

Carbohydrate-binding Agents Cause Deletions of Highly Conserved Glycosylation Sites in HIV GP120

A NEW THERAPEUTIC CONCEPT TO HIT THE ACHILLES HEEL OF HIV*

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Jan Balzarini^{†1}, Kristel Van Laethem[‡], Sigrid Hatse[‡], Matheus Froeyen[‡], Willy Peumans[§], Els Van Damme[§], and Dominique Schols[‡]

From the [†]Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven and the [§]Department of Molecular Biotechnology, Ghent University, B-9000 Ghent, Belgium

Mannose-binding proteins derived from several plants (*i.e.* *Hippeastrum* hybrid and *Galanthus nivalis* agglutinin) or prokaryotes (*i.e.* cyanovirin-N) inhibit human immunodeficiency virus (HIV) replication and select for drug-resistant viruses that show profound deletion of *N*-glycosylation sites in the GP120 envelope (Balzarini, J., Van Laethem, K., Hatse, S., Vermeire, K., De Clercq, E., Peumans, W., Van Damme, E., Vandamme, A.-M., Bolmstedt, A., and Schols, D. (2004) *J. Virol.* 78, 10617–10627; Balzarini, J., Van Laethem, K., Hatse, S., Froeyen, M., Van Damme, E., Bolmstedt, A., Peumans, W., De Clercq, E., and Schols, D. (2005) *Mol. Pharmacol.* 67, 1556–1565). Here we demonstrated that the *N*-acetylglucosamine-binding protein from *Urtica dioica* (UDA) prevents HIV entry and eventually selects for viruses in which conserved *N*-glycosylation sites in GP120 were deleted. In contrast to the mannose-binding proteins, which have a 50–100-fold decreased antiviral activity against the UDA-exposed mutant viruses, UDA has decreased anti-HIV activity to a very limited extent, even against those mutant virus strains that lack at least 9 of 22 (~40%) glycosylation sites in their GP120 envelope. Therefore, UDA represents the prototype of a new conceptual class of carbohydrate-binding agents with an unusually specific and targeted drug resistance profile. It forces HIV to escape drug pressure by deleting the indispensable glycans on its GP120, thereby obligatorily exposing previously hidden immunogenic epitopes on its envelope.

The glycoproteins GP120 and GP41 that are present on the envelope of HIV² mediate the entry of the virus into its host cells. The envelope glycoproteins bind sequentially to the cellular receptor protein CD4 and a co-receptor, mainly CXCR4 or CCR5. The receptor-binding events trigger conformational changes in the GP120 and GP41 that lead to membrane fusion and virus entry. After infection, the host cell synthesizes the viral envelope glycoproteins encoded by the HIV *env* gene. The

env precursor becomes *N*-glycosylated to form the GP160 glycoprotein by addition of preassembled Glc₃Man₉GlcNAc₂ entities in the rough endoplasmic reticulum. After oligomerization to a trimer, GP160 is cleaved in the Golgi apparatus by a cellular protease (leading to noncovalently linked GP120 and GP41), and then the glycans are further processed (3). A large fraction of the predicted accessible surface of GP120 in the trimer is composed of heavily glycosylated structures that surround the receptor-binding regions (4). In GP120 of HIV-1(III_B), all 24 potential *N*-linked glycosylation sites are utilized as follows: 13 sites containing complex-type oligosaccharides and 11 sites containing hybrid and/or high mannose-type structures (5, 6). In GP41, there are seven potential *N*-glycosylation sites but only four of them seem to be glycosylated. All *N*-linked glycoprotein carbohydrates in GP120 share a common pentasaccharide Man₃GlcNAc₂ linked to the amide nitrogen of asparagine through the reducing hydroxyl group of GlcNAc. In HIV-1 GP120, 33% of the oligosaccharides are of the high mannose type, 4% are of the hybrid type, and 63% are of the complex type, being predominantly (≥90%) fucosylated and/or sialylated (7, 8).

Several high mannose-binding proteins interact with the HIV GP120 envelope. Indeed, the HIV-1 GP120 is a potential site of attack by the innate immune system through the C-type mannose-binding lectin (MBL) (9). MBL is found as multimers in serum (10) with a subunit molecular mass of 31 kDa. It has been shown that MBL binds to the abundantly present high mannose glycans on GP120 (11). Therefore, MBL represents an important mechanism for recognition of HIV by the immune system. Another human mannose-binding lectin is DC-SIGN present on dendritic cells (12, 13). It functions in dendritic cell recognition and mediates uptake of pathogen leading to antigen presentation to T-cells (14). The neutralizing 2G12 monoclonal antibody (mAb) is also known to be directed to an epitope on GP120 containing *N*-linked *N*-acetylglucosamine/mannose glycans (4, 15). More specifically, the highly conserved Asn-295, Asn-332, and Asn-392 in GP120 have been identified to be involved in 2G12 binding, whereas the Asn-339 and Asn-360 glycans also seem to influence the antibody binding (16). The crystal structure of 2G12 bound to Man₃GlcNAc₂ and α(1,2)Man oligomers is available (17). In particular, the α(1,2)Man residues of the GP120 glycans are important for 2G12 mAb binding to its targeted glycosylation sites (18). The 11-kDa protein cyanovirin-N (CV-N) from the cyanobacterium *Nostoc ellipsosporum* (19) is also specific for α(1,2)mannose oligomers and inhibits HIV by binding to GP120 (20–22). Among the plant lectins, mannose-binding proteins derived from a variety of monocotyledon species (including GNA, HHA, concanavalin A, and several others) are known to bind the HIV glycoprotein GP120. They show pronounced anti-HIV activity in cell culture presumably by preventing the virus from entering the cells (Refs. 23–28 and for a review see Refs. 29 and 30). Most interestingly, the *N*-acetylglu-

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¹ To whom correspondence should be addressed: Rega Institute for Medical Research, Katholieke Universiteit at Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Tel.: 32-16-337352; Fax: 32-16-337340; E-mail: jan.balzarini@rega.kuleuven.be.

² The abbreviations used are: HIV, human immunodeficiency virus; HIV-1, HIV type 1; UDA, *U. dioica* agglutinin; CBA, carbohydrate-binding agents; MBL, mannose-binding lectin; GNA, *G. nivalis* agglutinin; HHA, *Hippeastrum* hybrid agglutinin; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; CV-N, cyanovirin; CA, *Cymbidium* agglutinin; NPA, *N. pseudonarcissus* agglutinin; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; Ag, antigen; SIV, simian immunodeficiency virus.

cosamine-recognizing protein from the stinging nettle *Urtica dioica* (UDA) has also been shown to inhibit HIV in cell culture (27) and represents the only GlcNAc-recognizing agent that shows a pronounced anti-HIV activity reported so far. UDA is an 8.5-kDa monomeric protein having two carbohydrate-binding sites with different affinities (31). It ranks among the smallest plant lectins ever reported (32, 33).

We demonstrated recently (1, 2) that mannose-binding proteins such as GNA, HHA, and CV-N select for mutant HIV-1 strains that contain deleted *N*-glycosylation sites in GP120 resulting in different degrees of resistance to the mannose-binding proteins. Because (GlcNAc)₂ is invariably present in all glycans of GP120, it would be of particular interest to investigate the inhibitory effect of the GlcNAc-binding UDA plant lectin against the mannose-specific plant lectin-resistant virus strains, to select for UDA resistance development against wild-type HIV-1, and to phenotypically and genotypically characterize such UDA-resistant viruses. We found that UDA also selects for deletions of *N*-glycosylation sites of GP120, but surprisingly, its antiviral activity was much more preserved against the mutated virus strains than that of the mannose-specific plant lectins, CV-N and the mAb 2G12. Given the numerous observations that deletion of glycosylation sites in HIV GP120 results in a higher susceptibility of these viruses to the neutralizing activity of antibodies (34–36), and the *in vivo* findings that SIV strains containing deletions in envelope glycosylation sites trigger the production of neutralizing antibodies resulting in markedly lower circulating SIV levels in Rhesus monkeys (37), we believe that we now can put forward an entirely new concept for therapeutic HIV treatment. This work represents the demonstration that carbohydrate-binding agents (CBA), in particular GlcNAc-binding compounds, are a novel and unique class of antivirals endowed with a highly specific and targeted resistance profile. CBAs can force HIV to delete highly conserved glycosylation sites in GP120.

EXPERIMENTAL PROCEDURES

Test Compounds—The mannose-specific plant lectins from *Galanthus nivalis* (GNA), *Hippeastrum* hybrid (HHA), *Narcissus pseudonarcissus* (NPA), *Cymbidium* hybrid (CA), *Listera ovata*, *Epipactis helleborigine*, and UDA were derived and purified from these plants, as described before (38–41). AMD3100 was from AnorMed (Langley, British Columbia, Canada), and dextran sulfate-5000 and polyvinylacrylate were from Sigma and Dr. Görög (Budapest, Hungary), respectively. T20 (pentafuside, enfuvirtide) was kindly provided by AIDS Research Alliance (Los Angeles, CA), and CV-N was from Dr. J. B. McMahon (National Institutes of Health, Bethesda) and Dr. A. Bolmstedt (Göteborg, Sweden). The monoclonal antibody 2G12 was obtained from the Medical Research Council, Potter Bar, Hertfordshire, UK. Nevirapine was a gift from Boehringer Ingelheim.

Cells—Human T-lymphocytic CEM cells were obtained from the American Type Culture Collection (Manassas, VA) and cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (Bio-Whittaker Europe, Verviers, Belgium), 2 mM L-glutamine, and 0.075 M NaHCO₃.

Viruses—HIV-1(III_B) was provided by Dr. R. C. Gallo and Dr. M. Popovic (at that time at the NCI, National Institutes of Health, Bethesda).

Selection and Isolation of UDA-resistant HIV-1 Strains—The procedure followed for the selection of drug-resistant virus mutants was essentially as described recently (1, 2). Briefly, HIV-1(III_B) was added to CEM cell cultures in 48-well plates in the presence of 20 µg/ml UDA. For the generation of drug-resistant virus mutants, an increased drug

concentration was administered when full cytopathicity was obtained in the previous cell culture. The drug resistance selection schedule is depicted in Fig. 1.

Antiretrovirus Assays—The methodology of the anti-HIV assays has been described previously (1, 2). Briefly, CEM cells (4.5×10^5 cells per ml) were suspended in fresh culture medium and infected with HIV-1 at 100 CCID₅₀ (cell culture infective dose-50) per ml of cell suspension. Then 100 µl of the infected cell suspension were transferred to microplate wells, mixed with 100 µl of the appropriate dilutions of the test compounds, and further incubated at 37 °C. After 4–5 days, giant cell formation was recorded microscopically in the CEM cell cultures. The 50% effective concentration (EC₅₀) corresponded to the compound concentrations required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures.

Antiviral Activity of Test Compounds against HIV-1 Clade Isolates in PBMC—Primary clinical isolates representing different HIV-1 clades and an HIV-2 isolate were all kindly provided by Dr. L. Lathey from BBI Biotech Research Laboratories, Inc., Gaithersburg, MD, and their co-receptor use (R5 or X4) was determined by us on the astrogloma U87.CD4 cell line transfected with either CCR5 or CXCR4 as described previously (42). The following clinical isolates were included in the study: UG273 (clade A, R5), US2 (clade B, R5), ETH2220 (clade C, R5), UG270 (clade D, X4), ID12 (clade E, R5), BZ163 (clade F, 5R), BCF-DIOUM (clade G, R5), BCF06 (clade O, X4), and HIV-2 BV-5061W (X4).

Antiviral testing of these isolates in PBMC was as follows. PBMC from healthy donors were isolated by density gradient centrifugation and stimulated with PHA at 2 µg/ml (Sigma) for 3 days at 37 °C. The PHA-stimulated blasts were washed twice with phosphate-buffered saline and counted by trypan blue. The cells were then seeded at 0.5×10^6 cells per well into a 48-well plate containing varying concentrations of compound in cell culture medium (RPMI 1640) containing 10% fetal calf serum and interleukin-2 (25 units/ml, R & D Systems Europe, Abingdon, UK). The virus stocks were diluted in medium and added at a final dose of 250 pg of p24 or p27/ml as determined by a viral core antigen (Ag)-specific enzyme-linked immunosorbent assay. Cell supernatant was collected at day 12, and HIV-1 core Ag in the culture supernatant was analyzed by a p24 Ag enzyme-linked immunosorbent assay kit (PerkinElmer Life Sciences). For HIV-2 p27 Ag detection, the INNOTEST from Innogenetics (Temse, Belgium) was used.

Genotyping of the HIV-1 Env Region—Proviral DNA was extracted from cell pellets using the QIAamp blood mini kit (Qiagen, Hilden, Germany) as described recently (43). Both the GP120 and GP41 genes were covered in this assay.

Viral Load Determination—The p24 antigen was determined using the commercial assay kit from PerkinElmer Life Sciences.

RESULTS

Selection and Genotypic Characterization of UDA-resistant HIV-1(III_B) Strains—HIV-1(III_B)-infected CEM cell cultures were exposed to UDA at ~5 µg/ml (~0.6 µM), which is an ~2–4-fold higher concentration than its EC₅₀ (Fig. 1). Subcultivations were performed by transferring the virus-infected cell suspensions to freshly prepared CEM cell cultures (ratio ~1:10 to 1:5). Syncytia formation was used as the parameter to estimate virus breakthrough. Only when the giant cell formation was abundantly visible in the drug-exposed, virus-infected cell cultures was the UDA concentration increased stepwise. A substantial number of subcultivations were required before virus breakthrough was observed at higher concentrations than the initial UDA concentration.

FIGURE 1. Selection pathway for UDA-, HHA-, GNA-, and nevirapine-resistant HIV-1 strains. The drug concentrations were increased when marked viral cytopathicity was observed in the cell cultures. Each subcultivation was performed every 4 or 5 days. About 100–200 μ l of the virus-infected drug-exposed CEM cell cultures were added to 800–900 μ l of fresh uninfected cell cultures at each passage.

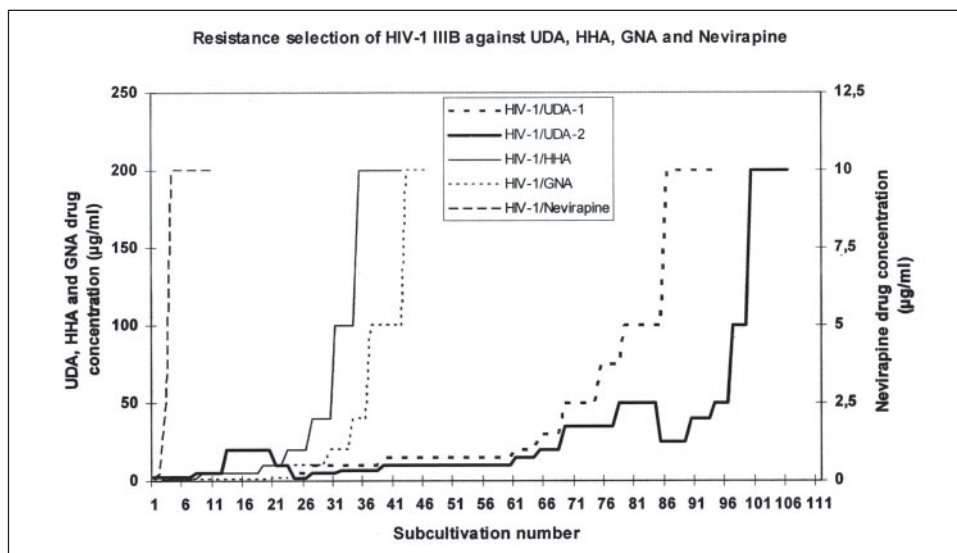


TABLE ONE

Glycosylation sites affected in HIV-1(IIIB) GP120 under UDA pressure in CEM cell cultures

Glycosylated asparagines ^{a,b} in HIV-1(IIIB) GP120	Mutant HIV-1(IIIB) strains that emerged under escalating UDA exposure ^c						
	UDA-20	UDA-25-1	UDA-50-1	UDA-15-2	UDA-50-2	UDA-100-2	UDA-200-2
⁸⁸ NVT ⁹⁰	Thr/Ile-90	Ile-90	Ile-90	Ile-90	Ile-90	Ile-90	Ile-90
¹³⁶ NDT ¹³⁸							
¹⁴¹ NSS ¹⁴³							
¹⁵⁶ NCS ¹⁵⁸							
¹⁶⁰ NIS ¹⁶²		<u>Asp-160</u>	<u>Asp-160</u>				
¹⁸⁶ NDT ¹⁸⁸							
¹⁹⁷ NTS ¹⁹⁹							
²³⁰ NKT ²³²		Thr/Met-232	Thr/Met-232	Asp-230	Asp-230	Asp-230	Asp-230
²³⁴ NGT ²³⁶		Ile-236	Ile-236	Ile-236	Ile-236	Ile-236	Ile-236
²⁴¹ NVS ²⁴³							
²⁶² NGS ²⁶⁴							
²⁷⁶ NFT ²⁷⁸							
²⁸⁹ NQS ²⁹¹					Thr-289	Thr-289	Thr-289
²⁹⁵ NCT ²⁹⁷							
³⁰¹ NNI ³⁰³		<u>Tyr-301</u>	<u>Tyr-301</u>	<u>Tyr-301</u>	<u>Tyr-301</u>	<u>Tyr-301</u>	Tyr-301
³³² NIS ³³⁴		Asn-334	Asn-334		Asn-334	Asn-334	Asn-334
³³⁹ NNT ³⁴¹		<u>Asp-339</u>	<u>Asp-339</u>				
³⁵⁶ NKT ³⁵⁸							
³⁸⁶ NST ³⁸⁸							
³⁹² NST ³⁹⁴		Ile-394	Ile-394	Thr/Ile-394	Ile-394	Ile-394	Ile-394
³⁹⁷ NST ^{399d}							
⁴⁰⁶ NNI ⁴⁰⁸					<u>Tyr-406</u>	<u>Tyr-406</u>	Tyr-406
⁴⁴⁸ NIT ⁴⁵⁰		<u>Asn/Lys/Tyr-448</u>	<u>Asn/Lys/Tyr-448</u>		<u>Asp-448</u>	<u>Asp-448</u>	Asp-448
⁴⁶³ NES ^{465e}							

^a Amino acid sequence numbering is according to Kwong *et al.* (44).

^b High mannose type glycosylation sites are indicated by an asterisk. The other glycosylation sites are complex mannose-type (according to Leonard *et al.* (5)).

^c Mutant HIV-1(IIIB) strains that emerged under stepwise escalating UDA concentrations are shown, starting from 20 to 200 μ g/ml. Underlined mutated amino acids represent mutations at the asparagine residues that are part of a glycosylation motif. Not underlined mutated amino acids represent mutations at threonine/serine residues that are part of a glycosylation motif.

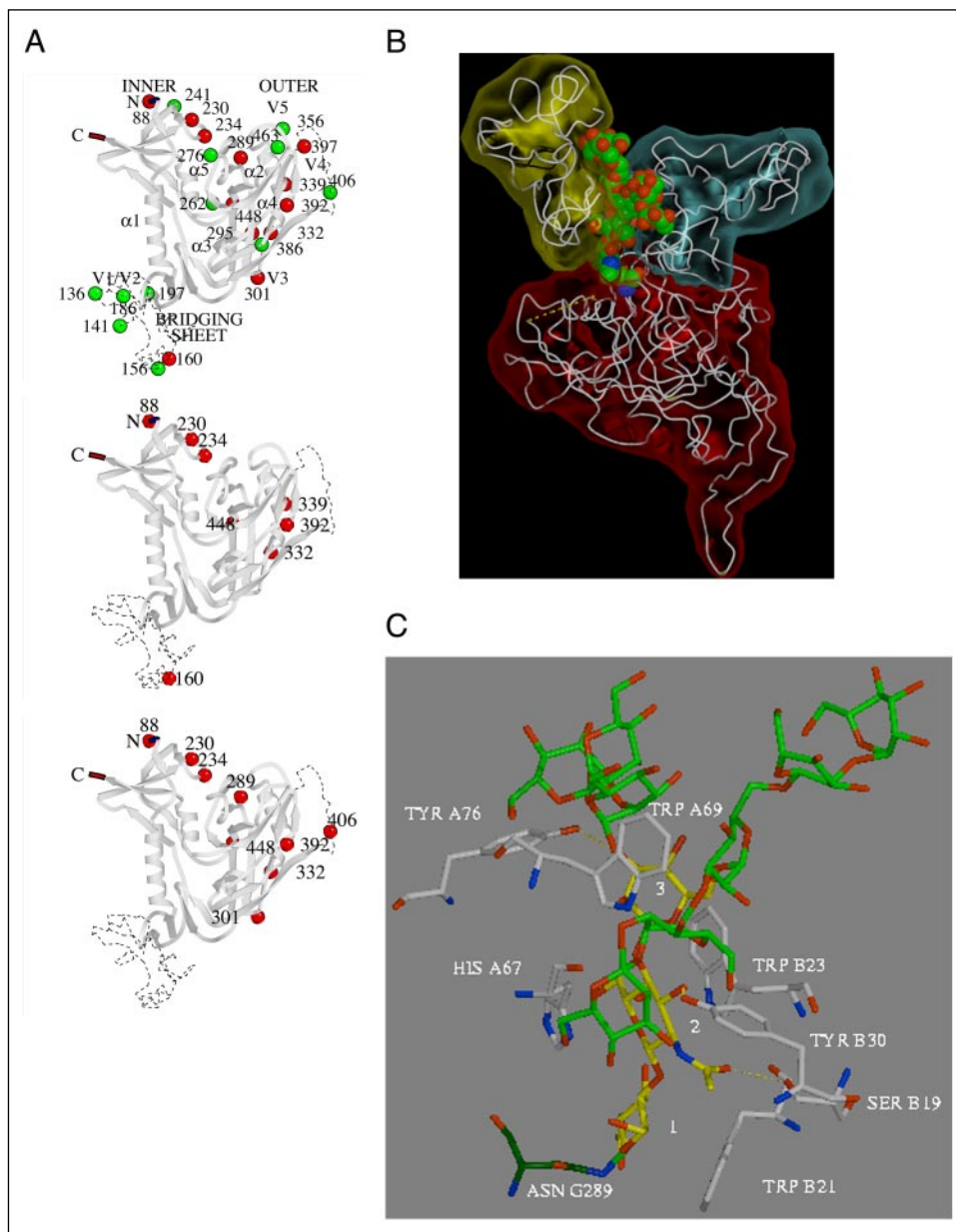
^d The ³⁹⁷NST³⁹⁹ glycosylation site is deleted in HIV-1(IIIB).

^e The ⁴⁶³NES⁴⁶⁵ glycosylation site does not exist in the HIV-1(IIIB) strain used in our experiments because of the presence of a ⁴⁶³NGP⁴⁶⁵ sequence.

At least 60 subcultivations (4 or 5 days/subcultivation) were required before the drug concentration could be increased without loss of the virus from the infected cell cultures. It took up to 80–100 subcultivations (≥ 9 months) before the virus could replicate in the presence of markedly higher UDA concentrations. The extremely long period required to effect marked virus breakthrough is in contrast to the exten-

sive drug resistance development of NRTIs (*i.e.* lamivudine, 3TC) (data not shown) and NNRTIs (*i.e.* nevirapine) that easily select for highly resistant virus strains within 5 or less subcultivations (Fig. 1). Moreover, there is clearly a much higher restricted genetic barrier to achieve UDA-resistant virus strains than to obtain NRTI and NNRTI-resistant virus strains. When UDA is added to the virus-infected cell cultures at con-

FIGURE 2. Mapping of the deleted glycosylation sites in GP120 of HIV-1 strains isolated under escalating UDA concentrations in CEM cell cultures and interaction of UDA with glycans of HIV-1 GP120. The graphs were generated by Bobscript, Molscript, and Raster 3D packages (54–56). **Panel A**, GP120 (according to Kwong *et al.* (44)) contains 24 numbered *N*-glycosylation sites (circles). The *N*-glycosylation sites that were found to be deleted in any of the seven UDA-exposed virus strains were colored in red. The *N*-glycosylation sites that were not found mutated were colored in green. **Panel B**, model of a $\text{Man}_9\text{GlcNAc}_2$ moiety (green/red ball representation) bound to GP120 (red/brown) at Asn-289 and sandwiched by two UDA molecules in yellow (left) and in blue (right). The two UDA molecules clamp a sequence of two GlcNAc residues and one Man residue. The model is created starting from the x-ray structure of GP120 in Protein Data Bank entry 1gc1 (38), a complex of tri-*N*-acetylchitotriose sugar with UDA in Protein Data Bank entry 1ehh (32), and a high mannose sugar tree ($\text{Man}_9\text{GlcNAc}_2$) from Protein Data Bank entry 1op5 (17). The GlcNAc-92 sugar next to Trp B21 in 1ehh was fit onto the GlcNAc sugar bound to Asn-289 of GP120. The GlcNAc was allowed to rotate around the bonds connecting it to Asn-289 of GP120 to ensure lack of any clashes between GP120 and the UDA proteins. The original GlcNAc attached to Asn-289 was then removed. In a following step the Man_3 residue of the mannose sugar tree was fitted onto residue GlcNAc-90 in code 1ehh (next to Trp A69 of UDA). The GlcNAc-90 was then removed. Finally, all dihedral angles connecting the mannose residues in the carbohydrate tree were manually rotated to remove atomic overlaps with the UDA molecules. **Panel C**, molecular structure of the UDA carbohydrate interaction for two UDA molecules with the $\text{GlcNAc}_2\text{Man}$ (yellow) oligomer. The remaining Man residues in the high mannose glycan are colored green. The individual carbohydrate rings are designated 1, 2, and 3. The amino acids from UDA that interact with $\text{GlcNAc}_2\text{Man}$ are shown in gray. A denotes the interacting amino acids of the first UDA molecule, and B denotes the interacting amino acids from the second UDA molecule.



centrations that are only 2–3-fold the EC_{50} , the virus is easily lost upon further subcultivations. For the NRTI lamivudine (data not shown) and for the NNRTI nevirapine, a 10-fold higher EC_{50} drug concentration still results in (mutant) virus breakthrough within 4–5 passages (Fig. 1). Also, mannose-specific proteins such as HHA and GNA allow abundant virus breakthrough to be much easier (~20–30 subcultivations) (2) than UDA (Fig. 1). During the UDA resistance selection process, a total of seven virus strains have been isolated, grown, and genetically and phenotypically characterized. The GP120 and GP41 genes of a total of eight (one control and 7 drug-treated) different virus strains were isolated during the UDA resistance selection process. A variety of amino acid changes occurred in GP120 of the drug-selected virus strains (TABLE ONE). More importantly, virtually all mutations found in GP120 were located at *N*-glycosylation sites or at Thr/Ser sites that are part of a glycosylation motif. Although only one mixture of mutated amino acid with wild-type has been found in GP120 of the virus isolate that was obtained after a few passages in the presence of 20 $\mu\text{g}/\text{ml}$ UDA, prolonged exposure of the virus-infected cell cultures at fixed UDA

concentrations as low as 25 $\mu\text{g}/\text{ml}$ (selection series-1) or 15 $\mu\text{g}/\text{ml}$ (selection series-2) resulted in the appearance of virus isolates that contained at least 9 (7 pure and 2 mixtures with wild-type (series-1)) or 5 (4 pure and 1 mixture with wild-type (series-2)) deletions at potential *N*-glycosylation sites in GP120. A further stepwise increase of the UDA concentration in the cell cultures resulted in the isolation of virus strains containing eight different glycosylation site deletions in the series-1 of drug exposure. The recovery of virus strains in the series-2 of drug exposure contained nine different glycosylation site deletions that differed slightly from those found in the series-1 of drug exposure (TABLE ONE). The virus strain that was used to start the UDA resistance selections contained 22 of the 24 potential glycosylation sites reported by Kwong and co-workers (44). In total, 11 of 22 glycosylation sites of HIV-1(III_B) GP120 have been affected by prolonged UDA exposure in cell culture. These mutations represent 7 of 11 high mannose- or hybrid-type and 4 of 11 complex mannose-type glycosylation sites in the GP120. Most interestingly, in GP41, it was observed that a novel *N*-glycosylation site appeared by mutating Lys to Asn at amino acid position

TABLE TWO

Sensitivity of mutant HIV-1(III_B) strains to the inhibitory activity of test compounds

The molecular weights of the lectins are as follows: UDA (8,700); GNA (50,000); HHA (50,000); LOA (25,000); NPA (33,000); CA (25,000); EHA (25,000); and CV-N (11,000).

	EC ₅₀ ^a (μM)							
	HIV-1(III _B)	UDA-20	UDA-25-1	UDA-50-1	UDA-15-2	UDA-50-2	UDA-100-2	UDA-200-2
UDA	0.14 ± 0.04	0.094 ± 0.0	1.5 ± 0.16	1.8 ± 0.83	0.41 ± 0.11	0.65 ± 0.33	1.2 ± 0.34	3.3 ± 0.05
GNA	0.018 ± 0.0	0.006 ± 0.0	0.55 ± 0.07	0.60 ± 0.28	0.11 ± 0.06	0.70 ± 0.14	0.80 ± 0.40	0.70 ± 0.14
HHA	0.006 ± 0.003	0.005 ± 0.02	0.50 ± 0.14	0.50 ± 0.14	0.09 ± 0.05	0.80 ± 0.35	0.66 ± 0.31	0.70 ± 0.14
LOA	0.004 ± 0.0	0.005 ± 0.002	0.13 ± 0.09	0.13 ± 0.04	0.04 ± 0.004	0.41 ± 0.34	0.63 ± 0.30	0.32 ± 0.0
NPA	0.009 ± 0.0	0.033 ± 0.004	0.18 ± 0.0	0.79 ± 0.60	0.15 ± 0.06	0.30 ± 0.08	0.23 ± 0.02	0.39 ± 0.18
CA	0.031 ± 0.01	0.06 ± 0.0	1.3 ± 0.76	1.4 ± 0.85	1.0 ± 0.28	1.2 ± 0.74	1.0 ± 0.03	1.4 ± 0.56
EHA	0.004 ± 0.0		0.15 ± 0.09	0.54 ± 0.37		0.41 ± 0.34	0.49 ± 0.32	0.93 ± 0.23
CV-N	0.003 ± 0.0	0.001 ± 0.0003	0.13 ± 0.07	0.11 ± 0.0	0.004 ± 0.0006	0.17 ± 0.03	0.15 ± 0.80	0.25 ± 0.03
mAb 2G12 ^b	1.7 ± 0.46			50				>50
DS-5000	0.08 ± 0.03	0.15 ± 0.07	0.09 ± 0.01	0.07 ± 0.01	0.25 ± 0.07	0.13 ± 0.07	0.13 ± 0.04	0.25 ± 0.07
Polyvinylacryl sulfate	0.01 ± 0.007		0.025 ± 0.0	0.03 ± 0.008		0.02 ± 0.003	0.03 ± 0.009	0.024 ± 0.018
AMD 3100	0.096 ± 0.0	0.048 ± 0.007	0.043 ± 0.007	0.06 ± 0.0	0.072 ± 0.001	0.039 ± 0.028	0.043 ± 0.007	0.030 ± 0.012
T-20	0.054 ± 0.023	0.076 ± 0.009	1.48 ± 0.49	0.18 ± 0.06	0.126 ± 0.063	0.162 ± 0.072	0.45 ± 0.36	1.0 ± 0.18

^a EC₅₀ or 50% effective concentration that is required to inhibit HIV-1 replication in CEM cell cultures by 50%.

^b Data are expressed in μg/ml.

126 in the HIV-1 strains UDA-50-2, UDA-100-2, and UDA-200-2 and in the virus strains UDA-25-1 (mixture with wild-type) and UDA-50-1.

Mapping of the UDA-selected Amino Acid Mutations in the Three-dimensional Structure of HIV-1 GP120—The *N*-glycosylation sites that were deleted under UDA drug pressure were mapped on the three-dimensional structure of GP120 determined by Kwong *et al.* (44) (Fig. 2). It shows the 24 putative glycosylation sites represented by circles and their accompanying amino acid number. The red circles in Fig. 2 indicate the *N*-glycosylation site deletions that emerge under UDA drug treatment in all isolates. As can be seen, the deleted glycosylation sites occur scattered rather than clustered at certain positions in GP120. Although Asn-88, Asn-230, and Asn-234 are closely located at the GP41-binding interface of GP120, the other deleted glycosylation sites were located between the V1 and V2 domains (Asn-160), in the V3 domain (Asn-301), closely near the V3 domain (Asn-289 and Asn-332), and in the V4 domain (Asn-392 and Asn-406). The Asn-448 glycosylation site is located between C4 and V5 (5).

Drug Sensitivity/Resistance Spectrum of the Mutated Virus Strains—The mutant virus strains isolated at different time points during the UDA selection process have been phenotypically characterized for their susceptibility to the inhibitory potential of the *N*-acetylglucosamine-specific UDA; the α(1,3)/α(1,6)-mannose-specific plant lectins HHA, GNA, *L. ovata*, NPA, CA, and *E. helleborigine*; the α(1,2)-mannose-specific prokaryotic CV-N; the polyanions DS-5000 and polyvinylacryl sulfate; the CXCR4 antagonist AMD3100; and the GP41-targeting fusion peptide T-20 (TABLE TWO). The HIV-1/UDA-20 strain virtually displays a similar drug sensitivity spectrum as wild-type virus. For all other mutant virus strains, UDA showed a modestly decreased inhibitory activity (with one exception, only 3–12-fold). In contrast, although the α(1,3)/α(1,6)-mannose-specific plant lectins had an ~10–30-fold decreased antiviral activity against the HIV-1/UDA-15-2 virus strain that contains five deletions in the glycosylation sites of GP120, they invariably lost 40–200-fold of their antiviral potency against the other virus strains that contained nine deleted glycosylation sites. Also the α(1,2)-mannose-binding cyanovirin showed (except for the HIV-1/UDA-15-2 strain) a 50–100-fold decreased antiviral activity, which was fairly comparable with the loss of antiviral activity of the mannose-specific plant lectins (TABLE TWO). Also, the mAb 2G12 markedly lost antiviral activity against the UDA-exposed viruses. In sharp contrast,

none of the other entry inhibitors, affecting three different sites of interaction in the entry process (*i.e.* GP120 for the polyanions, CXCR4 for AMD3100 and GP41 for T20), showed a significantly decreased antiviral activity against the mutant virus strains selected in the presence of escalating UDA concentrations (TABLE TWO).

Inhibitory Activity of UDA against HIV-1(III_B) Isolates Selected under Escalating Concentrations of Mannose-binding Proteins—Recently, a variety of HIV-1(III_B) strains has been selected under escalating concentrations of the mannose-binding plant lectins GNA and HHA. The locations of the deleted glycosylation sites in GP120 have been determined (1, 2). The number of GP120 glycosylation sites in GP120 affected in the virus isolates ranked from 2 (isolate 2) to 8 (isolates 10 and 12) (TABLE THREE). The degree of decreased sensitivity of these viruses against the inhibitory activity of the mannose-binding HHA and GNA closely correlated with an increasing number of deleted glycosylation sites in HIV-1 GP120. The decreased inhibitory activities of HHA and GNA amounted to 600–700-fold for the highest mutated virus strains (isolate 12) (TABLE THREE). In sharp contrast, the sensitivity of the virus strains for UDA were at most 1.5–2.5-fold decreased for the highest mutated virus strains, but in the majority of cases, UDA fully kept its antiviral potency against the mutant virus strains (TABLE THREE).

Inhibitory Activity of UDA and Mannose-binding Proteins against Isolates of Distinct HIV-1 Clades Containing Varying Numbers of Glycosylation Sites in GP120—UDA and also the mannose-specific GNA, HHA, CA, and CV-N were evaluated for their inhibitory activity against the replication of virus strains that belong to different HIV-1 clades (TABLE FOUR). The mannose-specific compounds showed widely varying degrees of inhibitory activities against the different clade isolates. The α(1,2)-mannose-binding CV-N kept a much more consistent antiviral potency than the mannose-specific plant lectin congeners (EC₅₀ range between 0.15 and 1.8 μg/ml) (0.013–0.16 μM) (TABLE FOUR), but still showed up to 12-fold variation in its EC₅₀ values. Remarkably, UDA kept a consistent antiviral inhibitory potential irrespective of the nature of the HIV clade (EC₅₀ range between 4.9 and 8.9 μg/ml) (0.56–1.0 μM) (TABLE FOUR).

Infectivity (Virulence) of UDA-resistant Mutant HIV-1 Strains—The virulence (infectivity potential) of HIV-1/UDA-50-1 and HIV-1/UDA-200-2 was determined in comparison with wild-type parental HIV-1(III_B). When CEM cell cultures were infected at equal p24 Ag amounts

TABLE THREE

Antiviral activity of UDA and mannose-binding proteins against HIV-1 strains mutated in GP120 glycosylation sites

N-Glycosylation sites ^a affected in GP120	Mutated HIV-1 (III _B) strains ^b											
	1	2	3	5	7	9	8	6	4	11	10	12
88				+/-	+/-					+/-		+
186												+
230				+/-		+/-	+/-			+	+	
234		+/-	+/-	+/-	+	+/-	+	+	+	+	+	+
276							+/-		+/-		+/-	
289			+/-	+/-	+/-	+	+/-	+	+/-	+/-		+
295												+
301		+	+	+	+	+	+	+	+	+	+	+
332											+	+
339			+	+	+	+	+/-	+	+	+	+	+
386												+
392						+/-	+		+	+	+	
Compound	EC ₅₀ ^c (μM) in CEM cell cultures											
GNA ^d	0.008	0.020	0.056	0.24	0.58	0.98	0.62	0.40	0.11	3.1	0.52	5
HHA ^d	0.006	0.024	0.040	0.20	0.52	0.74	0.38	0.22	0.12	2.1	1.1	3.8
UDA	0.129	0.141	0.18	0.21	0.19	0.21	0.15	0.18	0.11	0.18	0.29	0.16

^a N-Glycosylation sites in GP120 were deleted in the mutant HIV-1 strains with +. The +/- means that DNA sequencing revealed a mixture between wild-type and mutated(deleted) glycosylation site.

^b Mutant HIV-1 strains that were isolated under escalating concentrations of the mannose-binding proteins GNA and HHA are shown. The isolation and characterization of the virus strains were described by Balzarini *et al.* (1, 2). The code numbers correspond to following designations in the references as follows: 1, wild type; 2, GNA-1.0; 3, HHA-1.1; 4, HHA-2.1; 5, GNA-1.1a; 6, HHA-1.3; 7, GNA-2.1a; 8, HHA-2.2; 9, GNA-1.2; 10, GNA-500CS; 11, HHA-500CS; and 12, HHA-500SN.

^c 50% effective concentration of the antiviral agents in CEM cell cultures is shown.

^d Data were taken from Balzarini *et al.* (1, 2) (μg/ml values converted to μM values).

TABLE FOUR

Antiviral activity of UDA and mannose-binding proteins against a variety of HIV-1 clade isolates, laboratory HIV-1 strains, and HIV-2 in PBMC

Compound	EC ₅₀ ^a (μM)											
	HIV-1 clade isolate (with co-receptor preference)								HIV-2	HIV-1 laboratory strains		
	A, UG273 R5	B, US2 R5	C, ETH2220 R5	D, UG270 X4	E, ID12 R5	F, BZ163 R5	G, BCF-DIOUM R5	O, BCF06 X4	BV-5061W X4	III _B ^b X4	NL4.3 X4	BaL R5
UDA	0.97	0.56	1.0	1.0	0.62	0.61	1.0	1.0	1.0	0.052	0.56	1.0
HHA	0.58	0.11	0.88	0.98	0.24	0.09	0.82	0.024	0.18	0.022	0.072	0.12
GNA	0.54	0.34	≥2.0	>0.4	0.38	0.50	≥2.0	0.038	0.19	0.066	0.028	0.13
CA	0.44	0.23	0.96	0.42	0.096	0.50	0.74	0.26	0.088	0.029	0.018	0.19
CV-N	0.13	0.038	0.16	0.03	0.10	0.10	0.077	0.013	0.013	0.002	0.014	0.16

^a 50% effective concentration or compound concentration was required to inhibit viral p24 (for HIV-1) or p27 (for HIV-2) production by 50%.

^b Data were obtained in CEM cell cultures.

in the absence of UDA, no marked differences were observed in the replication kinetics of HIV-1/UDA-200-2 and wild-type HIV-1 (followed for 4 days after which full cytopathicity was observed). In contrast, HIV-1/UDA-50-1 showed a 2–3-fold lower p24 production, compared with wild-type HIV-1 at all time points evaluated (data not shown). Moreover, an HIV-1/UDA-200-1 strain that was isolated at the end of the UDA drug selection series-1 was heavily compromised in its replication and infection efficacy. This resulted in a very poor p24 viral Ag production in the infected cell cultures. The viral amount was so low that no sufficient amplification product for GP120 sequencing could be generated.

Modeling of the UDA Interaction with the GlcNAc₂Man₁ Moiety of a Glycan on HIV-1 GP120—By using the UDA coordinates from the crystal complex with a GlcNAc oligomer (Protein Data Bank entry 1EHH), we have modeled two UDA molecules to bind the GlcNAc₂Man₁ moiety linked to the asparagines on GP120 in a similar sandwiched manner as in the crystal structure (Fig. 2, panel B). Our x-ray structure-based modeling shows that UDA can efficiently interact with the GlcNAc₂Man₁ moiety of the glycans on GP120 without affording a steric clash with either GP120 or the remaining carbohydrates in the

glycan (Fig. 2, panel B). A closer look at the molecular interactions of UDA with the glycan carbohydrates revealed efficient stacking of Trp-21 with the first GlcNAc (ring A) that is directly bound to Asn-289 in GP120, and of Trp-69 with the Man (ring C) that is bound to the second GlcNAc (ring B) (Fig. 2, panel C). This second GlcNAc is sandwiched between Trp-23 and His-67. Thus, the face-to-face contacts of the pyranose rings with aromatic side chains of the UDA molecules is identical as found in the crystal structures of the UDA-GlcNAc₃ complexes (32). In contrast with an additional five hydrogen bonds that further stabilize the UDA-GlcNAc₃ complex, only three hydrogen bonds were retained in the UDA/GP120 interaction (*i.e.* Tyr-76 to the 3-OH of ring C, Tyr-30 to the 3-OH of ring B, and Ser-19 to the carbonyl from 2NAc of ring B), suggesting only a slightly less efficient interaction of UDA with GP120 glycans than in the UDA-GlcNAc₃ complex.

DISCUSSION

A pre-assembled glycan entity consisting of two GlcNAc, nine mannose, and three glucose molecules ((Glc₂Man₉GlcNAc₂) (5, 45)) is linked on the amide function of asparagines in GP120 through a β1 linkage with the terminal GlcNAc. However, extensive modifications of

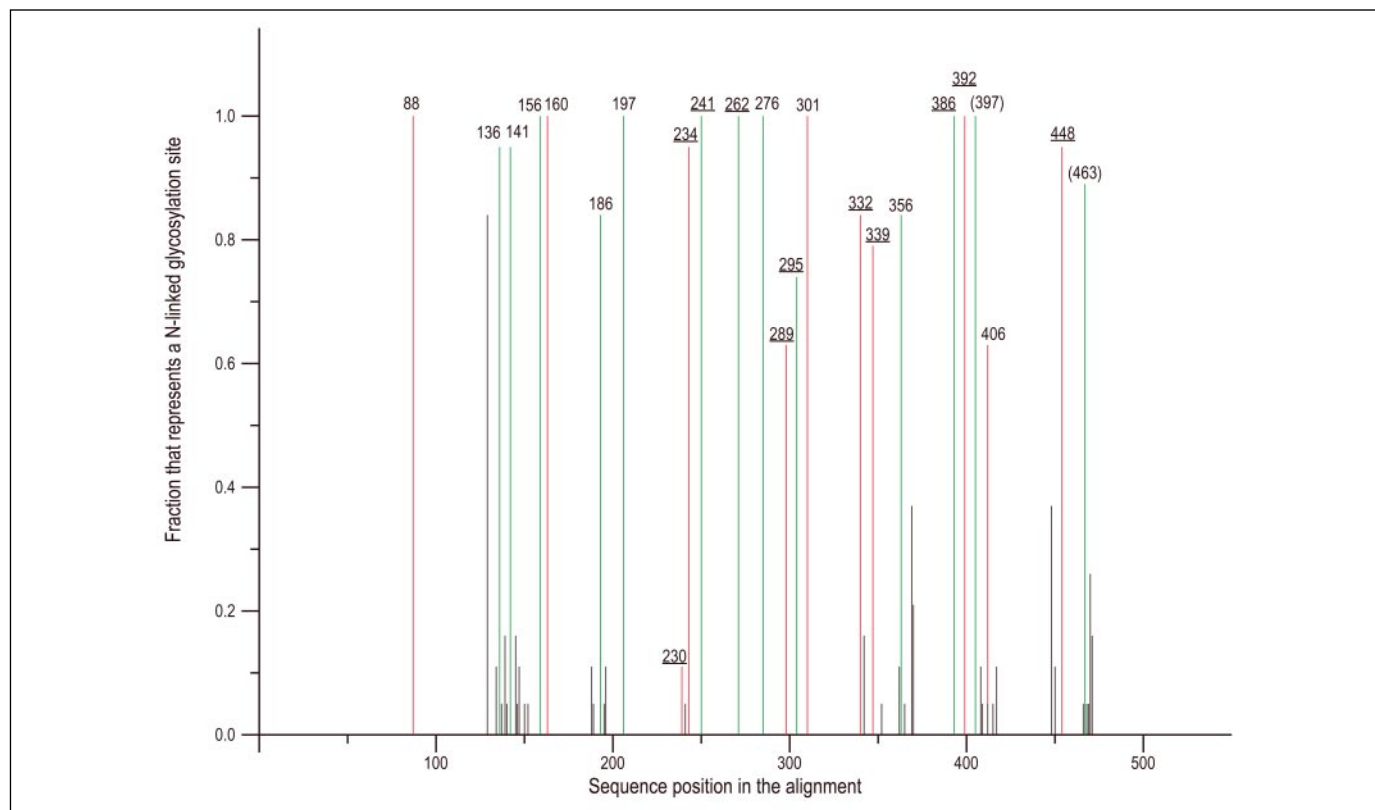


FIGURE 3. An alignment of consensus sequences (2004) was obtained from the HIV sequence data base (hiv-web.lanl.gov) and subjected to analysis with the program *N*-glycosite (hiv-web.lanl.gov) for the allocation of putative *N*-glycosylation sites. The alignment contained consensus sequences for HIV subtypes A1, A2, B, C, D, F1, F2, G, H, CRF01, CRF02, CRF03, CRF04, CRF06, CRF08, CRF10, CRF11, CRF12, and CRF14. Based on these results, the fraction that represents an *N*-linked glycosylation site at each sequence position in the alignment was calculated and visualized. The consensus of the consensus sequences and the ancestral sequences were omitted in the calculations. The numbers at the top of each of the vertical lines represent the asparagine positions in GP120 according to Kwong *et al.* (44). Underlined numbers represent high mannose type glycosylation sites. The other positions are complex-mannose type. Numbers between parentheses represent glycosylation sites that were not present in the HIV-1(III_B) strain that was used in our experiments. Vertical lines are green-colored if present in HIV-1(III_B) but not found mutated, and are red-colored when mutated in at least one of the isolates that were obtained after resistance selection experiments with UDA (see TABLE ONE).

the *N*-linked oligosaccharides are performed as soon as the glycan block has been added to the target protein, contributing to the heterogeneity of the resulting glycoproteins (3). The HIV envelope GP120 is among the most heavily glycosylated proteins known. Up to 50% of the 120-kDa molecular mass of GP120 consists of *N*-linked carbohydrates. In particular, HIV GP120 contains 20–29 *N*-glycosylation sites depending on the nature of the viral isolate and the type of virus clade. However, a number of these sites are very well conserved among all clades. We calculated the level of conservation of the different glycosylation sites in GP120 of a wide variety of HIV clade isolates (consensus sequences) (Fig. 3). At least 11 (out of 24) *N*-glycosylation sites that are found in HIV-1(III_B) GP120 were fully conserved, and 4 additional *N*-glycosylation sites were conserved in 95% of the consensus strains. Thus, almost 50% of the total number of *N*-glycosylation sites found in HIV-1(III_B) GP120 (11 of 24) were fully conserved among all HIV-1 clades. Sixteen percent of the glycosylation sites in HIV-1(III_B) GP120 (4 of 24) were conserved in 95% of the virus strains. It is important to notice that more than one-third of the fully conserved *N*-glycosylation sites were deleted in the UDA-resistant virus strains that were isolated during the two independent series of UDA-1 and UDA-2 drug selections (Asn-88, Asn-160, Asn-301, and Asn-392). Also, 2 of 4 (50%) of the *N*-glycosylation sites that showed 95% conservation were deleted in the UDA-resistant virus strains (Asn-234 and Asn-448). It thus seems that almost half of the *N*-glycosylation sites that are highly conserved ($\geq 95\%$) among all subtypes of clinical HIV-1 isolates can be deleted under UDA pressure (also see Fig. 2 for a three-dimensional view of the deleted *N*-glycosyla-

tion sites in GP120). Most interestingly, these deletions occur almost at the very beginning of the UDA exposure period and remain deleted during the whole drug selection process (TABLE ONE). Given the fact that these glycosylation sites are among the most conserved in GP120, hiding underlying amino acid epitopes of GP120 by these glycans seems very crucial for the virus to maintain its *in vivo* virulence and/or to effectively protect the underlying amino acid epitopes against the neutralizing activity of the immune system. The resistance selection experiments in this study have been performed with the laboratory HIV-1(III_B) strain. However, we also selected for resistance of the clinical HIV-1/HE isolate against the plant lectin GNA in CEM cell cultures (2). As found for the HIV-1(III_B) resistance selection experiments, the glycosylation pattern of GP120 of the drug-exposed clinical virus isolate was also affected (2). Also, resistance to UDA slowly arises after the clinical HIV-1/HE isolate had been exposed to the drug for at least 20 subcultivations. Thus, it seemed that primary HIV-1 isolates may adapt in the same way as the laboratory HIV-1(III_B) strain to the plant lectin pressure.

Wei *et al.* (46) and others have demonstrated that HIV-1 continuously changes its glycan shield to escape the immune system in virus-infected individuals to avoid neutralization. Moreover, a variety of studies has shown that deletion of one or several glycosylation sites in HIV-1 GP120 results in a markedly higher susceptibility of these viruses to the neutralizing activity of antibodies (34–36). It is therefore very likely that forcing the virus to remove these glycans from its envelope GP120 by mutating the glycosylation sites under selective pressure of UDA may

compromise survival of the virus in the presence of an active immune surveillance. Strong evidence that a deleterious effect of the immune system on deglycosylated virus strains does also occur *in vivo* is provided by Reitter *et al.* (37), who infected Rhesus monkeys with SIV strains that contained mutations at a few (two) glycosylation sites in its envelope glycoprotein. Such infection resulted in formation of higher titers of neutralizing antibodies directed against the previously hidden epitopes at the deglycosylated region of the *env* glycoprotein. It also resulted in substantially lower levels of circulating mutant SIV in the plasma of these animals. It is clear that the highly dense carbohydrate shield on GP120 causes a relatively low antigenicity and low immunogenicity of GP120 and protects the virus against the immune system (37, 47, 48). These results demonstrate a crucial role for *N*-linked glycosylation in limiting the neutralizing antibody response to SIV and in shielding the virus from immune recognition. As a consequence, it is also evident that the virus needs a minimum degree of glycans on its GP120 to avoid an efficient antibody response against silent (protected) areas on GP120. Pressure of a drug like UDA on the virus would necessarily lead to sequential deletion of an increasing amount of glycosylation sites in GP120. These events would result in not only an increased exposure of the viral envelope to a detrimental antibody response but eventually also to a lesser infectivity (virulence) of the virus. We indeed found that an HIV-1 strain that was selected in the presence of escalating UDA concentrations, and that was isolated from a virus pool lacking at least eight to nine glycosylation sites in the GP120, markedly lost its infectious potential. Another highly mutated HIV-1 strain was also shown to have a lower replication potential.

Our findings indicate that CBAs such as *N*-acetylglucosamine-binding compounds may delete glycosylation sites in its GP120 envelope in an attempt to escape the CBA pressure. However, such virus strains may become increasingly vulnerable to a more efficient suppression/neutralization by the immune system and increasingly less infectious (virulent) for the host cells. In addition, a lower efficiency of virus transmission may also occur because of the fact that the innate DC-SIGN and mannose-binding lectin that bind the HIV particles by adhering through the mannose residues of the HIV GP120 glycans before transmitting and exposing the virus to T-lymphocytes will markedly lose their HIV binding capacity and transmission because of selective and progressive glycan depletion in GP120.

Leonard *et al.* (5) have demonstrated that all 24 *N*-linked glycosylation sites in GP120 of HIV-1(III_B) are utilized and that 13 of the sites contain complex-type oligosaccharides, although 11 contain hybrid and/or high mannose structures (TABLE ONE). Generally, high mannose-type oligosaccharides typically have 2–6 mannose residues attached to the core pentasaccharide Man₃GlcNAc₂, whereas the complex oligosaccharides have different numbers and types of carbohydrate residues in their outer branches. Hybrid oligosaccharides contain elements of both structures. More importantly, in contrast to the glycoproteins of HIV-1 GP120, mammalian cell surface or serum glycoproteins rarely contain terminal mannose residues (49). Indeed, in mammals, the presence of a terminal mannose moiety on a protein triggers binding to hepatic lectin receptors and fast removal from the plasma (50). However, HIV-1 uses the terminal mannoses to its advantage for reducing its antigenicity against the human immune system (16). It is of interest to note that during the UDA selection process more high mannose/hybrid-type glycan-containing glycosylation sites (7 of 11) were deleted compared with complex mannose-type glycans of GP120 (4 of 11) (TABLE ONE). A similar phenomenon was observed in the virus strains that were selected in the presence of escalating concentrations of mannose-specific GNA and HHA (1, 2). Thus, both the man-

nose- and GlcNAc-specific CBAs have a similar preference for annihilation of high mannose-type glycans in GP120.

The 2G12 antibody is one of the few broadly neutralizing antibodies directed against HIV-1. It recognizes an epitope around the C4/V4 region of GP120 with nanomolar affinity (4, 15, 18). Except for one clade, it neutralizes a wide spectrum of different HIV-1 isolates from all known virus clades (15, 51). Very recently, it was shown that administration of 2G12, together with two other neutralizing antibodies, was able to delay virus rebound in HIV-1-infected individuals after termination of antiretroviral therapy. However, it was also shown in the same study that the eventual appearance of virus under 2G12 treatment proved to have lost its sensitivity to 2G12 (52). 2G12 likely binds to the high mannose-type residues in a highly conserved epitope containing Asn-295, Asn-332, and Asn-392 (4, 18, 53). The Asn-339 and Asn-386 glycans may indirectly influence 2G12 binding. Not surprisingly, 2G12 markedly or even completely lost its inhibitory activity against the HIV-1 strains that emerged under UDA treatment and in which the Asn-332 and Asn-392 glycosylation sites were deleted. In contrast, these virus strains only marginally lost their sensitivity to the inhibitory effect of UDA. Thus, neutralizing antibodies such as 2G12, whose sugar-recognizing epitopes consist of at most 2–3 spatially and closely located glycan sites, are easily vulnerable to loss of neutralizing activity upon mutation of one or two glycans of these sites, although sensitivity to UDA is virtually not affected by a few glycosylation site deletions because this compound is thought to be bound at numerous sites of GP120. This is also shown for the $\alpha(1,2)$ -mannose-binding CV-N that lost marked inhibitory activity against virus strains that have deleted glycosylation sites at Asn-332, Asn-339, and Asn-448 in their GP120 envelope protein (2). Consequently, (neutralizing) antibody epitopes represent relatively small and specific areas on GP120 that can be easily mutated (*i.e.* by the evolving glycan shield). Glycan deletions in this area of GP120 may then result in significant loss of neutralizing activity of the envelope antibodies. The GlcNAc-binding UDA needs a much higher number of glycan deletions before significant loss of antiviral activity. The equal antiviral effectiveness of UDA, irrespective of the nature of the clade, is in agreement with our findings of virtual independence of the virus suppression potential of UDA on the number and type of glycosylation sites in GP120. Thus, CBAs like UDA are endowed with a very high genetic barrier in terms of resistance development, and the long duration of virus breakthrough and the very modest loss of sensitivity of HIV to UDA are in agreement with each other.

It is yet unclear why the GlcNAc-binding UDA is endowed with much less variation in its antiviral profile than mannose-binding proteins such as 2G12, the mannose-specific plant lectins, and CV-N. One contributing factor may be the size of the protein. Although the 2G12 antibody has a heavy molecular mass and the plant lectins GNA and HHA consist of tetramers with a molecular mass of 50,000 Da, UDA is a rather unique plant lectin that exists as monomer with a molecular mass as low as 8,500 Da (~89 amino acids) (32, 33). Perhaps sugar-binding proteins with a lower molecular weight, and thus a smaller size, can more easily interact with parts of the GP120 glycans because of a lesser steric hindrance at the sub-surface areas of the GP120 glycans. In this respect, it should be mentioned that CV-N, which also occurs as a rather "low molecular weight" (11,000 Da) monomer generally keeps a higher sensitivity profile than the higher molecular weight plant lectins GNA, HHA, and NPA against the different virus clade isolates (TABLE FOUR). Another contributing factor that may be even more important is the sugar specificity of these carbohydrate-binding agents. Indeed, although CV-N recognizes the $\alpha(1,2)$ -mannose residues on the surface of the GP120 glycans, and although the mannose-binding plant lectins

HHa and GNA predominantly recognize $\alpha(1,3)$ - and/or $\alpha(1,6)$ -mannose residues that are particularly located between the surface of the glycans and the top of the pentasaccharide core, UDA represents a compound with an entirely different sugar specificity (GlcNAc) profile (31–33). A GlcNAc₂ oligomer is invariably present at the basis of the pentasaccharide core in each of the glycosylation sites in GP120. Moreover, in the complex and hybrid mannose-type glycans in GP120 that are present in ~63% of the total average number of glycosylation sites (7, 8), at least one or several additional GlcNAc units can be present. Thus, UDA may have the opportunity to interact with the GP120 glycans not only at each *N*-glycosylation site on GP120 but even at several locations within each of the GP120-bound oligosaccharides. It is therefore interesting to have a closer look to the crystal structure of UDA (31). It resembles a dumbbell-shape molecule consisting of two domains analogous to hevein and connected to each other by a four-amino acid sequence. Sugar-binding sites are present at both ends of the UDA molecule. Each domain (~42 amino acids) is approximately double the size of a glycan on GP120. In the crystal complex of UDA with GlcNAc₃, one GlcNAc oligomer is sandwiched between two different UDA molecules in a head-to-tail mode, and another GlcNAc oligomer is also sandwiched by the first UDA molecule and second UDA (32). The GlcNAc binding is mainly performed by three aromatic amino acid residues and one serine through hydrogen bonds and amino acid stacking interactions. The binding is quite specific for *N*-acetylglucosamine moieties. We hypothesized that, given the relatively low molecular weight of UDA and the flexibility between the two carbohydrate-binding domains in the UDA molecule, it may well be possible that UDA is attached to the GlcNAc₂ moiety of the glycans that link the asparagines of GP120 with the complex/high mannose tree. Our computer-assisted modeling indeed revealed that two UDA molecules can efficiently interact with the GlcNAc₂ and the next mannose molecule of the glycans on GP120 (Fig. 2, B and C). Because many UDA molecules may concomitantly interact with GP120, it becomes understandable that a substantial amount of glycosylation sites in GP120 need to be deleted before significant resistance to such a molecule may occur (high genetic barrier). Only a few glycosylation site deletions in GP120 will have a very marginal effect on UDA inhibition of HIV, as demonstrated in our studies. Therefore, GlcNAc-binding agents may represent an advantage over mannose-binding agents.

Because mannose- and also GlcNAc-binding plant lectins may be randomly bound to many *N*-linked glycosylated (cellular) proteins, potential toxic side effects of GlcNAc-binding agents can be expected. However, it was shown previously that the mannose-specific GNA and HHA lack acute toxicity upon intravenous administration to adult mice at 50 mg/kg (28). We could also ascertain for UDA that it did not afford visible toxic side effects when given intravenously to mice at doses of 5, 10, and 25 mg/kg. It has also been reported that UDA did not agglutinate human red blood cells at concentrations that were substantially higher than its antiviral activity in cell culture and that UDA was poorly mitogenic (29). However, it should be kept in mind that CBAs can potentially show general or long term side effects because of interaction with cellular glycoproteins.

In conclusion, the GP120-targeted CBA concept fundamentally differs from all currently available therapeutic approaches. (i) Although the cellular glycosylation machinery is responsible for proper GP120 *N*-glycosylation, CBAs such as UDA do not exert any metabolic drug pressure on these cellular events. Therefore, cellular resistance to escape drug pressure for the virus will be unlikely. (ii) In contrast with other existing therapeutic interventions, resistance development of HIV against CBAs may allow efficient immunological suppression of virus

replication and virus clearance from the systemic circulation because of the exposure of previously hidden immunogenic epitopes on GP120. CBAs may therefore represent the first available drugs for which chemotherapy may act in concert with an immunological response. (iii) Although the current drugs are targeted against viral proteins (*i.e.* reverse transcriptase, protease, GP41) in a stoichiometric manner (one drug molecule binds to one target molecule), several CBA molecules likely bind to each single GP120 molecule of the virus at the same time, resulting in the requirement of multiple mutations ($\gg 5$) before decreased drug susceptibility becomes evident (high genetic barrier). Each glycosylation site deletion may result in a specific neutralizing antibody response. The monkey infection experiments of Reitter *et al.* (37), in which SIV strains were used that contained deleted envelope glycosylation sites, provide strong evidence for the antiviral potential of the CBA concept *in vivo*. Our findings indicate that the glycosylation of HIV GP120 may be the "Achilles heel" of the virus. Targeting the carbohydrate moieties on HIV GP120 by direct therapeutic intervention with CBAs may represent an entirely new conceptual approach with far-reaching applications, including vaccine development combined with chemotherapy. Such a concerted action between chemotherapy and immune surveillance would thus far be unprecedented in anti-HIV therapy. This concept should be further explored not only for HIV but also for other chronic infections by enveloped viruses that contain heavily glycosylated envelope proteins such as hepatitis B and C viruses and perhaps also for more acute infections by enveloped viruses such as influenza viruses, respiratory syncytial virus, coronaviruses, and several flaviviruses.

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