Exposure of HIV-1 to a combination of two carbohydrate-binding agents markedly delays drug resistance development and selects for virus strains with compromised fitness

Leen Mathys and Jan Balzarini*

Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10 B-3000 Leuven, Belgium

*Corresponding author. Tel: +32-16-337367; Fax: +32-16-337340; E-mail: jan.balzarini@rega.kuleuven.be

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Objectives: We investigated the effect of combining two potent carbohydrate-binding agents (CBAs) with a complementary resistance profile (based on different N-glycan deletion selections in the HIV envelope glycoprotein gp120) on drug resistance development and viral fitness.

Methods: A long-term selection procedure was used to obtain virus strains resistant to the mannose-specific Hippeastrum hybrid agglutinin (HHA), the mannose-recognizing monoclonal antibody 2G12 and the combination of both compounds. The env genes of the mutant viruses were sequenced and the infectivity potential and phenotypic resistance profile of the virus strains were examined in CD4+ T lymphocyte C8166 cell cultures.

Results: The long-term exposure of HIV-1 to CBAs resulted in the selection of virus isolates containing deletions of several high-mannose-type N-glycans in their envelope. The generation of virus strains phenotypically resistant to the combination of both CBAs took markedly longer than the generation of virus strains resistant to the single compounds. The mutant HIV strains derived from the HHA/2G12 combination exposure showed much lower genotypic and phenotypic resistance than those isolated from the virus selection experiments with the single compounds. It was further shown that the CBA-resistant viruses had significantly decreased infectivities.

Conclusions: The data revealed that CBAs are interesting anti-HIV drug candidates with an increased antiviral potential upon internal combination.

Keywords: CBAs, HIV, envelope gp120

Introduction

The HIV particle is surrounded by a lipid bilayer, the viral envelope, which contains trimeric spikes of the virus-encoded glycoproteins gp120 and gp41, which are non-covalently associated. The surface envelope glycoprotein, gp120, is particularly highly glycansylated. In total, ∼50% of the mass of gp120 is contributed by N-linked glycans, of which 56%–73% are suggested to be high-mannose-type glycans and 27%–44% to be complex-type glycans. This high percentage of high-mannose-type glycans on gp120 can be exploited to differentiate host glycoproteins from foreign viral glycoproteins, since human glycoproteins contain mostly complex-type N-linked glycans and the dense clustering of the high-mannose-type glycans on HIV gp120 is also quite unusual. Carbohydrate-binding agents (CBAs) with a specificity for mannose-containing glycans will therefore be expected to have a pronounced selectivity to interact with HIV glycoproteins.

Several mannose-specific CBAs have been studied and described in detail, and were shown to be able to block at least four pathways involved in HIV infection and transmission. They are able to block cell-free virus infection of the main target cells of HIV, namely CD4+ T lymphocytes and macrophages, and inhibit the synctium formation between HIV-infected and non-infected CD4+ T lymphocytes. CBAs are also able to block the capturing of viral particles by cells expressing dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) and inhibit the transmission of captured virus to CD4+ T lymphocytes. The dendritic cells are among the first cells the virus encounters upon transmission during sexual intercourse. DC-SIGN is a C-type lectin with a preference for high-mannose-type glycans. Binding of gp120 by DC-SIGN results in capture of the viral particle, after which the DC-SIGN+ cell will migrate to the lymphoid tissues to signal to CD4+ T lymphocytes that an intruder has been found. By directly blocking viral infection and transmission in different manners, CBAs offer great potential as candidate antiretroviral compounds, especially for microbical application.

As with all antiretroviral drugs, HIV mutants that have a decreased susceptibility towards the inhibitory CBAs arise upon drug pressure. It has been shown that these mutant viral strains may lack several (high-mannose-type) N-glycans due to the mutation...
(deletion) of N-glycosylation sites in gp120 (reviewed in Balzarini). However, some of the CBAs are endowed with a high genetic barrier and several concomitant mutations in N-glycosylation motifs of the viral envelope are often required to result in significant phenotypic resistance. The deletion of N-glycans from gp120 is believed to create ‘holes’ in the glycan shield of HIV gp120 that normally covers the immunogenic peptide epitopes. Therefore, CBAs may be also act indirectly by inducing specific immune responses upon CBA-triggered deletions of N-linked glycans in the HIV envelope.

In this study, we focused on two mannos-specific CBAs: the plant lectin Hippeastrum hybrid agglutinin (HHA) derived from Hippeastrum hybrid and the broadly neutralizing monoclonal antibody 2G12.

HHA is a tetramer with a total molecular weight of 50 kDa. It has a binding specificity for α(1,3)- and α(1,6)-linked mannoside residues and can bind to gp120 with a high affinity (K<sub>D</sub> in the low nanomolar range). It has been shown previously that HHA-exposed HIV-1-infected cell cultures select for HIV-1 strains that contain deletions of several N-linked glycans in the gp120 envelope, mostly high-mannose-type glycans (Asn-228, Asn-232, Asn-287 and Asn-337). The complex-type N-glycans that were also deleted were positioned at Asn-274, Asn-299 and Asn-395. It was also shown that this CBA has a broad antilentiviral activity, with inhibitory capacities towards HIV-1 (X4, R5 and X4R5), HIV-2, SIV and FIV.

2G12 is a broadly neutralizing human antibody directed to HIV-1 gp120. The antibody shows an unusual swapping of the variable heavy-chain domains. This CBA has a molecular weight of 285 kDa and contains four antigen recognition sites, of which two are created by unusual domain swapping. It has a binding specificity (K<sub>D</sub> in the nanomolar range) for clustered high-mannose-type glycans, with a pivotal role for the terminal α(1,2)-mannose residue.

The epitope of 2G12 is located at the C1/C2/C4/V4 region of gp120 and is solely covered by high-mannose-type glycans. In particular, the high-mannose-type N-glycans on Asn-293, Asn-330, Asn-337 and Asn-390 are known to play an instrumental role in the binding to HIV-1 gp120 and the neutralizing activity of 2G12. These glycans, which were identified in the gp120–2G12 complex to interact with 2G12, were also selected to be mutated (deleted) when the virus was exposed to 2G12 in cell culture at suboptimal concentrations.

Since the mutant resistance spectrum (i.e., the locations of the selected N-glycan mutations in gp120) was different between HHA and 2G12, and since it has previously been shown that combining two CBAs may often result in synergistic antiviral activity, it would be of particular interest to perform HIV-1 drug selection experiments in the concomitant presence of such a rationally selected pair of CBAs in comparison with drug resistance selections in the presence of the single compounds, to reveal differences in the mutational patterns and resistance profiles of virus isolates that were exposed to the single versus combined CBAs.

Materials and methods

Compounds

The mannose-specific plant lectins derived from Hippeastrum hybrid (HHA) and Galanthus nivalis (GNA) and the N-acetylglucosamino-specific plant lectin Urtica dioica agglutinin (UDA) were kindly provided by Professor E. Van Damme (Ghent, Belgium). The mannose-specific lectin actinohivin (AH) from the actinomycete K97-0003 was provided by H. Tanaka (Toyama, Japan). The monoclonal antibody 2G12 was purchased from Polymun Scientific (Vienna, Austria).

Cells

Human CD4+ T lymphocytic C8166 cells were obtained from ATCC (Manassas, VA, USA) and were grown in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium), supplemented with 10% fetal calf serum (FCS) (Sigma, Bornem, Belgium), 2 mM L-glutamine and 0.002% gentamicin (Invitrogen).

Human embryonal kidney cells (HEK 293T) were obtained from ATCC. Microbial U87.CD4.CCR4.CCR5 cells were provided by Professor D. Schols (Leuven, Belgium) and their construction and characterization have been described previously. Both cell lines were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FCS (Sigma), 75 mM NaHCO₃ and 0.002% gentamicin (Invitrogen). For the U87.CD4.CCR4.CCR5 cells, 0.0001% pyruvate (Invitrogen) and 0.02% geneticin (Invitrogen) were added.

Viruses

HIV-1NL4.3 was recombinantly produced by cotransfecting HEK 293T cells with a DNA construct encoding gp160 and XbaI-digested pNL4.3 ΔEnv_eGFP. This results in homologous recombination, inserting the gp160 gene in the pNL4.3 eGFP backbone. Therefore, infected cells express enhanced green fluorescent protein (eGFP). The construct pNL4.3 ΔEnv_eGFP was kindly provided by Dr M. E. Quinones Mateu (Lerner Research Institute, Cleveland, OH, USA).

The produced wild-type (WT) virus was used to infect U87.CD4.CCR4.CCR5, leading to an increased viral yield.

Selection and isolation of HHA-, 2G12- and HHA/2G12-resistant HIV-1 strains

Starting the selection procedure, 6 × 10⁴ C8166 cells were added into a 48-well plate, together with complete RPMI-1640 culture medium, a defined concentration of compound and 1000 pg of p24 HIV-1NL4.3, adding up to a volume of 1 ml per well. The initial compound concentrations were 2 nM HHA (HHA condition), 1.75 nM 2G12 (2G12 condition) and the combination of 2 nM HHA and 1.75 nM 2G12 (HHA/2G12 condition). In parallel, virus was also passaged in the absence of any compound to correct for randomly occurring mutations (WT condition).

Every 4–5 days, the cytopathic effect of the virus was evaluated microscopically and the eGFP expression upon infection was examined using a fluorescence microscope. Next, 6 × 10⁴ fresh uninfected C8166 cells were added to a new well, together with RPMI-1640 culture medium, an appropriate concentration of the test compound and 50 μL of culture supernatant of the previous passage. The compound concentrations were increased once sufficient viral breakthrough was achieved, which was defined as three consecutive passages with full giant-cell formation or increased once sufficient viral breakthrough was achieved, which was defined as three consecutive passages with full giant-cell formation or eGFP expression. At regular timepoints, supernatants and cell suspension samples were harvested and stored for further analysis. This procedure was repeated until pronounced phenotypic drug resistance was achieved. The viral isolates were then grown at a larger scale for further analysis.

Genotyping of the proviral env region

Cell pellets of HIV-1-infected C8166 cells, growing under increasing compound concentrations, were used to isolate genomic DNA with the DNeasy Blood & Tissue Kit (Qiagen). Next, two consecutive PCRs were used to amplify the proviral env gene. In the first PCR, a fragment of 3876 bp was amplified using the Platinum Taq DNA Polymerase High Fidelity Kit and the primers KVL008 and KVL009. The second PCR was used to amplify a 2346 bp fragment encoding gp120 (primers AV317 and AV323) and a 2048 bp fragment encoding gp41 (primers AV318 and AV319). These fragments were then sequenced using the ABI PRISM BigDye
The CBA-exposed cultures were directly related to CBA exposure. Therefore, it could be assumed that the mutations that occurred and a few random mutations occurred in the gp120/gp41 complex, but were later found to be the predominant mutations after prolonged drug exposure without detection of any WT amino acid sequence (Figures 1a, 2a and 3a).

In the absence of CBAs, a few random mutations in (WT) gp41 occurred and a few random mutations occurred in the gp120/gp41 of the drug-exposed isolates (Figure 4). However, none of these mutations affected the glycosylation profile of the viral envelope. Therefore, it could be assumed that the mutations that appeared in the N-glycosylation motifs of the virus isolates from the CBA-exposed cultures were directly related to CBA exposure.

The prolonged exposure of HIV-1 env to HHA led to the deletion of several N-linked glycans of gp120 by mutation of N-glycosylation sites (Table 1, Figure 2a and b, and Figure 4). In most cases, the serine or threonine that is part of the glycosylation motif was mutated (S162N, T230K, S289F, T339A and S463P). In one glycosylation motif, the asparagine was mutated (N384D). All N-glycan deletions occurred in gp120 and none of the N-glycans in gp41 was affected. Most glycan deletions in gp120 involved high-mannose-type glycans, as previously determined (Asn-228, Asn-287, Asn-337 and Asn-384). Two deleted glycans were previously found to be complex glycans (Asn-160 and Asn-461). Mutation of Ser-162 to an asparagine resulted in the deletion of the glycosylation motif that determines the glycan from

Results

Selection of HIV-1 strains in the presence of escalating concentrations of HHA, 2G12 and the HHA/2G12 drug combination

CD4+ T lymphocyte C8166 cells were infected with HIV-1, and cultured in the presence of HHA, 2G12 or a combination of both compounds (HHA/2G12). At the start, compound concentrations near the EC50s of HHA and 2G12 were used (1.75–2 nM). In parallel, virus-infected C8166 cells were also cultured in the absence of the compounds, to account for randomly occurring mutations. Every 4–5 days, fresh uninfected C8166 cells were exposed to virus-containing supernatant from the previous cell culture passage, in the presence of freshly added compound. As soon as three consecutive passages were observed to have full viral breakthrough (giant cell formation), the compound concentrations were stepwise increased. This procedure was repeated until the compound concentrations exceeded the EC50s by at least 30–1000-fold (HHA: >1000-fold; 2G12: >100-fold; HHA and 2G12 in the combination experiment: >100- and >30-fold, respectively) (Figures 1a, 2a and 3a). To reach these high drug concentrations, 34 passages (>5 months) were needed for 2G12 and 43 passages (>6 months) were needed for HHA and the HHA/2G12 combination.

Sequence analysis of the env gene of the CBA-resistant mutant virus strains

At regular timepoints during the selection of mutant virus strains, genomic DNA was isolated from the HIV-1-infected cell cultures. The genome of these cell cultures contained at least one copy of the viral DNA and could therefore be used to determine the amino acid sequence of the proviral env gene. Following isolation of the genomic DNA, two PCR-driven amplifications and a subsequent sequencing reaction were performed to obtain the nucleotide sequence of the entire env gene. Analysis of the nucleotide sequence using the appropriate software was then performed to compare the obtained nucleotide and amino acid sequences of the viral envelope between HIV-1 NL4.3 cultured in the absence of CBA or in the presence of HHA, 2G12 and the HHA/2G12 combination (Figure 4). A variety of mutations were found in the multiple virus isolates that predominantly affected the env N-glycosylation pattern (Table 1). Several amino acid mutations initially coexisted with the WT amino acid, but were later found to be the predominant mutations after prolonged drug exposure without detection of any WT amino acid sequence (Figures 1a, 2a and 3a).

In the absence of CBAs, a few random mutations in (WT) gp41 occurred and a few random mutations occurred in the gp120/gp41 of the drug-exposed isolates (Figure 4). However, none of these mutations affected the glycosylation profile of the viral envelope. Therefore, it could be assumed that the mutations that appeared in the N-glycosylation motifs of the virus isolates from the CBA-exposed cultures were directly related to CBA exposure.

The prolonged exposure of HIV-1 NL4.3 to HHA led to the deletion of several N-linked glycans of gp120 by mutation of N-glycosylation sites (Table 1, Figure 2a and b, and Figure 4). In most cases, the serine or threonine that is part of the glycosylation motif was mutated (S162N, T230K, S289F, T339A and S463P). In one glycosylation motif, the asparagine was mutated (N384D). All N-glycan deletions occurred in gp120 and none of the N-glycans in gp41 was affected. Most glycan deletions in gp120 involved high-mannose-type glycans, as previously determined (Asn-228, Asn-287, Asn-337 and Asn-384). Two deleted glycans were previously found to be complex glycans (Asn-160 and Asn-461). Mutation of Ser-162 to an asparagine resulted in the deletion of the glycosylation motif that determines the glycosylation of Asn-160, but led to the creation of a new glycosylation motif (162NTS164). Therefore, deletion of the glycan at Asn-160 could merely be considered as a repositioning of the glycan from
Figure 1. Pathway of resistance selection for HHA with indication of the changes in the Env glycosylation pattern as found at different timepoints. Every 4–5 days, viral breakthrough was evaluated microscopically and the virus was passaged in fresh cell cultures. The compound concentrations were increased whenever three consecutive passages presented with maximal viral breakthrough. Mutations shown between parentheses existed, at that timepoint, as a mixture with the WT amino acid. (a) Selection of HHA-resistant virus. The concentration of HHA at the start of the selection was 2 nM. (b) Two-dimensional structure of HIV-1 gp120 with indication of the N-linked glycans that were deleted during resistance development. Figure adapted from Leonard et al.\textsuperscript{23}
Figure 2. Pathway of resistance selection for 2G12 with indication of the changes in the Env glycosylation pattern as found at different timepoints. Every 4–5 days, viral breakthrough was evaluated microscopically and the virus was passaged in fresh cell cultures. The compound concentrations were increased whenever three consecutive passages presented with maximal viral breakthrough. Mutations shown between parentheses existed, at that timepoint, as a mixture with the WT amino acid. (a) Selection of 2G12-resistant virus. The concentration of 2G12 at the start of the selection was 1.75 nM. (b) Two-dimensional structure of HIV-1 gp120 with indication of the N-linked glycans that were deleted during resistance development. Figure adapted from Leonard et al.23
Figure 3. Pathway of resistance selection for the combination of HHA and 2G12 with indication of the changes in the Env glycosylation pattern as found at different timepoints. Every 4–5 days, viral breakthrough was evaluated microscopically and the virus was passaged in fresh cell cultures. The compound concentrations were increased whenever three consecutive passages presented with maximal viral breakthrough. Mutations shown between parentheses existed, at that timepoint, as a mixture with the WT amino acid. (a) Selection of HIV-1 NL4-3 resistant to both HHA and 2G12. The starting concentrations were 2 nM HHA and 1.75 nM 2G12. (b) Two-dimensional structure of HIV-1 gp120 with indication of the N-linked glycans that were deleted during resistance development. Figure adapted from Leonard et al.23

Exposure of HIV-1 to a combination of two CBAs
Table 1. Amino acid alignment of the envelope gp120/gp41 glycoproteins obtained at the end of the selection procedure. Mutations involving N-linked glycosylation sites are indicated in black boxes; other mutations are indicated in grey boxes. A vertical line is used to make a distinction between the gp120 and the gp41 sequence, with gp120 upstream of the line and gp41 downstream.

Figure 4. Amino acid alignment of the envelope gp120/gp41 glycoproteins obtained at the end of the selection procedure. Mutations involving N-linked glycosylation sites are indicated in black boxes; other mutations are indicated in grey boxes. A vertical line is used to make a distinction between the gp120 and the gp41 sequence, with gp120 upstream of the line and gp41 downstream.
Asn-160 to the newly established Asn-162 amino acid position. At one timepoint early during the selection process, a second newly established N-glycosylation site was found (i.e. Asn-358), but this mutation converted again into WT upon further subcultivations. Aside from mutations directly affecting N-glycosylation sites in gp120, four other mutations were found (Figure 4). However, their role in the eventual CBA res phenotype is currently unknown.

The selection of 2G12-resistant virus strains eventually resulted in four mutations, of which only one affected an N-linked glycosylation site (Figure 4). However, the selection of 2G12-res virus strains initially resulted in mutations in three glycosylation motifs (Table 1). In all cases, the asparagine of the N-glycosylation motif was altered into a serine (N293S, N337S and N390S) and the deleted glycans were invariably reported before to be high-mannose-type glycans.23 Whereas after 24 passages, N293S, N337S and N390S were found to be mutated, only N390S remained mutated after 30 passages (Figure 2aa n db).

When HHA and 2G12 were combined in the selection process, the speed of development of virus strains that could replicate at higher drug concentrations was clearly delayed (Figure 3a). During the selection procedure, two glycan deletions that initially occurred after 18 or 28 passages (Asn-390 and Asn-395) were absent in the viral strains obtained after 34 and 43 passages of the experiment (Table 1 and Figure 3a and b). These two glycans were the high-mannose-type glycan on Asn-390 (mutation N390I) and the complex-type glycan on Asn-395 (mutation N395I).23 Eventually, the viral strains obtained after 43 passages contained only three glycan deletions in gp120 [Asn-160 (due to S162N), Asn-337 (due to T339A) and Asn-384 (due to N384Y)], but surprisingly also one additional glycan deletion in gp41 (N672D) had occurred. The deleted glycans at Asn-337 and Asn-384 were reported to be high-mannose-type glycans, while the glycans at Asn-160 and Asn-672 were described previously as complex-type glycans (Figure 3b).23 As was also observed for HHA-res virus strains, the deletion of a glycan at Asn-160 was the result of the mutation of Ser-162 into an asparagine. This mutation disrupted the original glycosylation motif 160NIS162, but resulted in the creation of the new glycosylation motif 162NTS164. Aside from mutations in N-glycosylation sites, two other mutations were found in gp120. It is currently not known whether they played a role in the resistance development for the combination of HHA and 2G12.

At the end of the selection procedure, the viruses were grown on a larger scale in the absence of compound to obtain high viral titres sufficient to further characterize these virus isolates. Because up-scaling took only one more passage, the chance of the appearance of revertants in gp120 was thought to be limited. However, afterwards, the harvested viral strains were sequenced again to confirm the identity of the mutated env genes. In the case of the HHA/2G12-exposed virus isolate that was scaled up in the absence of HHA and 2G12, we found that all mutations in the env gene were retained, except for the glycan deletion in gp41 (N672D) that was reverted to WT again. Therefore, the original viral strain was scaled up again in the presence of the HHA/2G12 combination, which resulted in virus that had an env gene completely identical to the mutant virus obtained at the end of the selection procedure (thus keeping the N672D mutation). To investigate the role of this glycan deletion in gp41, both viral strains were further characterized in parallel and are denoted as HHA/2G12-res.N672(WT) (revertant to WT N672) and HHA/2G12-res.N672D (retained mutation at N672D).

### Resistance and cross-resistance of the mutant virus strains to CBAs

The obtained viral strains [WT, HHA-res, 2G12-res, HHA/2G12-res.N672(WT) and HHA/2G12-res.N672D] were examined using an antiretroviral assay in which the susceptibility to several CBAs was evaluated. The EC50 values and the fold resistance

**Table 1. Overview of the glycan deletions that occurred during CBA resistance selection**

<table>
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<tbody>
<tr>
<td>Deleted glycan</td>
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<tr>
<td>gp120</td>
<td></td>
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<tr>
<td>N160</td>
<td>S162N</td>
<td></td>
<td>S162N</td>
<td>S162N</td>
</tr>
<tr>
<td>N228*</td>
<td>T230K</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>N287*</td>
<td>S289F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N337*</td>
<td>T339A</td>
<td></td>
<td>(N293S)</td>
<td>(N293S)</td>
</tr>
<tr>
<td>N384*</td>
<td>N384D</td>
<td></td>
<td>T339A</td>
<td>N384Y</td>
</tr>
<tr>
<td>N390*</td>
<td>N390S</td>
<td></td>
<td>(N390I)</td>
<td>(N390I)</td>
</tr>
<tr>
<td>N395</td>
<td>N461</td>
<td>S463P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gp41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N672</td>
<td>N672D</td>
<td></td>
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<td>(N672D)</td>
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<tr>
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<tr>
<td>N358</td>
<td>(S358N)</td>
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</tbody>
</table>

Mutations that were observed during the selection procedure, but were reversed again to WT or became undetectable in the final resistant viral strains, are indicated between parentheses.

*High-mannose-type N-glycans as originally determined by Leonard et al.23*
were calculated based on the microscopic evaluation of giant cell formation and are listed in Table 2.

For the WT virus, it was shown that most CBAs (HHA, GNA, AH, UDA and 2G12) had EC$_{50}$S in the low nanomolar range. PRM-S had a somewhat lower anti-HIV activity, with an EC$_{50}$ in the low micromolar range.

The HHA-res viral strain showed EC$_{50}$S for the CBAs that were significantly increased compared with those observed for WT virus. A 62-fold phenotypic resistance for HHA and a $>57$-fold resistance for 2G12 were observed. Furthermore, the virus was also shown to have a decreased susceptibility to the other tested CBAs (i.e. GNA), although the degree of resistance was rather moderate for AH, UDA and PRM-S (3–8-fold) (Table 2).

The 2G12-res virus was $>57$-fold less susceptible to the inhibitory effect of 2G12, but had no cross-resistance towards any of the other CBAs, including HHA (Table 2).

The HHA/2G12-res.N672D virus, which still contained the N-glycan deletion in gp41, was shown to have a high degree of phenotypic resistance to 2G12 ($>57$-fold) and to have cross-resistance to some of the other CBAs. However, the degree of phenotypic cross-resistance was generally found to be lower than observed for the HHA-res strain. Interestingly, the HHA/2G12-res.N672(WT) virus, which no longer contained the glycan deletion in gp41, also had a $>57$-fold resistance towards 2G12, but was found to have a consistent trend to lower levels of cross-resistance to the other CBAs, compared with HHA/2G12-res.N672D (Table 2).

**Viral infectivity of the CBA-resistant mutant viral strains**

CD4$^+$ T lymphocyte C8166 cells were infected with equal p24 amounts of the viral isolates that were recovered at the passage that contained the highest concentration of the test compounds [WT, HHA-res, 2G12-res, HHA/2G12-res.N672(WT) and HHA/2G12-res.N672D]. For 8 consecutive days, cell suspensions were harvested from the cultures and the production of new viral particles was quantified by p24 ELISA. It was shown that the infectivity of the viral strain originating from the WT control condition was significantly higher than the infectivities of the other viruses. Among the CBA-exposed viruses, the strain resistant to HHA seemed to have a somewhat higher infectivity than the virus strains grown in the presence of 2G12 or the combination HHA/2G12 (Figure 5). Furthermore, the HHA/2G12-res.N672(WT) viral strain that no longer contained the gp41 glycan deletion was shown to have a higher infectivity compared with the HHA/2G12-res.N672D viral strain in which the N672D mutation was still present.

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**Table 2. Phenotypic susceptibility of WT and mutant HIV-1NL4.3 towards CBAs**

<table>
<thead>
<tr>
<th>Agent</th>
<th>WT EC$_{50}$ (nM)</th>
<th>HHA-res EC$_{50}$ (nM)</th>
<th>Fold resistance$^b$</th>
<th>2G12-res EC$_{50}$ (nM)</th>
<th>Fold resistance$^b$</th>
<th>HHA/2G12-res.N672D EC$_{50}$ (nM)</th>
<th>Fold resistance$^b$</th>
<th>HHA/2G12-res.N672(WT) EC$_{50}$ (nM)</th>
<th>Fold resistance$^b$</th>
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</thead>
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<tr>
<td>HHA</td>
<td>10±2</td>
<td>551±44</td>
<td>62</td>
<td>14±3</td>
<td>1</td>
<td>167±58</td>
<td>16</td>
<td>91±17</td>
<td>10</td>
</tr>
<tr>
<td>GNA</td>
<td>11±2</td>
<td>394±64</td>
<td>39</td>
<td>6.4±0</td>
<td>1</td>
<td>98±22</td>
<td>10</td>
<td>49±13</td>
<td>5</td>
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<tr>
<td>AH</td>
<td>41±11</td>
<td>100±18</td>
<td>3</td>
<td>34±5</td>
<td>1</td>
<td>84±36</td>
<td>2</td>
<td>33±7</td>
<td>1</td>
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<tr>
<td>UDA</td>
<td>130±33</td>
<td>748±117</td>
<td>8</td>
<td>106±24</td>
<td>1</td>
<td>214±83</td>
<td>2</td>
<td>201±24</td>
<td>2</td>
</tr>
<tr>
<td>2G12</td>
<td>5.3±1</td>
<td>&gt;351</td>
<td>&gt;57</td>
<td>&gt;351</td>
<td>&gt;57</td>
<td>&gt;351</td>
<td>&gt;57</td>
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<tr>
<td>PRM-S</td>
<td>5337±0</td>
<td>26683±0</td>
<td>5</td>
<td>5192±145</td>
<td>1</td>
<td>24510±724</td>
<td>5</td>
<td>15129±2906</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$EC$_{50}$ or compound concentration required to inhibit virus-induced cytopathicity in C8166 cell cultures (±SEM). Data are the means of at least three independent experiments.

$^b$Fold resistance or ratio of EC$_{50}$HIV,res/EC$_{50}$HIV.WT.

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**Figure 5.** Infectivity of WT and CBA-resistant virus. At day 0, C8166 T cells were infected with equal amounts (p24 values) of the viral strains. Next, samples were harvested for 8 consecutive days. The amount of virus produced was determined using a p24 ELISA and was used as a measurement of infectivity. Data are the means of three independent experiments.
Incorporation of envelope glycoproteins in CBA-resistant viral particles

A western blot was used to reveal whether the obtained differences in viral infectivity among the CBA-resistant viruses were related to a decrease in the expression or incorporation of gp120 or gp41 in the mutant viral particles. Equal p24 amounts of the different (mutant) viruses were analysed [WT, HHA-res, 2G12-res, HHA/2G12-res.N672(WT) and HHA/2G12-res.N672D]. The mutant viral strains showed no marked decreases in gp120/gp41 expression or incorporation into the viral particles, except the HHA-res virus strain that contained approximately half the gp120 levels compared with WT, but ~4-fold higher gp41 levels (Figure 6a and b).

Whereas the other mutant virus strains contained gp120 levels in their virus particles that were close to those observed in WT, the gp41 levels were consistently increased compared with WT. Nevertheless, there seems to be no correlation between decreased viral infectivity and gp120/gp41 levels in the virus particle.

Discussion

There is an urgent need for the development of microbicides to prevent HIV transmission/infection upon sexual intercourse. CBAs are prime-candidate compounds that may qualify as potential microbicide drugs, because they have shown to be able to inhibit four different events that may play a role in virus transmission/infection, including cell-free virus infection of target cells, syncytium formation between virus-infected and uninfected cells, virus particle capture by DC-SIGN-expressing cells and subsequent transmission of the captured virus to uninfected CD4+ T lymphocytes. As has been shown with highly active antiretroviral therapy that requires administration of a cocktail of at least three to four drugs, a successful microbicide will also most likely need to consist of a mixture of several drugs in one formulation/application. Recently, Féris et al. have shown that a synergistic antiviral activity occurs when tenofovir, an established HIV-1 reverse transcriptase inhibitor, is combined with CBAs. Interestingly, further studies have also demonstrated that several antiviral CBAs can be internally combined to afford synergistic antiviral activity, a phenomenon that is rather unusual when compounds belonging to the same structural/functional class of HIV inhibitors are combined. For these reasons, it was of particular interest to study the resistance development when two CBAs were combined. The combination of HHA and 2G12 was chosen because the N-glycan deletions in HIV gp120 that have been shown to occur in drug selection experiments proved to be different for the individual agents.

Several new observations have been made. After a similar time period of selection in cell culture (i.e. passage 43), CBA resistance development was clearly delayed when HHA and 2G12 were combined, compared with resistance development in the presence of the single drugs. Thus, the synergistic activity of combined CBAs against virus infection shown in previous studies could now also be observed for their resistance development when the two CBAs used in this study were combined. Whereas single CBA (HHA) exposure to HIV-1 resulted in the selection of virus strains that contained at least six different N-glycan deletions (plus the movement of one N-glycan to a nearby amino acid location) in gp120, the virus that has been exposed to the HHA/2G12 drug combination contained only three N-glycan deletions in its gp120. These three deletions were also found in the HHA-selected virus strain. The lower number of N-glycan deletions in the presence of both HHA and 2G12 is probably due to the fact that the compound concentrations at the end of the selection procedure were markedly lower in the drug combination experiments compared with the final compound concentrations when only a single CBA was used. Additionally, it can be assumed that the exposure of virus to two compounds with distinct binding sites will make it harder for the virus to escape drug pressure, resulting in a slower resistance development and a lower frequency of accumulation of mutations.

Figure 6. Levels of expression of gp120, gp41 and p24 in WT and CBA-resistant viral strains, as determined by western blotting. For each viral strain, 37.5 ng of p24 was loaded onto the gel. After blotting to a PVDF membrane, gp120, gp41 and p24 were visualized. Relative protein levels were quantified using ImageJ software and are presented relative to the p24 levels.
Interestingly, the Asn-390 glycan deletion that was eventually selected in 2G12-exposed HIV-1-infected cell cultures as the sole N-glycan deletion in gp120 was not found in the final HHA/2G12-res virus strain; instead, in the latter strain, deletion of another N-glycan that was previously shown to be involved in 2G12 interaction with gp120 (Asn-337)\textsuperscript{14} was observed. This mutation has transiently been observed during the single 2G12 selection process, but was not retained in the final virus strain at the end of the selection period in the presence of single 2G12.

When the virus strains were examined for phenotypic resistance/susceptibility to HHA, 2G12 and several other CBAs, it was of interest to observe that: (i) the HHA-selected strain was highly resistant to HHA, GNA and 2G12; (ii) the 2G12-selected strain (containing only one deleted N-glycan) was solely resistant to 2G12, but not to the other CBAs; and (iii) the virus strain selected by the HHA/2G12 CBA combination (HHA/2G12-res.N672D) showed clearly lower resistance to HHA and the other CBAs, but still showed full resistance to 2G12. The lower degree of CBA resistance could probably be explained by the lower number of N-glycan deletions in its gp120 and, thus, is in agreement with the genotypic characterization of this virus isolate.

Our findings also demonstrated that the monoclonal antibody 2G12 loses substantial inhibitory capacity in the presence of a sole N-glycan deletion, whereas the other CBAs such as HHA clearly require the accumulation of several N-glycan deletions before showing significantly decreased antiviral potential. This latter property of non-antibody CBAs is favourable from a microbiologic viewpoint, because a variety of virus strains that may lack one of the 2G12-specific N-glycans may turn out to be ‘naturally’ resistant to 2G12 inhibition at the time of transmission. Thus, these observations reveal that antibodies might be too specific in their interaction with HIV gp120, allowing a single mutation to be sufficient for a marked loss of neutralizing activity. The non-antibody CBAs might act against HIV-1 gp120 in a somewhat less specific manner, providing them with a higher genetic barrier than antibody-based CBAs.

In this study, we could also, for the first time, demonstrate that an N-glycan deletion in HIV-1 gp41 had an effect on phenotypic CBA resistance development. The virus strain (HHA/2G12-res.N672D) showed a trend to a higher level of resistance to several CBAs (such as HHA, GNA, AH and PRM-S), compared with the virus strain [HHA/2G12-res.N672D(WT)] that had lost the N672D mutation in gp41. The appearance of N-glycan mutations in HIV-1 gp41 upon CBA exposure has seldom been observed to occur in cell culture, and this is the first time that it could be demonstrated that such mutations in gp41 also affect phenotypic resistance to some CBAs and may suggest at least some communication between gp41 and gp120 through their N-glycans. In this respect, it would be of particular interest to explore the role of the other N-glycans of HIV-1 gp41 in the phenotypic resistance of CBAs to virus strains with N-glycan deletions in gp120.

The determination of the replication capacity of the mutant virus strains also showed that these mutant strains containing N-glycan deletions in their envelope glycoprotein gp120 have a markedly compromised replication potential, when compared with WT virus. It has been observed previously that viral replication is generally more compromised when the number of N-glycan deletions in gp120 is higher.\textsuperscript{15} In agreement with our findings, the Asn-160 glycan deletion that occurred early in the HHA selection process, but later also in the HHA/2G12 selection process, had previously also been shown to have a substantially compromised replication capacity as a single mutant (N160Q).\textsuperscript{25}

Since the Asn-672 glycan-deleted HHA/2G12-res.N672D strain (containing three N-glycan deletions in gp120) was severely compromised in its replication efficacy, it was not surprising that the N672D mutation was easily lost when this virus was further cultivated in the absence of CBA pressure. The reversion to its WT amino acid in this glycosylation motif may therefore be also regarded as a compensatory mutation to partially restore its replication capacity. It is also interesting to notice that this gly41-N-glycan mutant only emerged in the HHA/2G12 combination selection, but not in the single CBA (HHA or 2G12) selection processes. CBAs generally bind to gp120 and are believed to freeze its conformation,\textsuperscript{8,9} thereby hindering the exposure of gp41 that can only happen upon conformational changes in gp120. This may explain why CBAs are only rarely found to select for glycan deletions in gp41. Nonetheless, the mutation found in this resistance selection experiment (N672D) might be of importance for the resistance to the combination of HHA and 2G12 and might not be just a coincidence. A detailed analysis of the role of gp41 N-linked glycans for CBA susceptibility, infectivity and transmission will shed more light on this. Our preliminary data, using mutant virus obtained by site-directed mutagenesis, revealed that the N672D mutation alone had only a modest detrimental influence on the viral infectivity, as compared with WT HIV-1\textsubscript{NL4-3}.

In this study, we also revealed one novel N-glycan deletion in gp120 that had never been observed before to emerge in the presence of CBAs (Asn-461). Additionally, we found the deletion of the N-glycan on Asn-384 in the presence of HHA, which was never described before in HHA resistance selection experiments. These findings reveal that the virus can choose several drug resistance pathways to the same drug, probably depending on subtle differences in the drug selection process, virus pool and/or cell conditions and stress the flexibility of HIV to select for different mutational pathways for N-glycan deletions in an attempt to escape CBA pressure.

The resistance selection experiments were performed using the laboratory-adapted HIV-1\textsubscript{NL4-3} strain. However, it has been shown before that most CBAs have similar suppressive effects on primary clinical viral isolates, compared with laboratory-adapted viral strains.\textsuperscript{11,16,26–28} Moreover, one CBA resistance selection experiment using the clinical isolate HIV-1/HE in parallel with the laboratory-adapted strain HIV-1\textsubscript{NL4-3} was performed previously. In that study, it was shown that both viruses responded to the long-term exposure to HHA or GNA by deleting N-linked glycans in gp120.\textsuperscript{29} Therefore, we assume that laboratory-adapted and clinical isolates may respond to CBA pressure in a similar manner.

In conclusion, all data taken together, it could be demonstrated that combining two CBAs (HHA and 2G12) results in a delay in the appearance of genotypic/phenotypic drug resistance selection compared with the single CBAs and also results in a lower degree of resistance compared with that of the singly administered CBAs. N-glycans of gp41 may also contribute to phenotypic CBA resistance, resulting in an overall higher degree of phenotypic resistance once deleted. The CBA-exposed virus strains had a markedly compromised replication capacity compared with WT virus, which would be advantageous in the in vivo setting after CBA exposure.
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Transparency declarations
None to declare.

Supplementary data
Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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