Minireview

# α-Glucosidase inhibitors as potential broad based anti-viral agents

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**Abstract** ***N*-Linked oligosaccharides play many roles in the fate and functions of glycoproteins. One function is to assist in the folding of proteins by mediating interactions of the lectin-like chaperone proteins calnexin and calreticulin with nascent glycoproteins. These interactions can be prevented by inhibitors of the α-glucosidases and this causes some proteins to be misfolded and retained within the endoplasmic reticulum. In human immunodeficiency virus (HIV) and hepatitis B virus (HBV) the misfolding of key viral envelope glycoproteins interferes with the viral life cycle. It has been demonstrated in an animal model of chronic HBV that glucosidase inhibitors can alter glycosylation and have anti-viral activity. As the mechanism of action of α-glucosidase inhibitors is the induction of misfolded or otherwise defective viral glycoproteins, such inhibitors may be useful therapeutics for many viruses, especially those which bud from the endoplasmic reticulum (where protein folding takes place). For example bovine viral diarrhea virus, a pestivirus akin to hepatitis C virus, is also extremely sensitive to glucosidase inhibition.**



*Key words:* α-Glucosidase inhibitor; Antiviral agent; Endoplasmic reticulum

## 1. Introduction

*N*-Linked glycosylation is initiated by the cotranslational transfer of a 14 residue oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$) to certain polypeptides which contain the Asn-xaa-Ser/Thr glycosylation sequon [1]. After transfer the glycan chain is modified by a series of reactions within the endoplasmic reticulum (ER) and Golgi apparatus (Fig. 1). The first processing event is the stepwise removal of the terminal glucose residues by the ER α-glucosidases. α-Glucosidase I removes the outer α1,2-linked glucose residue while the inner two α1,3-linked glucose residues are removed in two steps by α-glucosidase II. In contrast to many of the other glycan processing events in the secretory pathway, the removal of the glucose residues is highly efficient. Cells do not normally secrete glycoproteins containing glucosylated glycan structures. Indeed, many cell types contain an additional pathway, the Golgi-endomannosidase pathway, which can remove glucose residues from glycoproteins that have not been fully processed within the ER [2]. In the presence of α-glucosidase inhibitors, this pathway can allow for further Golgi processing events to occur.

## 2. ER glucosidase processing allows interactions with chaperone proteins

The glucosidase reactions allow glycoproteins to interact with the ER chaperones calnexin and calreticulin [3]. Recent work has determined that calnexin is a lectin which binds exclusively to mono-glucosylated glycoproteins independent of protein conformation (Fig. 2) [4]. Interactions with calnexin facilitate the correct folding of some but not all glycoproteins. Monoglucosylated glycoproteins can be formed either by the sequential action of α-glucosidase I and II or by the action of the UDP-Glc:glycoprotein glucosyltransferase (Fig. 2) [5], which can reglucosylate misfolded glycoproteins that have been processed by α-glucosidase II [6]. This process allows further interactions with calnexin to occur, and can continue until the native conformation of the protein is achieved and the glycoprotein is no longer reglucosylated by the glucosyltransferase. For some proteins, such as the vesicular stomatitis virus (VSV) G protein and influenza HA, preventing a calnexin interaction with inhibitors of the α-glucosidases causes their misfolding and prolonged retention within the ER [7]. However, as not all proteins require a calnexin pathway to achieve their native conformation, inhibitors of the α-glucosidases can be used to specifically target the folding of proteins which require an interaction with calnexin.

## 3. α-Glucosidase inhibitors as anti-viral agents

Many animal viruses contain an outer envelope which is composed of one or more viral glycoproteins. These glycoproteins are often *essential* proteins in that they are required in the viral life cycle, either in virion assembly and secretion and/or infectivity. As processing of these glycoproteins occurs through the cellular machinery, inhibitors of the *N*-glycan processing pathway have been used to study the role of the *N*-glycans in several viral systems including human immunodeficiency virus (HIV-1 [8]), human hepatitis B virus (HBV [9]), human cytomegalovirus virus (HCMV [10]), influenza [11], Sinbis virus [12], and VSV [13]. Two examples which highlight *N*-linked glycan processing as a target of anti-viral intervention are the HIV model and the HBV model.

### *3.1. Glucosidase inhibitors prevent the fusion of HIV*

HIV-1, the causative agent of acquired immunodeficiency syndrome (AIDS), encodes two essential envelope glycoproteins (gp120 and gp41) through endoproteolytic cleavage of a precursor protein (gp160) within the *cis*-Golgi apparatus. Although proteolytically cleaved, gp120 remains non-covalently attached to the lumenal portion of the transmembrane

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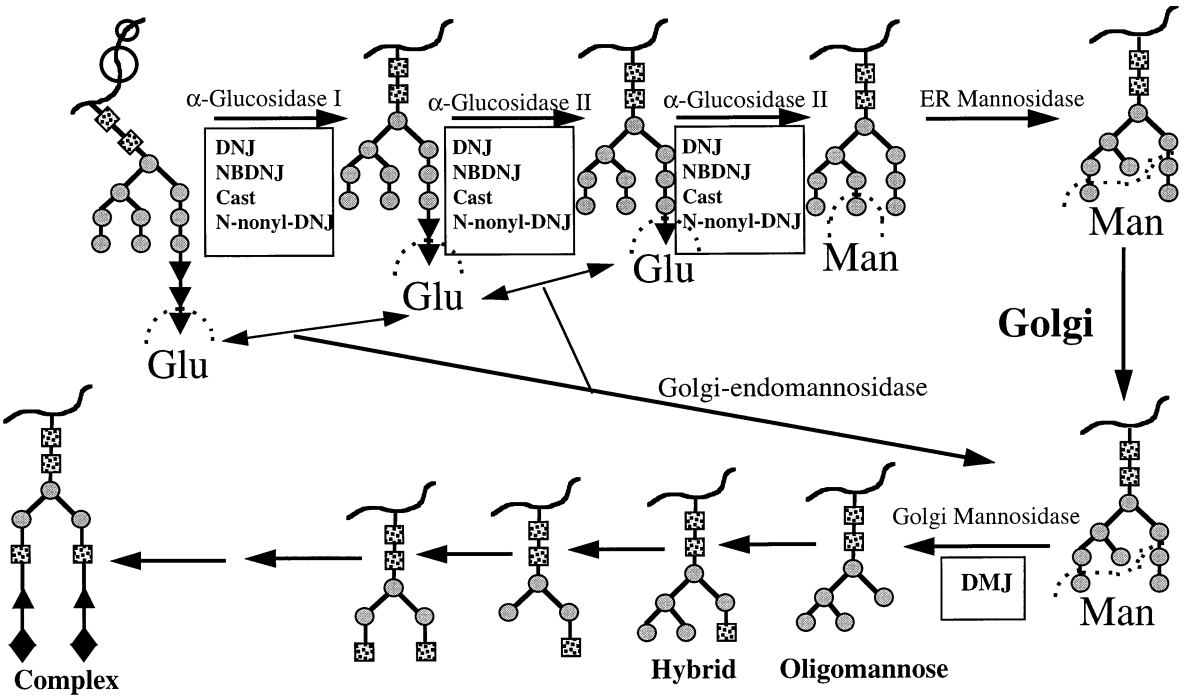


Fig. 1. Diagram of some of the major processing events in the biosynthesis of *N*-linked glycans. *N*-Linked glycosylation is initiated by the co-translational transfer of a 14 sugar superstructure to the growing polypeptide. The formation of this superstructure can be inhibited by tunicamycin (not shown). Other inhibitors of glycan processing events are shown in the boxes. After transfer to the Golgi, reactions can terminate at several steps, leading to the secretion of differentially glycosylated proteins (glycoforms). The three major classes of *N*-linked glycans are shown at the bottom (complex, hybrid, and oligomannose). Abbreviations are as follows: Glu, glucose; Man, mannose; DNJ, deoxynojirimycin; NBDNJ, *N*-butyl-deoxynojirimycin; *N*-nonyl-DNJ, *N*-nonyl-deoxynojirimycin; Cast, castanospermine; DMJ, deoxymannojirimycin. For the glycan structures (dotted squares): *N*-acetylglucosamine (GlcNAc); (gray circles): mannose; ▼: glucose; ▲: galactose; ◆: sialic acid.

gp41 through conserved regions within the amino and carboxy terminus. Therefore, gp120 is fully exposed on the outer face of the viral envelope, with the transmembrane gp41 acting as an anchor for the complex [14]. During infection gp120 binds to the CD4 surface antigen (the cellular receptor) and undergoes a conformational change and cleavage which ex-

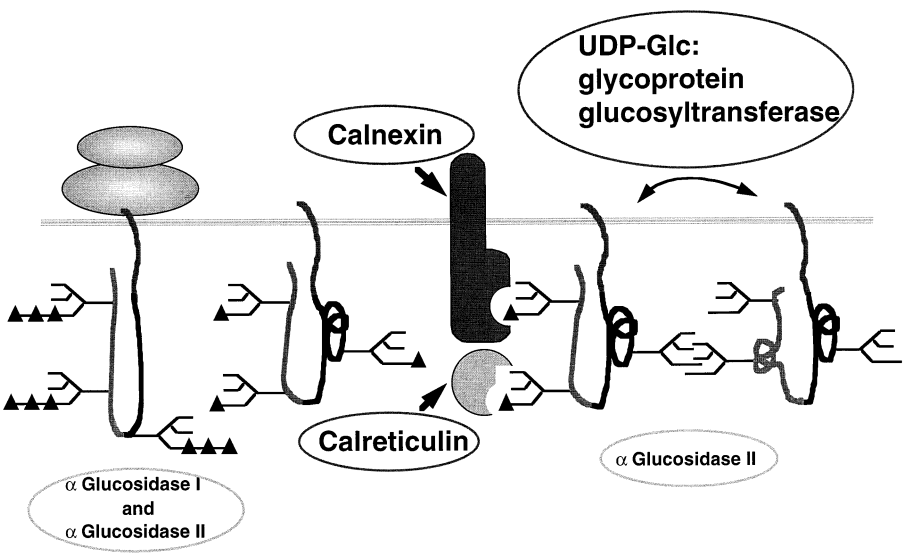


Fig. 2. The actions of glucosidase I and II enable the interactions between folding glycoproteins and the ER chaperones calnexin and calreticulin to occur. α-Glucosidase I cotranslationally removes the terminal α1-2 glucose residue which is followed by the removal of the exposed α1-3 residue by glucosidase II. The resulting monoglucosylated glycoprotein is recognized by and binds to calnexin and/or calreticulin. Removal of the last α1-3 linked glucose residue mediates release from calnexin. Misfolded proteins can be reglucosylated by the UDP-Glc:glycoprotein glucosyltransferase which allows them to rebind calnexin. Although some glycoproteins appear to require an interaction with calnexin/calreticulin for their folding and transport out of the ER [7], many do not [28]. In the glycan structures, triangles represent glucose residues.

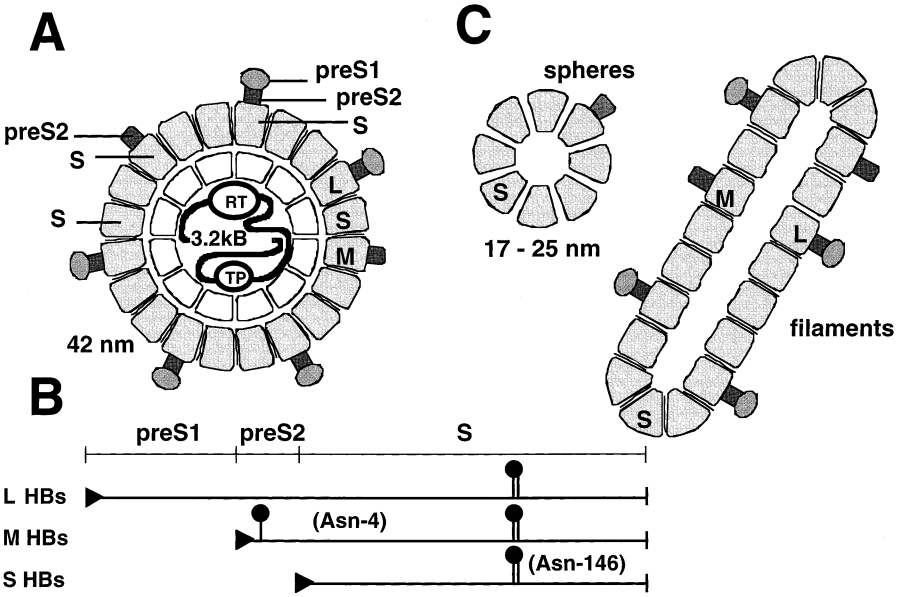


Fig. 3. The hepatitis B virus particles and envelope proteins. A: The HBV viral particle consists of a DNA containing nucleocapsid shell which is enveloped by the HBV envelope proteins. The specific viral envelope glycoproteins are indicated. B: The three HBV envelope proteins with the site of *N*-glycan attachment in the pre-S2 and S domains are indicated. The L protein consists of three domains: the pre-S1 domain, pre-S2 domain, and the S domain. M contains the pre-S2 and S domains while the S protein contains only the S domain. All three proteins have a common *N*-linked glycosylation site at Asn-146 of the S domain with the M protein containing an additional site at Asn-4 of the pre-S2 domain. The Asn-146 glycan site is partially occupied in all three envelope proteins while the pre-S2 site is fully occupied in M but unoccupied in the L protein [20]. The Asn-4 site on M has been shown to interact with calnexin [24]. C: The hepatitis B surface antigens (HBsAgs) or the HBV sub-viral particles as they are also called. The shape of the sub-viral particles is dependent upon the relative proportions of the S, M and L proteins. Spheres are composed predominantly of the S protein with small amounts of the M and L protein. Filaments contain more L protein and less S protein.

poses gp41. The exposure of gp41 allows fusion with the cellular membrane, thus mediating viral entry into the cell [15].

Both gp41 and gp120 are heavily *N*-glycosylated and normally contain a mixture of complex and oligomannose type glycans [14]. Treatment of HIV-1 infected cells with *N*-butyldeoxynojirimycin (NB-DNJ), an inhibitor of the α-glucosidases, inhibits syncytium formation and the formation of infectious virus [16]. In contrast, inhibition of post-ER glycosylation steps (DMJ, see Fig. 1) had no effect on the secretion of infectious virus, confirming that α-glucosidase processing is required for proper interactions with calnexin and/or calreticulin [1].

The reduction in secretion of infectious virus caused by NB-DNJ is the result of impairments in post-CD4 binding steps [17]. Although binding to CD4 occurs, the conformational shift and cleavage of gp120 that results in the exposure of gp41 does not. Thus, the process of viral fusion is prevented. Consistent with this is the finding that in the presence of NB-DNJ there is regional misfolding of gp120. Analysis of gp120 by a panel of conformation-dependent antibodies revealed structural changes within the V1/V2 loop region of gp120 [18]. Although these changes in gp120 do not prevent the transport to the plasma membrane or viral budding, they are sufficient to inhibit the viral fusion process, a crucial step in the HIV life cycle.

### 3.2. *Alterations in the pre-S2 glycan on the HBV M proteins, using site-directed mutagenesis, tunicamycin or NB-DNJ, prevents the secretion of hepatitis B virus*

In contrast to the HIV envelope glycoproteins, which contain 30 sites of *N*-linked glycosylation [14], the envelope proteins of hepatitis B virus contain only two glycosylation sites. However, just as with the HIV envelope proteins, the HBV glycoproteins are sensitive to inhibitors of the *N*-linked glycosylation pathway.

Hepatitis B virus, which infects over 350 million people worldwide, is a major etiological agent of liver disease and hepatocellular carcinoma [19]. The HBV genome encodes three envelope proteins: large (L), middle (M) and small (S). These are derived from a single open reading frame through the utilization of alternative translational start sites (see Fig. 3) [20]. In addition to being the major component of the viral envelope, these proteins are secreted in the form of smaller non-infectious sub-viral particles. These particles, which lack DNA, are secreted in vast excess compared to

Table 1
HBV viral and sub-viral particle secretion from cells treated with inhibitors of the *N*-linked glycosylation pathway or transfected with the glycan mutants

| | Virions | Sub-viral particles |
|---|---|---|
| Treatment/glycan mutant | | |
| Tunicamycin[a] | No | Yes |
| NB-DNJ[b] | No | Yes; No M protein |
| DMJ[a] | Yes | Yes |
| Glycan mutant[c] | | |
| $Sg^-$,$Mg^-$ | No | Yes |
| $Sg^-$ | Yes | Yes |
| $Mg^-$ | No | Yes |

[a]As in [21].
[b]As in [9].
[c]$Sg^-$ vector has glycan site Asn-146 removed; $Mg^-$ vector has glycan site Asn-4 removed; $Sg^-$,$Mg^-$ has both N-linked glycosylation sites removed (see Fig. 3, [22]).

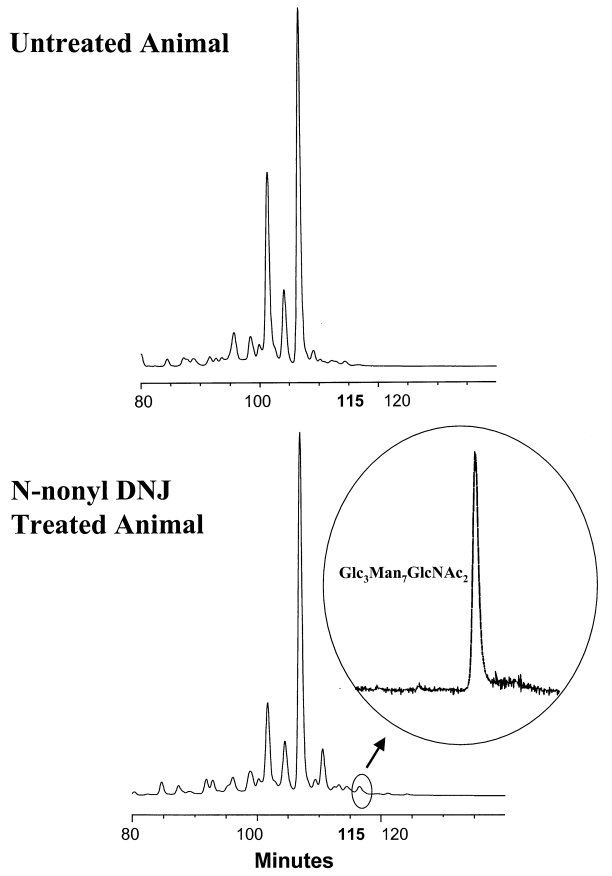


Fig. 4. Normal phase HPLC profile of serum glycans from an untreated animal and a *N*-nonyl-DNJ treated animal 4 weeks after treatment. The *x*-axis shows the retention time of the glycans on the HPLC column. Note that while after 115 min there are no peaks in the untreated animal, the treated animal has a small peak at approximately 116.5 min. This peak, which has been shown by oligosaccharide sequencing to be the $Glc_3Man_7GlcNAc_2$ structure, is indicated. Glucosidase inhibition does not lead to a 100% production of the $Glc_3Man_7GlcNAc_2$ structure due to the presence of a shunt pathway [2].

the viral particle, often outnumbering the virion a million to one.

The role of the *N*-glycans of HBV have been probed with site-directed mutagenesis and inhibitors of the *N*-linked glycosylation pathway (Fig. 1). These include tunicamycin (*N*-linked glycosylation) [21], NB-DNJ (glucosidase I and II) [9], and DMJ (mannosidase I) [21]. The results are summarized in Table 1.

Treatment of an HBV secreting cell line (Hep G2.2.15 cells) with NB-DNJ prevents the secretion of enveloped DNA [9] and causes the intracellular accumulation of viral DNA and particles containing excessive amounts of the M protein [22,23]. In contrast, sub-viral particles containing the L and S proteins are secreted with a full array of complex glycan structures which have been processed through the Golgi-endomannosidase pathway. In the presence of the glucosidase blockade, the Golgi-endomannosidase will remove all three glucose residues in one step [2]. These data show that neither ER glucosidase processing nor interaction with chaperones like calnexin, which only recognize monoglucosylated glycoproteins, is required for the correct folding of the S or L proteins.

Site-directed mutagenesis provides evidence that the pre-S2 glycosylation site on M plays an important role in virion morphogenesis [22]. Viral glycoproteins that lack the pre-S2 glycan site ($Mg^-$) are unable to secrete virus. In contrast, removal of the common site in the S domain ($Sg^-$) has no effect on viral secretion. These results, in conjunction with those seen in Table 1, indicate that the proper folding of the M protein requires the processing of the pre-S2 glycan to a $Glc_1Man_9GlcNAc_2$ structure, which allows it to interact with calnexin [24]. When this monoglucosylated structure is prevented from being formed, either by site-directed mutagenesis, tunicamycin or NB-DNJ, the M protein does not fold correctly and viral envelopment is prevented. Consistent with this is the lack of effect on virus secretion when using DMJ, a mannosidase inhibitor which prevents the formation of complex glycan structures, but does not interfere with the removal of the terminal glucose residues by the α-glucosidases nor with the correct folding of the protein (Table 1 and Figs. 1 and 2). The finding that the inhibition of glycan processing of a single site can so profoundly influence HBV secretion provides a powerful target for anti-viral intervention.

### *3.3. Glucosidase inhibitors can be used in an animal model of HBV to prevent the secretion of virus*

Woodchuck hepatitis virus (WHV) is a naturally occurring hepadnavirus pathogen of woodchucks [25]. WHV shares biochemical properties with human HBV such as (i) glycosylated envelope proteins, (ii) sensitivity to glucosidase inhibitors in vitro (unpublished data) and (iii) the capacity to establish chronic infections in woodchucks which resemble the human HBV infection in causing chronic hepatitis and hepatocellular carcinoma [25]. Woodchucks with chronic WHV infection are well studied and recognized to be good animal models to test anti-viral agents with potential for treating the human disease.

Recent work has shown that the glucosidase inhibitor *N*-nonyl-deoxynojirimycin (*N*-nonyl-DNJ, see Fig. 1) reduced the viremia in chronically infected woodchucks in a dose dependent manner [26]. *N*-Nonyl-DNJ, a 9 carbon alkyl derivative of deoxynojirimycin (DNJ), is 100–200 times more potent than *N*-butyl-deoxynojirimycin (NB-DNJ) in inhibiting HBV in cell based assays (manuscript in preparation). The mechanism of action of the drug, i.e. the inhibition of glucosidase processing, was observed in *N*-nonyl-DNJ treated animals (Fig. 4). Interestingly, at *N*-nonyl-DNJ concentrations sufficient to prevent WHV secretion the glycosylation of most serum glycoproteins appears unaffected (Fig. 4), suggesting that this class of therapeutics is highly selective against HBV.

Fig. 5 shows that the amount of hyperglucosylated glycan in the serum correlated with the change in viremia in *N*-nonyl-DNJ treated animals. Animals with the highest levels of hyperglucosylated glycan were those with the greatest reduction in viremia. The presence of the hyperglucosylated glycan represents a biochemical marker which highlights the extreme sensitivity of HBV to glucosidase inhibitors and can be used as a measure of drug efficacy.

## 4. Conclusions and further work

Inhibitors of glycosylation processing for the treatment of human disease have been used previously [27]. For example, NB-DNJ has been shown to have anti-HIV properties in vitro

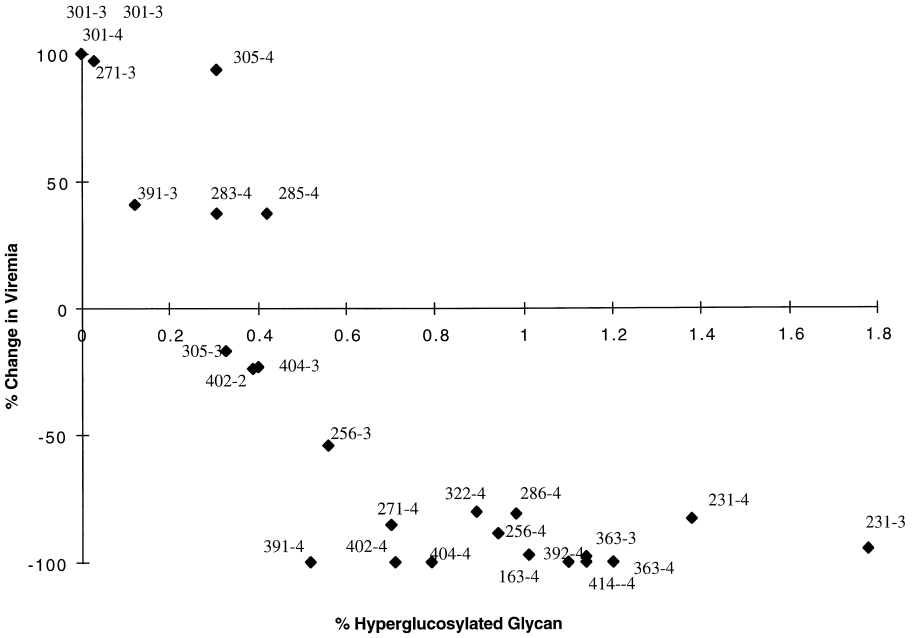


Fig. 5. The $Glc_3Man_7GlcNAc_2$ structure acts as a surrogate marker for drug efficacy. Woodchucks chronically infected with woodchuck hepatitis virus (WHV) were treated with N-nonyl DNJ for 4 weeks with varying amounts of drug [26]. The amount of enveloped virus at each week of treatment was determined and its change, as compared to pre-treatment levels, is shown on the X-axis. The amount of hyperglucosylated glycan in the serum of treated animals is shown along the Y-axis. Individual animals are indicated by the animal number followed by the week of glycan analysis (i.e. 231-3 is animal #231 at 3 weeks of treatment). Clearly, it is seen that animals with a greater amount of hyperglucosylated glycan have the greatest reduction in viremia.

[28] and is tolerated in people [29]. However, problems in achieving the therapeutic serum concentrations of NB-DNJ needed to inhibit glucosidase sufficiently in humans may limit the usefulness of the drug as an anti-HIV agent. Furthermore, glucosidase inhibition does not prevent the secretion of HIV but rather interferes with the infectivity process. Since one competent gp120 molecule on the virion surface may be sufficient to mediate the infection process, total glucosidase inhibition would be required to disrupt the viral life cycle. In HBV, glucosidase inhibition prevents the formation and secretion of the virus through the disruption of the viral envelope. In this case only a few misfolded viral glycoproteins are sufficient to prevent virion formation, and therefore only a mild degree of glucosidase inhibition is required in vivo to prevent the formation and secretion of virus (see Figs. 4 and 5). In addition, the use of a derivative, such as *N*-nonyl-DNJ, which has significantly enhanced anti-HBV activity, provides confidence that glycan processing may be a viable therapeutic target against HBV.

The process of *N*-linked glycosylation is a dynamic event which plays many roles in the fate and function of proteins. One important function is to assistant protein folding through enabling the interaction with lectin-like chaperones in the ER. The use of glucosidase inhibitors to prevent these interactions and have anti-viral activity may be especially promising in cases where the virus buds through the ER (as in HBV). By causing the misfolding of only a small number of envelope glycoproteins, these inhibitors sufficiently prevent proper virus envelopment in the ER and, hence, secretion of virions. Preliminary data obtained with another ER-budding virus (bovine viral diarrhea virus, BVDV) supports this hypothesis (manuscript in preparation). BVDV is extremely sensitive to glucosidase inhibitors (Fig. 6). This is particularly interesting as BVDV is in the same viral family as hepatitis C virus (HCV) and it is often used as a model for the latter. BVDV and HCV have a high degree of genomic homology, common replication strategies, and it is believed the same sub-cellular location for viral envelopment [30]. If their similarities extend to the same dependence on a functional glucosidase for the proper folding of their viral envelope glycoproteins, it may lead to the exciting possibility that glucosidase inhibitors could be used as broad based anti viral-hepatitis agents. Clearly, a single drug against HBV and HCV (and possibly hepatitis delta virus which uses the HBV glycoproteins) which together chronically infect over 400 million people worldwide would be of great therapeutic value.

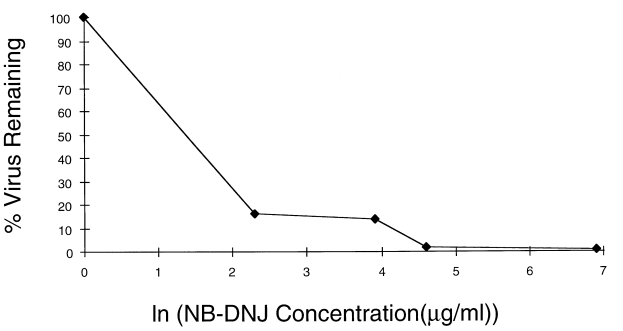


Fig. 6. NB-DNJ prevents plaque formation by bovine viral diarrhea virus (BVDV). The growth sensitivity of BVDV to the glucosidase inhibitor NB-DNJ was determined by plaque reduction assay. The relationship between the number of plaques (as compared to untreated) in % of virus remaining (*y*-axis) and amount of inhibitor used as a function of the natural log (ln) of the concentration of drug (in μg/ml, *x*-axis) is shown. An 84% reduction in plaques was achieved using 10 μg/ml (45 μm) of NB-DNJ. The $IC_{50}$ is approximately 6 μg/ml (27 μm). 98% of plaque reduction was achieved with 100 μg/ml NB-DNJ and even at the highest inhibitor concentration no cytotoxicity was observed.

## References


[1] Elbein, A.D. (1991) Semin. Cell Biol. 2, 309–317.
[2] Moore, S.E. and Spiro, R.G. (1990) J. Biol. Chem. 265, 1304–13112.
[3] Helenius, A. (1994) Mol. Biol. Cell. 5, 253–265.
[4] Zapun, A., Petrescu, S.M., Rudd, P.M., Dwek, R.A., Thomas, D. and Bergeron, J.J.M. (1997) Cell 88, 621–630.
[5] Pertrescu, A., Butters, T.B., Petrescu, S.M., Reinkensmeir, G., Dwek, R.A., Platt, F.M. and Wormald, M.R. (1997) EMBO J. 16 (in press).
[6] Sousa, M.C., Ferrero-Garcia, M.A. and Parodi, A.J. (1992) Biochemistry 31, 97–105.
[7] Hammond, C., Braakman, I. and Helenius, A. (1994) Proc. Natl. Acad. Sci. USA 91, 913–917.
[8] Karpas, A., Fleet, G.W.J., Dwek, R.A., Petursson, S., Namgoon, S., Ramsden, N.G., Jacob, G.S. and Rademacher, T.W. (1988) Proc. Natl. Acad. Sci. USA 85, 9229–9233.
[9] Block, T., Platt, F., Lu, X., Gerlich, W., Foster, G., Blumberg, B. and Dwek, R.A. (1994) Proc. Natl. Acad. Sci. USA 91, 2235–2239.
[10] Taylor, D.L., Fellows, L.E., Farrar, G.H., Nash, R.J., Taylor-Robinson, D., Mobberlay, M.A., Ryder, T.A., Jeffries, D.J. and Tyms, A.S. (1988) Antiviral Res. 10, 11–26.
[11] Datema, R., Olofsson, S. and Romero, P. (1987) Pharmacol. Ther. 33, 221–286.
[12] Schlesinger, S., Koyama, A.H., Malfar, C., Gee, S.L. and Schlesinger, M.J. (1985) Virus Res. 2, 139–149.
[13] Schlesinger, S., Miller, C. and Schlesinger, M.J. (1984) J. Biol. Chem. 259, 7597–7601.
[14] Hansen, J.-E.S. (1992) APMIS 100, (Suppl. 27) 96–108.
[15] McCune, J.M., Rabin, L.B., Feiberg, M.B., Lieberman, M., Kosek, J.C., Reyes, G.R. and Weissmann, I.L. (1988) Cell 53, 55–67.
[16] Fisher, P.B., Collin, M., Karlsson, G.B., James, W., Butters, T.D., Davis, S.J., Gordon, S., Dwek, R.A. and Platt, F.M. (1995) J. Virol. 69, 5791–5797.
[17] Fisher, P.B., Karlsson, G.B., Butters, T.D., Dwek, R.A. and Platt, F.M. (1996) J. Virol. 70, 7153–7160.
[18] Fisher, P.B., Karlsson, G.B., Butters, T.D., Dwek, R.A. and Platt, F.M. (1996) J. Virol. 70, 7143–7152.
[19] Beasley, R.P. (1988) Cancer 61, 1942–1956.
[20] Heermann, K.-H. and Gerlich, W.H. (1992) in: Molecular Biology of HBV (Maclachlan, A., Ed.), CRC Press, Boca Raton, FL.
[21] Lu, X., Mehta, A., Butters, T., Dwek, R.A. and Block, T.M. (1995) Virology 213, 660–665.
[22] Mehta, A., Lu, X., Block, T.M., Blumberg, B.S. and Dwek, R.A. (1997) Proc. Natl. Acad. Sci. USA 94, 1822–1827.
[23] Lu, X., Mehta, A., Dadmarz, M., Dwek, R.A., Blumberg, B.S. and Block, T.M. (1997) Proc. Natl. Acad. Sci. USA 94, 2380–2385.
[24] Werr, M. and Prange, R. (1998) J. Virol. 72, 778–782.
[25] Tennant, B.C. and Gerin, J.L. (1994) in: The Liver: Biology and Pathobiology, 3rd edn. (Arias, I.M., Faustto, N., Jacoby, W.B., Boyer, J.L., Schacter, D.A. and Shafritz, D.A., Eds.), pp. 1455–1466, Raven Press, New York.
[26] Block, T.M., Lu, X., Mehta, A., Blumberg, B., Tennant, B., Ebling, M., Korba, B., Lansky, D.M., Jacob, G.S. and Dwek, R.A. (1998) Nature Med. 4, 610–615.
[27] Jacob, G.S. (1995) Curr. Opin. Struct. Biol. 5, 605–661.
[28] Karpas, A., Fleet, G.W.J., Dwek, R.A., Petursson, S., Namgoon, S., Ramsden, N.G., Jacob, G.S. and Rademacher, T.W. (1988) Proc. Natl. Acad. Sci. USA 85, 9229–9233.
[29] Fischl, M., Resnick, L., Coombs, R., Kremer, A. and Potage, J. et al. (1994) J. AIDS 7, 139–147.
[30] Collet, M.S. (1992) Comp. Immun. Micro. Infect. Dis. 15, 145–155.