Tetrahedron: *Asymmetry* 20 (2009) 897–901

Contents lists available at ScienceDirect

Tetrahedron: *Asymmetry*

journal homepage: www.elsevier.com/locate/tetasy

Improved cellular inhibitors for glycoprotein processing α-glucosidases: biological characterisation of alkyl- and arylalkyl-N-substituted deoxynojirimycins

Dominic S. Alonzi, Raymond A. Dwek, Terry D. Butters *

Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

ARTICLE INFO

Article history:
Received 4 February 2009
Accepted 10 March 2009
Available online 3 April 2009

Special edition of Tetrahedron: Asymmetry in honour of Professor George Fleet's 65th birthday

ABSTRACT

A series of *N*-alkyl- and *N*-arylalkyl-DNJ compounds have been evaluated for their efficacy for inhibition of endoplasmic reticulum resident α-glucosidases in cells. A recently developed free oligosaccharide (FOS) assay allowed the products of glucosidase inhibition to be quantified and compounds compared for relative inhibitory activity. A *N*-alkyl chain of one to six carbon atoms provided a flexible linker between deoxynojirimycin (DNJ) and a phenyl, cyclohexyl or cyclopentyl group to explore the requirements for glucosidase inhibition. The most effective compounds were those in which the linker contained four to six carbon atoms and a phenyl group. These compounds all significantly inhibited α-glucosidase I at concentrations of 100 μM following addition to cells for 24 h whereas DNJ was without effect. Inhibition of α-glucosidase II was evident by all inhibitors, consistent with a previously identified mechanism of action of imino sugar inhibitors in cells.

1. Introduction

Over 20 years ago, one of the first indications that chemically synthesised derivatives of naturally occurring polyhydroxylated piperidine alkaloids could have a dramatic effect on the infectivity of a human virus, HIV,[1] led to an increased interest in imino sugar chemistry and biology. More than 600 imino sugars that have potential therapeutic benefit have been described to date and their impact on the field has been observed as just the 'tip of the iceberg'.[2]

The cellular mechanism that generates non-infectious virions following imino sugar treatment is becoming clearer. Biogenesis of viral coat glycoproteins depends on the host cell glycosylation machinery and a key intermediate, a mono-glucosylated N-linked oligosaccharide, is required for chaperone recognition to assist protein folding.[3] Imino sugar glucosidase inhibitors prevent endoplasmic reticulum (ER) glucosidases, especially glucosidase I, from revealing this cryptic intermediate, leading to viral glycoprotein misfolding and loss of coat integrity.

Prevention of ER-glucosidase I activity has led to the evaluation of imino sugars, particularly the deoxynojirimycin family of piperidine heterocycles, for their effects on enzyme activity in vitro,[4] glycoprotein oligosaccharide synthesis[5,6] and viral infectivity.[7–10] However, the correlation between in vitro glucosidase inhibition and viral infectivity assumes pharmacological equivalence in cells and tissues where membrane permeability, ER entry, residency time and enzyme on/off rates all influence potency.[11,12]

N-alkylation of deoxynojirimycin **1** has a profound effect on cellular activity and less so on in vitro inhibition of α-glucosidase I[13] that is postulated to relate to hydrophobicity of the alkyl chain dictating ER membrane localisation. *N*-Nonyl-DNJ was 10-fold more effective in reducing bovine viral diarrhoea virus (BVDV) infectivity when compared to DNJ.[14]

We have recently developed novel methods for the evaluation of ER-glucosidase inhibition in cells by quantifying the amount of free oligosaccharide produced in response to glycoprotein misfolding and clearance via the endoplasmic reticulum-associated degradation (ERAD) pathway.[12,15] In this paper we have compared a series of DNJ compounds modified with *N*-alkyl and *N*-arylalkyl groups to probe their inhibitory effects on α-glucosidase in cells (see Fig. 1).

2. Results and discussion

The initial screening of the DNJ analogues in HL60 cells at 100 μM for 24 h using the FOS assay resulted in α-glucosidase I inhibition in all cases (Fig. 2). All compounds were non-cytotoxic at this concentration. The level of α-glucosidase I inhibition was determined by the amount of $Glc_3Man_5GlcNAc_1$ produced in the cytosol as a result of ERAD. This FOS species is the major tri-glucosylated FOS species produced in response to α-glucosidase inhibition in the ER as a result of retrotranslocation via an ERAD pathway and the actions of PNGase and cytosolic α-mannosidase.[15]

* Corresponding author. Tel.: +44 (0)1865 275725; fax: +44 (0)1865 275216.
E-mail address: terry.butters@bioch.ox.ac.uk (T.D. Butters).

0957-4166/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2009.03.007

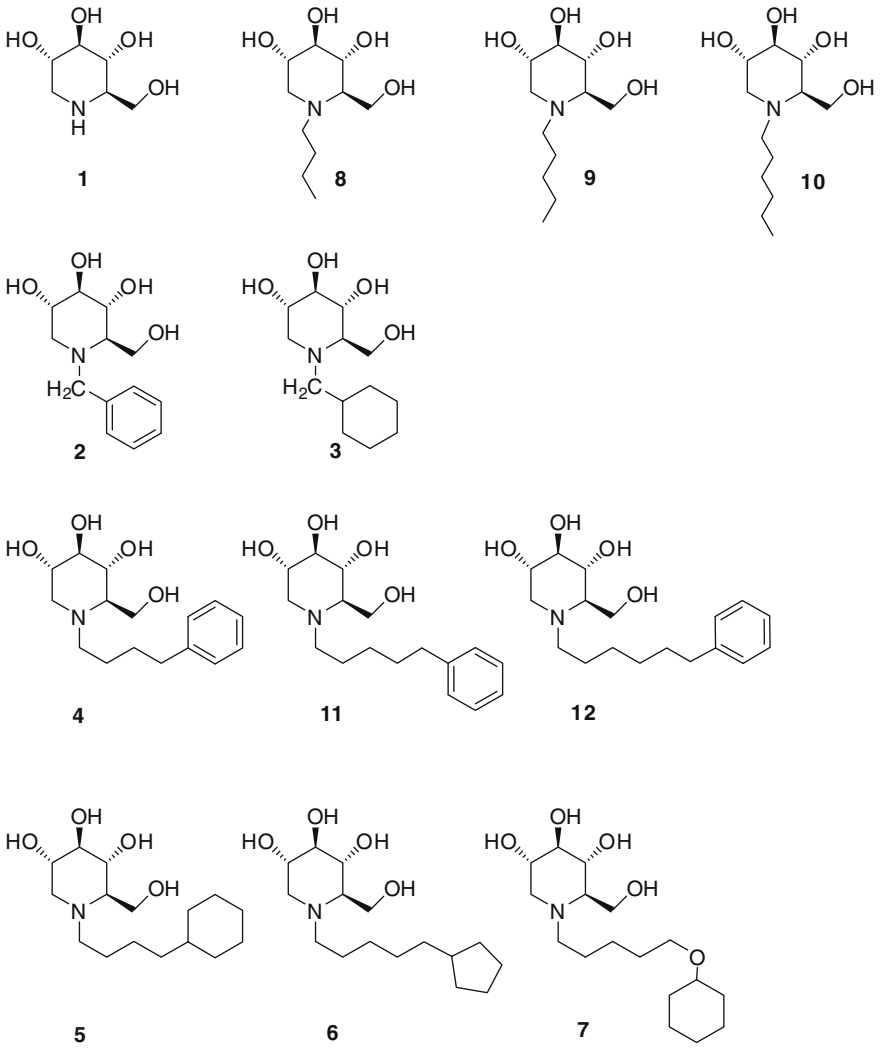

Figure 1. Structure of DNJ α-glucosidase inhibitors evaluated in this study. Compound **1**, DNJ; **2**, *N*-benzyl-DNJ; **3**, *N*-methylcyclohexyl-DNJ; **4**, *N*-butylphenyl-DNJ; **5**, *N*-butylcyclohexyl-DNJ; **6**, *N*-pentylcyclopentyl-DNJ; **7**, *N*-pentyl-(5-cyclohexoxy)-DNJ; **8**, *N*-butyl-DNJ; **9**, *N*-pentyl-DNJ; **10**, *N*-hexyl-DNJ; **11**, *N*-pentylphenyl-DNJ and **12**, *N*-hexylphenyl-DNJ.

The FOS species $Glc_3Man_5GlcNAc_1$ eluted from the TSKgel amide-80 column under the chromatographic condition was utilised in the study at approximately 37.5 min with a glucose unit (GU) value of 8.26 consistent with our previously published data[15] (Fig. 2). The structure of all FOS species was confirmed by α-glucosidase digestion and mass spectrometry as reported (results not shown).[15]

In control, untreated HL60 cells, no $Glc_3Man_5GlcNAc_1$ was produced and using 100 μM DNJ **1** treatment for 24 h no tri-glucosylated FOS species was observed (Fig. 2). A change in the FOS profile was obtained however, which was the result of α-glucosidase II inhibition and the appearance of $Glc_1Man_4GlcNAc_1$ (eluted at approximately 27.2 min, GU value 5.31) and an increase in $Glc_1Man_5GlcNAc_1$ (elutes at approximately 32.2 min, GU value 6.61). This discriminatory effect of glucosidase inhibitors on glucosidase I and II has been shown previously using low concentrations of DNJ inhibitors. The slower hydrolysis of mono-glucosylated glycans by α-glucosidase II, as part of the ERAD process, provides a more favourable environment for weak inhibitors to prevent enzyme-mediated hydrolysis.[15]

The *N*-arylalkyl-substituted DNJ compounds **2**, **3**, **4**, **5**, **6** and **7** were more effective cellular inhibitors than DNJ as determined by the generation of tri-glucosylated FOS species (Fig. 2). To obtain quantitative data, the peak area corresponding to $Glc_3Man_5GlcNAc_1$ was obtained, converted to pmol and standardised to the amount of protein used for FOS analysis (Table 1). These data reveal that the greatest inhibition was achieved by *N*-butylphenyl-DNJ **4** (448 ± 16 pmol/mg). When compounds **2** and **3** or **4** and **5** were compared it was apparent that in both cases the phenyl substituent of the alkyl chain was more inhibitory to glucosidase I than the cyclohexyl group, respectively. In addition, an increase in the alkyl chain length linker also resulted in an improvement in the amount of inhibition, as shown by the comparison between compounds **2** and **4** or **3** and **5**.

This increase in chain length appeared to have such a beneficial effect on α-glucosidase I inhibition that further structural/activity relationships were warranted. This resulted in the synthesis of *N*-pentylphenyl-DNJ **11** and *N*-hexylphenyl-DNJ **12**. The effect of these compounds analysed following treatment of HL60 cells at 100 μM concentrations for 24 h using the FOS assay and then compared to their respective *N*-alkyl-DNJ compounds; *N*-butyl-DNJ **8**, *N*-pentyl-DNJ **9** and *N*-hexyl-DNJ **10** (Fig. 3). In all cases the phenyl-substituted DNJ analogues were better than their corresponding N-alkylated DNJ inhibitors. The effect of increasing the alkyl chain length on glucosidase I inhibition was very similar with or without the phenyl group since there was a significant increase (more than twofold) in the amount of FOS species $Glc_3Man_5GlcNAc_1$

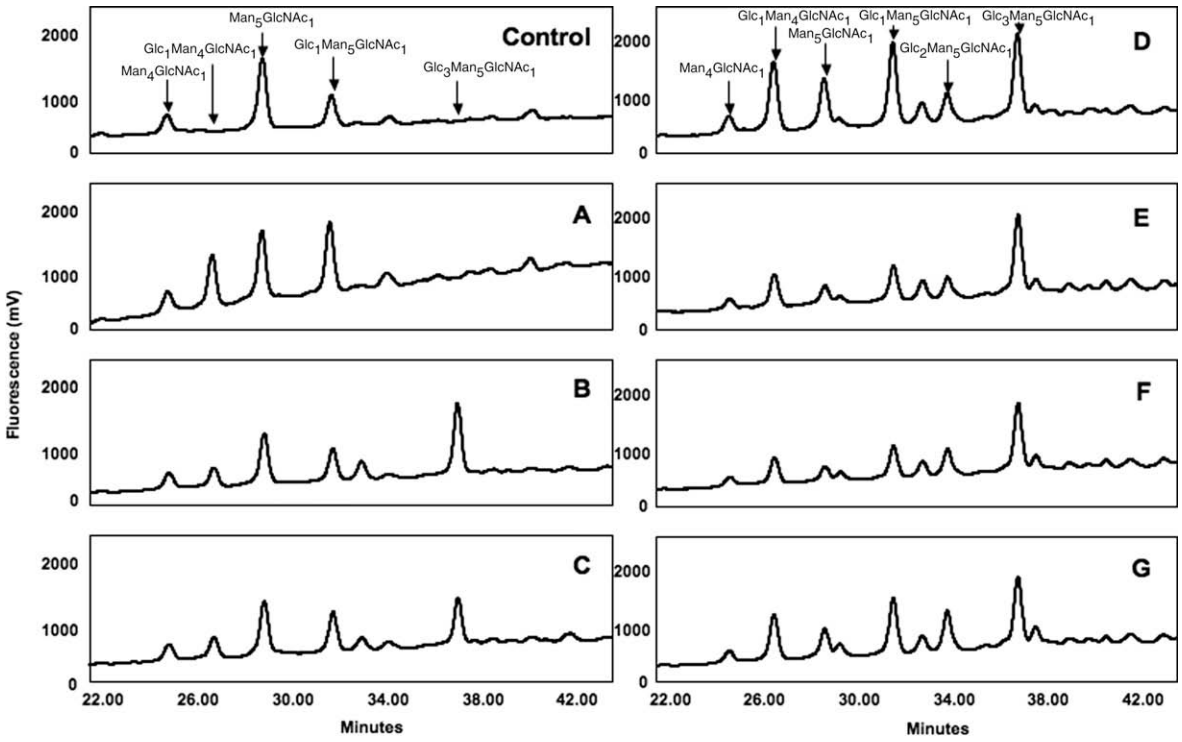

Figure 2. HPLC analysis of 2-AA fluorescently labelled FOS: Effect of *N*-arylalkyl-DNJ compounds on inhibition of glucosidase I in cells. HL60 cells were homogenised in water and FOS extracted, fluorescently labelled using 2-AA and purified as described in the Experimental. FOS species were analysed using NP-HPLC following inhibitor treatment at 100 μM for 24 h. Control, no inhibitor treatment; A, compound **1**; B, compound **2**, C, compound **3**, D, compound **4**; E, compound **5**; F, compound **6**; G, compound **7**.

for 4-carbon linkers **8** and **4**, the 5-carbon linkers **9** and **11** and the 6-carbon linkers **10** and **12** (Table 2). The improvement to inhibitory potency when an alkyl chain is substituted with a phenyl group appears more profound than just an increase in the alkyl chain length suggesting that both moieties contribute to the inhibitory potency.

The increase in ER-glucosidase inhibition with an increasing alkyl chain length has previously been attributed to the hydrophobicity of the alkyl chain allowing greater retention in ER due to interactions with the lipid membrane.[11] The more hydrophilic compound DNJ **1** may more easily diffuse out of the ER or being actively pumped out by a yet unknown mechanism. The addition of the aryl group to an alkylated DNJ compound may also aid membrane insertion due to a planar conformation but an alternative role may be to coordinate binding to an auxiliary site of the enzyme and increase enzyme inactivation. The presence of the DNJ moiety in arylalkyl–DNJ conjugates shown here is an absolute requirement of glucosidase activity for inhibition since the arylalkyl group alone has been shown to have no inhibitory effect.[16]

The therapeutic potential of imino sugars has never been fully realised mainly due to the inability to gain access to the ER.[11,12,17] The FOS assay provides a robust technique for examining α-glucosidase inhibition in the ER and provides an analytical snapshot of the inhibitory potential of compounds in a cellular context. The inhibitory activity of compounds was assessed at one concentration, which makes a comparison of efficacy simplified. However, there is a maximal level of inhibition of α-glucosidase I that can be achieved, determined by the level of $Glc_3Man_5GlcNAc_1$ produced.[15]

Table 1
Amount of $Glc_3Man_5GlcNAc_1$ free oligosaccharide (FOS) produced following inhibition of α-glucosidase I in HL60 cells: effect of *N*-alkylaryl-substitution

Inhibitor	[$Glc_3Man_5GlcNAc_1$] pmol/mg
Control	0.0
Compound **1**	0.0
Compound **2**	349 ± 8
Compound **3**	216 ± 4
Compound **4**	448 ± 16
Compound **5**	407 ± 10
Compound **6**	386 ± 19
Compound **7**	408 ± 8

Inhibition of ER resident α-glucosidase I results in the retrotranslocation of misfolded proteins by ERAD into the cytosol and the production of glucosylated FOS.[15] The major species is $Glc_3Man_5GlcNAc_1$ (see Fig. 2). The amount of this species was used as comparison for the level of ER α-glucosidase I inhibition in cells following DNJ compound (see Fig. 1) treatment. The peak area corresponding to $Glc_3Man_5GlcNAc_1$ was measured following HPLC analysis, converted to pmol using known amounts of 2-AA and normalised for different inhibitor treatments to cellular protein taken for FOS extraction and analysis.

Table 2
Table 1: Amount of $Glc_3Man_5GlcNAc_1$ free oligosaccharide (FOS) produced following inhibition of α-glucosidase I in HL60 cells: effect of chain length and phenyl group on relative potency

Inhibitor	[$Glc_3Man_5GlcNAc_1$] pmol/mg
Control	0.0
Compound **8**	168 ± 5
Compound **9**	208 ± 9
Compound **10**	210 ± 12
Compound **4**	448 ± 16
Compound **11**	481 ± 11
Compound **12**	477 ± 16

The major free oligosaccharide species, $Glc_3Man_5GlcNAc_1$ (see Fig. 3), was used as comparison for the level of ER α-glucosidase I inhibition in cells following DNJ compound (see Fig. 1) treatment. The peak area corresponding to $Glc_3Man_5GlcNAc_1$ was measured following HPLC analysis, converted to pmol using known amounts of 2-AA and normalised for different inhibitor treatments to cellular protein taken for FOS extraction and analysis.

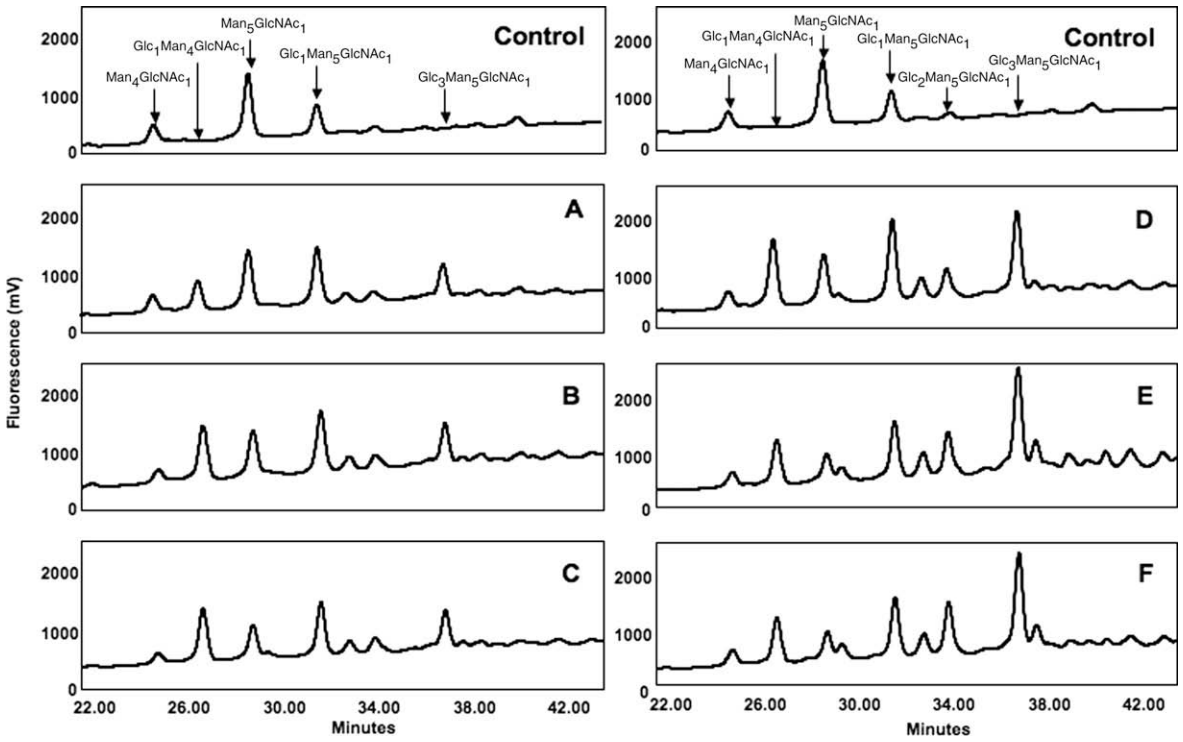

Figure 3. HPLC analysis of 2-AA fluorescently labelled FOS: Effect of *N*-alkyl chain length of DNJ compounds on inhibition of glucosidase I in cells. HL60 cells were treated with DNJ compounds containing different alkyl chains, with or without a phenyl group. HL60 cells were treated with inhibitors at 100 μM for 24 h prior to FOS extraction and analysis as described in the text. Control, no inhibitor treatment; A, compound **8**; B, compound **9**, C, compound **10**, D, compound **4**; E, compound **11** and F, compound **12**.

An interesting observation with compounds that are better α-glucosidase I inhibitors **4**, **5**, **6**, **7**, **11** and **12** is that complete inhibition of this enzyme has not been reached in the time and concentration used (Figs. 2 and 3). These conditions are sufficient for the inhibition of α-glucosidase II and results in the generation of FOS species $Glc_2Man_5GlcNAc_1$ (Figs. 2 and 3). A minor peak at this retention time in control cells (approximately 34 min) is a $Man_7GlcNAc_1$ species.[15] The complete dissection of glucosidase I and II activity in response to inhibition by potent compounds is possible using a compound concentration course.[15]

3. Conclusions

Arylalkyl-N-substituted DNJ compounds are excellent inhibitors of α-glucosidases in cells. The correlation between structure and activity studied in this paper allows us to design more effective inhibitors in a cellular context and could lead towards antiviral compounds with increased ER access and glucosidase inhibition.[3] The longer alkyl chain (at least 5 carbons) and the addition of a phenyl group provided DNJ compounds with increased inhibitory activity allowing further exploration of phenyl group substitutions to provide a potent area for further research.[16]

4. Experimental

4.1. Compounds

Reagents and chemicals were purchased from Sigma/Aldrich.

N-alkyl- and *N*-arylalkyl-DNJ compounds were synthesised from DNJ and the corresponding alkyl- or arylalkyl-aldehyde using sodium cyanoborohydride reductive amination as described previously.[13] All compounds were greater than 95% pure when analysed by 500 MHz NMR.

4.2. Cell culture

HL60 were cultured in RPMI media containing 10% foetal calf serum, 2 mM L-glutamine and 1% penicillin-streptomycin (Invitrogen).

4.3. Free oligosaccharides (FOS) isolation from cells

Cells were cultured to high density (1×107 cells mL^{-1}) before the medium was replaced with fresh medium containing inhibitor at varying concentrations and the cells were seeded at a lower density so as 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 homogenisation in water. The conditions for extraction of FOS were optimised to maximise recovery of FOS. Essentially, the homogenate was desalted and deproteinated by passage through a mixed bed ion exchange column [0.2 mL AG50W-X12 (H^+, 100–200 mesh) over 0.4 mL AG3-X4 (OH^-, 100–200 mesh)], pre-equilibrated with water (5×1 mL). The homogenate was added and collected with 4×1 mL water washes. The extracted purified FOS was then dried under vacuum.

4.4. Carbohydrate fluorescent labelling

The FOS were labelled with anthranilic acid as described previously.[15,18] Briefly, anthranilic acid (30 mg mL^{-1}) was dissolved in a solution of sodium acetate trihydrate (4%, w/v) and boric acid (2% w/v) in methanol. This solution was added to sodium cyanoborohydride (final concentration 45 mg mL^{-1}) and mixed to give the final labelling mixture. The dried FOS were dissolved in 30 μL water and 80 μL labelling mixture added before incubating at 80 °C for 1 h. The reaction was allowed to cool to room temperature, 1 mL acetonitrile/water (97:3, v/v) was added and the mixture was

vortexed. Labelled oligosaccharides were purified by chromatography through Spe-ed Amide 2 columns (Applied Separations, Allentown, USA). The columns were pre-equilibrated with 2×1 mL acetonitrile, 2×1 mL water followed by 2×1 mL acetonitrile. The samples were loaded using gravity flow and allowed to drip through the column. The column was washed with 2×1 mL acetonitrile/water (95:5, v/v) and labelled oligosaccharides eluted with 2×0.75 mL water.

4.5. Purification of fluorescently labelled FOS

Labelled oligosaccharides in 50 mM Tris/HCl buffer, pH 7.2 were purified through Concanavalin A (Con A)-Sepharose 4B column (100 μL packed resin). The column was pre-equilibrated with 2×1 mL water followed by 1 mL of 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ in water and finally 2×1 mL 50 mM Tris/HCl buffer, pH 7.2. The sample was added and washed with 2×1 mL 50 mM Tris/HCl buffer, pH 7.2. The bound FOS were then eluted with 2×1 mL hot (70 °C) 0.5 M methyl-α-D-mannopyranoside in 50 mM Tris/HCl buffer, pH 7.2.

4.6. Carbohydrate analysis by normal-phase high performance liquid chromatography (NP-HPLC)

ConA-Sepharose purified 2-AA-labelled oligosaccharides were separated by NP-HPLC using a 4.6×250 mM TSK gel Amide-80 column (Sigma, UK) according to previously published methods.[15] The chromatography system consisted of a Waters Alliance 2695 separations module and an in-line Waters 474 fluorescence detector set at Exλ 360 nm and Emλ 425 nm. All chromatography analyses were performed at 30 °C. Solvent A was acetonitrile. Solvent B was Milli-Q water. Solvent C was composed of 100 mM ammonium hydroxide, titrated to pH 3.85 with acetic acid, in Milli-Q water and was prepared using a standard 5.0 N ammonium hydroxide solution (Sigma, UK). Gradient conditions were as follows: time = 0 min ($t = 0$), 71.6% A, 8.4% B, 20% C (0.8 mL min^{-1}); $t = 6$, 71.6% A, 8.4% B, 20% C (0.8 mL min^{-1}); $t = 6$, 71.6% A, 8.4% B, 20% C (0.8 mL min^{-1}); $t = 40$, 52% A, 28% B, 20% C (0.8 mL min^{-1}); $t = 41$, 23% A, 57% B, 20% C (1.0 mL min^{-1}); $t = 43$, 23% A, 57% B, 20% C (1.0 mL min^{-1}); $t = 44$, 71.6% A, 8.4% B, 20% C (1.2 mL min^{-1}); $t = 59$, 71.6% A, 8.4% B, 20% C (1.2 mL min^{-1}); $t = 60$, 71.6% A, 8.4% B, 20% C (0.8 mL min^{-1}). Samples (<50 μL) were injected in Milli-Q water/acetonitrile (1:1, v/v).

Acknowledgements

The Glycobiology Institute is thanked for support. The authors thank United Therapeutics for the gift of some of the compounds used in this study.

References

1. Fleet, G. W.; Karpas, A.; Dwek, R. A.; Fellows, L. E.; Tyms, A. S.; Petursson, S.; Namgoong, S. K.; Ramsden, N. G.; Smith, P. W.; Son, J. C. *FEBS Lett.* **1988**, *237*, 128.
2. Compain, P.; Martin, O. R. *Imino Sugars: From Synthesis to Therapeutic Applications*; John Wiley & Sons Ltd, Chichester, 2007.
3. Dwek, R. A.; Butters, T. D.; Platt, F. M.; Zitzmann, N. *Nat. Rev. Drug Disc.* **2002**, *1*, 65.
4. Butters, T. D.; Wormald, M. R.; Dwek, R. A.; Platt, F. M.; Van Den Broek, L. A. G. M.; Fleet, G. W. J.; Krulle, T. M. *Tetrahedron: Asymmetry* **2000**, *11*, 113.
5. Romero, P. A.; Saunier, B.; Herscovics, A. *Biochem. J.* **1985**, *226*, 733.
6. Tan, A.; van den Broek, L.; van Boeckel, S.; Ploegh, H.; Bolscher, J. *J. Biol. Chem.* **1991**, *266*, 14504.
7. Block, T. M.; Lu, X.; Mehta, A. S.; Blumberg, B. S.; Tennant, B.; Ebling, M.; Korba, B.; Lansky, D. M.; Jacob, G. S.; Dwek, R. A. *Nat. Med.* **1998**, *4*, 610.
8. Lu, X.; Mehta, A.; Dwek, R.; Butters, T.; Block, T. *Virology* **1995**, *213*, 660.
9. Schul, W.; Liu, W.; Xu, H. Y.; Flamand, M.; Vasudevan, S. G. *J. Infect. Dis.* **2007**, *195*, 665.
10. Zitzmann, N.; Mehta, A. S.; Carrouee, S.; Butters, T. D.; Platt, F. M.; McCauley, J.; Blumberg, B. S.; Dwek, R. A.; Block, T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11878.
11. Mellor, H. R.; Neville, D. C.; Harvey, D. J.; Platt, F. M.; Dwek, R. A.; Butters, T. D. *Biochem. J.* **2004**, *381*, 861.
12. Mellor, H. R.; Neville, D. C.; Harvey, D. J.; Platt, F. M.; Dwek, R. A.; Butters, T. D. *Biochem. J.* **2004**, *381*, 867.
13. Mellor, H. R.; Nolan, J.; Pickering, L.; Wormald, M. R.; Platt, F. M.; Dwek, R. A.; Fleet, G. W.; Butters, T. D. *Biochem. J.* **2002**, *366*, 225.
14. Durantel, D.; Branza-Nichita, N.; Carrouee-Durantel, S.; Butters, T. D.; Dwek, R. A.; Zitzmann, N. *J. Virol.* **2001**, *75*, 8987.
15. Alonzi, D. S.; Neville, D. C.; Lachmann, R. H.; Dwek, R. A.; Butters, T. D. *Biochem. J.* **2008**, *409*, 571.
16. Rawlings, A. J.; Lomas, H.; Pilling, A. W.; Lee, M. J-R.; Alonzi, D. S.; Rountree, J. S.; Jenkinson, S. F.; Fleet, G. W. J.; Dwek, R. A.; Jones, J. H.; Butters, T. D. *ChemBioChem.* **2009**, in press.
17. van den Broek, L. A. G. M.; Vermaas, D. J.; van Kemenade, F. J.; Tan, M. C. C. A.; Rotteveel, F. T. M.; Zandberg, P.; Butters, T. D.; Miedema, F.; Ploegh, H. L.; van Boeckel, C. A. A. *Recl. Trav. Chim. Pays-Bas* **1994**, *113*, 507.
18. Neville, D. C.; Coquard, V.; Priestman, D. A.; Te Vruchte, D. J.; Sillence, D. J.; Dwek, R. A.; Platt, F. M.; Butters, T. D. *Anal. Biochem.* **2004**, *331*, 275.