundance are given in Table 2.

oligosaccharides in the absence or inhibition of glucosidase activity [19,22]. Thus α -glucosidase-II-deficient cells have been shown to secrete Glc₁Man and Glc₂Man into the medium in amounts equivalent to the non-glucosylated oligosaccharide formed by the cell during the same period [19]. However, the normal physiological role of the endomannosidase, when glucosidase activity is normal, remains to be clarified. It has been previously suggested that the enzyme may be required to de-glucosylate Glc₁Man₈ and Glc₁Man₇ oligosaccharides, which are more likely to reach the Golgi through being poor substrates for ER glucosidase II, but are good substrates for the endomannosidase [22].

Glucosylated GlcNAc₂-core-containing FOS (FOS-GlcNAc₂), trapped in the ER in glucosidase-I-deficient or castanospermine-treated HepG2 cells, follows the secretory pathway through the Golgi [23]. The glucosylated FOS-GlcNAc₂ is trimmed by the endomannosidase and is subsequently converted into complex sialic-acid-containing structures before secretion into the extracellular space. If the Glc₁₋₃Man₁ endomannosidase products we found were the result of Golgi processing of glucosylated FOS-GlcNAc₂, we would have expected to find equivalent intracellular amounts of both end products, i.e. Glc₁₋₃Man₁ and complex FOS-GlcNAc₂. However, we found no intracellular complex FOS in the present study. It is possible that this reflects cell-line-specific

processing differences, but implies that the $\text{Glc}_{1-3}\text{Man}_1$ structures that we measured were the products of endomannosidase action on glucosylated glycoproteins and not on glucosylated FOS.

Free polymannose oligosaccharides containing a di-*N*-acetylchitobiose moiety at their reducing termini are generated during glycoprotein biosynthesis in the lumen of the ER [24]. It was subsequently shown that FOS can be detected in the cytosol [25], and that ATP-provoked transport of FOS from the ER into the cytosol [26] occurs where FOS-GlcNAc₂ is degraded by a cytosolic neutral chitobiase [27] to form FOS-GlcNAc₁ structures. The cytosolic α -mannosidase, which only recognizes the structures with a single reducing terminal GlcNAc [28], further trims the FOS down to a limited $\text{Man}_5\text{GlcNAc}_1$ structure [25]. The $\text{Man}_5\text{GlcNAc}_1$ is subsequently translocated into the lysosome for degradation [29] by an ATP-dependent process, which is selective for this structure [30].

In addition to direct transport of non-glucosylated FOS-GlcNAc₂ from the ER to the cytosol, there is also evidence that some phosphorylated $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}_7\text{GlcNAc}_2$ may be generated at the cytosolic face of the ER by removal from dolichol by a pyrophosphatase [31,32]. FOS may also be generated through the action of a cytosolic peptide:N-glycanase (PNGase) [33] on misfolded glycoproteins exported from the ER for proteasomal degradation via the Sec61-containing channel in the ER membrane [34]. Other investigators have identified a cytosolic endoglucosaminidase in rat liver [35,36]. Di- and tri-glucosylated polymannose oligosaccharides fail to interact with the chaperones calnexin and calreticulin [37,38]. The tendency of certain viral envelope proteins to fold incorrectly when glucosidases are inhibited forms the basis for the antiviral activity of DNJ-based compounds [6]. The FOS effects we have observed could be explained by incorrect folding of certain glycoproteins due to retention of glucose residues on the N-linked oligosaccharides, leading to an increase in proteins trafficking to the proteasome from the ER. Unlike the ER-to-cytosol transport of FOS, the transport of glycoproteins from the ER to the cytosol is not dependent on the glycan structures [39,40] and therefore the degree of glucosylation. The cytosolic or ER-associated PNGase could release glucosylated FOS from the proteins at an as yet undetermined stage in this process.

The FOS-GlcNAc₁ structures identified in NB-DNJ-treated HL60 cells in the present study must be cytosolic. Glucosylated FOS ($\text{Glc}_3\text{Man}_8\text{GlcNAc}_2$ and $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) are not transported from the ER into the cytosol by the ATP-provoked mechanism [26], implying that the 'transporter' only recognizes non-glucosylated polymannose-type FOS-GlcNAc₂. If glucosylated FOS-GlcNAc₂ generated in the ER is not the source of the glucosylated FOS-GlcNAc₁ seen in the NB-DNJ-treated cells, this raises questions about the origin of this material and the reason for the overall increase in FOS.

Treatment of cells with the glucosidase inhibitor castanospermine has been shown to lead to rapid degradation of unassembled MHC class 1 molecules [41]. Pulse-chase labelling experiments have subsequently been used to demonstrate the cytosolic deglycosylation of newly synthesized glycoproteins, generating a pool of cytosolic FOS-GlcNAc₁ [42]. In the presence of castanospermine, the level of $\text{Glc}_3\text{-FOS-GlcNAc}_2$ in the ER remained constant, but the level of cytosolic FOS-GlcNAc₁ increased over the 6 h chase. The same profile of polymannose structures was found both on immature glycoproteins and on FOS-GlcNAc₁, suggesting a product-substrate relationship. However, in a previous study [43], the compartmentalization of ER-associated oligosaccharide release was studied using HepG2 cells infected with vesicular stomatitis virus, where all released oligosaccharides can be attributed to one protein (vesicular-stomatitis-virus glycoprotein).

Scheme 1 Overview of the effects of N-alkylated DNJ inhibition of carbohydrate processing in HL60 cells

N-alkylated DNJ analogues rapidly enter the cell (< 1 min). The N-butyl/N-nonyl derivatives gain access to the lumen of the ER and inhibit N-linked oligosaccharide trimming by α -glucosidases I and II. For glycoproteins that require calnexin/calreticulin for correct folding, retention of two or three glucose residues prevents the interaction of the glycoprotein with the chaperone(s). The misfolded protein may still be processed to the final destination or may be translocated to the cytosol via the Sec61 channel. Prior to proteasomal degradation of the polypeptide, the glucosylated FOS-GlcNAc₂ are released by a cytosolic PNGase. A cytosolic chitobiase generates FOS-GlcNAc₁ from FOS-GlcNAc₂. For glycoproteins that reach the Golgi, endomannosidase cleaves the $\alpha 2$ linkage between the glucose-substituted mannose residue and the remaining oligosaccharide, allowing circumvention of the block in oligosaccharide processing. Glucosylated FOS are found in cellular secretions, suggesting export from the Golgi. The sites of action of N-alkylated DNJs are shown, together with the relative rates of intracellular access. No information regarding the rate of ER entry is available. N-Alkyl-DNJ compounds also inhibit CGT that synthesizes glucosylceramide, the precursor for most GSLs.

Treatment with castanospermine led to the generation of almost entirely FOS-GlcNAc₂, indicating that N-deglycosylation of this protein occurs in the ER, leading to the consequent trapping of the released FOS within this organelle. This suggests that there may be both ER and cytosolic PNGases, which may be selective for certain peptides or may be expressed in a cell-line-specific manner. The generation of FOS and the site of action of alkylated DNJ compounds are summarized in Scheme 1.

Some interesting questions, which have emerged from the present study and the work of others, remain to be answered. Firstly, can the observed increase in FOS-GlcNAc₁ be directly related to the degradation of misfolded proteins? Second, what is the eventual fate of the glucosylated cytosolic FOS? Does long-term

glucosidase inhibition lead to intracellular accumulation of glucosylated FOS? Although the cytosol to lysosome transporter is selective for Man₅GlcNAc₁ [30], there is evidence from experiments with the glucosidase-I-deficient Lec23 cells to suggest that cytosolic Glc₃Man₅GlcNAc₁ is eventually cleared when these cells are cultured past confluency [23]. How this may be achieved is unknown, but the small amount of glucosylated FOS found in the control cells demonstrates that this is a normal occurrence. If the glucosylated FOS-GlcNAc₁ does reach the lysosome and is catabolized, it remains to be established which enzyme(s) remove the glucose residues.

The present study has further characterized the cellular effects of DNJ analogues and the selectivity the N-alkyl chain confers with respect to these effects. These investigations will facilitate the design of imino-sugar-based therapeutics for the numerous applications for which this class of small molecule shows promise.

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