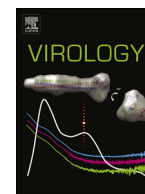




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Mechanisms of HIV-1 subtype C resistance to GRFT, CV-N and SVN

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ABSTRACT

We examined the ability of HIV-1 subtype C to develop resistance to the inhibitory lectins, griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN), which bind multiple mannose-rich glycans on gp120. Four primary HIV-1 strains cultured under escalating concentrations of these lectins became increasingly resistant tolerating 2 to 12 times their 50% inhibitory concentrations. Sequence analysis of gp120 showed that most had deletions of 1 to 5 mannose-rich glycans. Glycosylation sites at positions 230, 234, 241, 289 located in the C2 region and 339, 392 and 448 in the C3–C4 region were affected. Furthermore, deletions and insertions of up to 5 amino acids in the V4 region were observed in 3 of the 4 isolates. These data suggest that loss of glycosylation sites on gp120 as well as rearrangement of glycans in V4 are mechanisms involved in HIV-1 subtype C escape from GRFT, CV-N and SVN.

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Introduction

The surface of the HIV-1 envelope is populated with glycans that play an important role in protecting neutralization sensitive epitopes, promoting gp120 structural integrity and mediating interaction with cellular receptors (Geijtenbeek and Gringhuis, 2009; Li et al., 1993; Lin et al., 2003; Liu et al., 2004; Losman et al., 2001; Lue et al., 2002; Wei et al., 2003; Zhu et al., 2000). The majority of glycans on the HIV-1 envelope trimer are mannose-rich comprising 7 to 9 terminal mannose residues (Bonomelli et al., 2011; Doores et al., 2010) although the precise number and location remains undetermined. Complex glycans with terminal sialic acid residues are likely also present (Leonard et al., 1990). Mannose-rich glycans are targets for lectins or carbohydrate binding agents (CBAs) such as griffithsin (GRFT), cyanovirin-N (CV-N) and scytovirin (SVN) isolated from naturally occurring algae (Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005; Moulaei et al., 2007; Ziolkowska and Wlodawer, 2006). These lectins show potent and broad anti-HIV-1 activities

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in vitro and are, therefore, being investigated for use in HIV-1 prevention, mostly in the form of microbicides (Balzarini, 2005; Ferir et al., 2012; O'Keefe et al., 2009; Tsai et al., 2003, 2004).

Since the neutralization activity of lectins involves interaction with glycans, one potential mechanism of HIV-1 escape from these compounds is the deletion of glycosylation sites. Indeed studies on HIV-1 subtype B have shown deletion of mannose-rich glycans is a mechanism of resistance to CV-N (Balzarini et al., 2006; Hu et al., 2007). More specifically, a loss of mannose-rich glycans at positions 230, 289, 295, 332, 339, 386, 392 and 448 was associated with resistance in the laboratory-adapted strains HIV-1_{IIIB} and HIV-1_{NL-4.3} (Balzarini et al., 2006). In another study, the deletion of these glycans excluding those at positions 230 and 386 in HIV-1_{IIIB} cultured under escalating concentrations of CV-N, resulted in resistance to the lectin (Hu et al., 2007). In addition, HIV-1 resistance to the lectins *Galanthus nivalis* agglutinin and *Hippeastrum* hybrid agglutinin, was reported to occur via a partial loss of glycans on the envelope (Balzarini et al., 2004, 2005). Resistance to the broadly neutralizing antibody 2G12, that targets glycans on gp120, also involves the deletion of mannose-rich glycans. This is supported by the fact that most subtype C viruses are resistant to this antibody due to their lack of the 295 glycosylation site (Binley et al., 2004; Chen et al., 2005; Gray et al., 2007; Manrique et al., 2007).

The glycosylation pattern on the HIV-1 subtype C envelope differs from subtype B (Zhang et al., 2004), and the ability of these viruses to develop resistance to lectins is unknown. In the current study we describe the mechanism of resistance to CV-N among four subtype C primary viruses, which while similar to subtype B showed some differences. This involved the deletion of mannose-rich glycans on gp120 as well as 4–5 amino acids deletions or insertions in the V4 region. In addition, we studied HIV-1 escape from two other lectins, GRFT and SVN, and showed that it followed a similar pathway to CV-N although patterns varied between the lectins. Thus, changes of glycosylated and non-glycosylated amino acid sequences suggest multiple mechanisms of escape from these three lectins.

Results

Replication of HIV-1 subtype C isolates in the presence of sub-inhibitory concentrations GRFT, CV-N and SVN

HIV-1 subtype B has previously been shown to develop resistance after repeated passages in the presence of escalating concentrations of CV-N (Balzarini et al., 2004, 2005; Hu et al., 2007; Witvrouw et al., 2005). Since the glycosylation pattern of HIV-1 envelope differs by subtype (Zhang et al., 2004), we determined the ability of viruses from subtype C to develop resistance to CV-N and two other lectins, GRFT and SVN. Four subtype C primary isolates were cultured in CD8 depleted PBMC in the presence of increasing lectin concentrations for 11 to 22 weeks, starting with the concentration equal to the IC₅₀ for each compound (Table 1). Viral growth was measured weekly by p24 antigen ELISA. When the p24 levels in the lectin-containing cultures were lower than the control cultures (containing no lectin), the lectin concentrations were reduced in order to facilitate ongoing replication (Fig. 1). Of all four isolates, Du179 showed the highest levels of resistance, tolerating at least 10 times the starting concentration of each lectin (Table 1). The other 3 viruses, Du151, Du422 and COT9 grew at 3 times the starting concentrations of GRFT and SVN and 5 times the starting concentration of CV-N. Altogether, these data showed that the continuous growth of these four HIV-1 subtype C viruses under lectin selective pressure resulted in their ability to tolerate higher concentrations suggesting a level of resistance to these compounds.

Lectin-selected isolates showed decreased sensitivity and cross-resistance

We next determined whether viruses cultured in the presence of GRFT, CV-N and SVN showed reduced sensitivity to these compounds in the PBMC neutralization assay, using an 80% neutralization (IC₈₀) cut-off (Bures et al., 2002; Fenyo et al., 2009). Du179/GRFT.R, Du179/CV-N.R and Du179/SVN.R showed at least 5-fold increase in IC₈₀ compared to the control viruses, passaged in the absence of the lectin (Fig. 2A). For Du151 and Du422 there was an increase in IC₈₀ that

ran from 2 to 4 fold for all 3 lectins (Fig. 2B and C), while for COT9, the GRFT resistant virus showed a ~2 fold increase in IC₈₀ (Fig. 2D). However, there was no change in IC₈₀ for COT9 cultured under CV-N and SVN, despite the ability to grow under increased concentrations of the two lectins.

We next investigated whether viruses that were resistant to each compound also showed cross-resistance or decreased sensitivity to the other two lectins. We used Du179 for this study as this virus developed the greatest resistance to all three lectins. Du179/GRFT.R that was 10 fold resistant to GRFT showed a ~3 fold increase in resistance to CV-N and SVN neutralization (Fig. 3). A similar pattern was observed for Du179/CV-N.R and Du179/SVN.R. These data suggest that resistance to one lectin confers cross-resistance to the other; however resistance to the selecting agent was always the strongest.

Resistance was associated with amino acid changes and deletions at and around mannose-rich glycosylation sites

The full envelope sequences of both wild-type (viruses passaged in the absence of the lectin) and the corresponding resistant viruses were compared. Wild-type Du179, Du151 and COT9 each had 10 intact mannose-rich glycosylation sites while Du422 had nine. All four viruses lacked the 295 glycosylation site as is common among subtype C viruses (Zhang et al., 2004). Of the 12 selected viruses (four for each lectin), nine had deletions of glycans on gp120 with no changes in gp41 glycosylation patterns. Seven of the 11 glycosylation sites on gp120 that have been confirmed to contain mannose-rich glycans (Leonard et al., 1990), were involved in resistance to GRFT, CV-N and SVN (Fig. 4). Deletions of the 230, 392 and 448 glycans were observed among viruses selected by all three lectins with the loss of the 448 glycan observed in 6 out of the 12 selected viruses. Except for position 289, deletions at the seven sites occurred in response to more than one lectin.

Examination of glycan changes to individual lectins showed that the greatest number of deleted glycans was conferred by GRFT selection (Table 2). The loss of the glycan at position 339 occurred in three out of four GRFT selected viruses (Table 2 and Fig. 4), with those at position 230 and 234 occurring in two. The GRFT resistant Du179 also deleted the 442 glycan, predicted to be complex in studies conducted with monomeric gp120 (Kwong et al., 1998; Leonard et al., 1990). The loss of sensitivity to GRFT in Du422 restored the glycan at position 386 that was absent in the wild type virus (Table 2). For CV-N resistant viruses, the loss of the 448 glycan was the most common, occurring in half of these viruses (Table 2). However, we did not observe any changes in COT9 and Du422 sequences that accompanied their increased resistance to CV-N. Three out of four SVN selected viruses had the 448 deletion while two out four had the 339 glycan loss (Table 2). As with GRFT, selection with SVN restored the 386 glycan in Du422. Lastly, similar to CV-N, COT9 resistance to SVN was not associated with any apparent changes in glycans.

In addition to loss of glycans on gp120, we observed deletions and insertions of amino acid sequences near and within mannose-rich glycosylation sites located in the fourth variable (V4) region. GRFT resistance was associated with the deletion of four amino acids at position 400–403 and 396–399 in Du179 and COT9, respectively (Fig. 5A and D). However, in Du422 resistance to GRFT resulted in the insertion of five amino acids at position 398–402 (Fig. 5C). Similarly, CV-N resistance led to the deletion of four amino acids in Du179 at position 392–395 (Fig. 5A) that resulted in the loss of the 392 glycan. In Du422, SVN resistance resulted in the insertion of five amino acids at position 398–402, this was similar to GRFT (Fig. 5C). We observed no deletions or insertions of amino acids in Du151 under the selective pressure of any of the three lectins. In conclusion, our data using 4 subtype C primary isolates

Table 1
IC₅₀ values of GRFT, CV-N and SVN for the neutralization of HIV-1 isolates.

Virus	Pre-selection ^a IC ₅₀ (nM)			Fold increase after selection		
	GRFT	CV-N	SVN	GRFT	CV-N	SVN
Du179	37.8	102.2	134.1	10	10	12
Du151	40.3	41.2	128.5	3	5	3
Du422	39.5	82.1	215.6	3	5	2
COT9	85.8	77.1	449.5	3	4	2

^a 50% Inhibitory concentration.

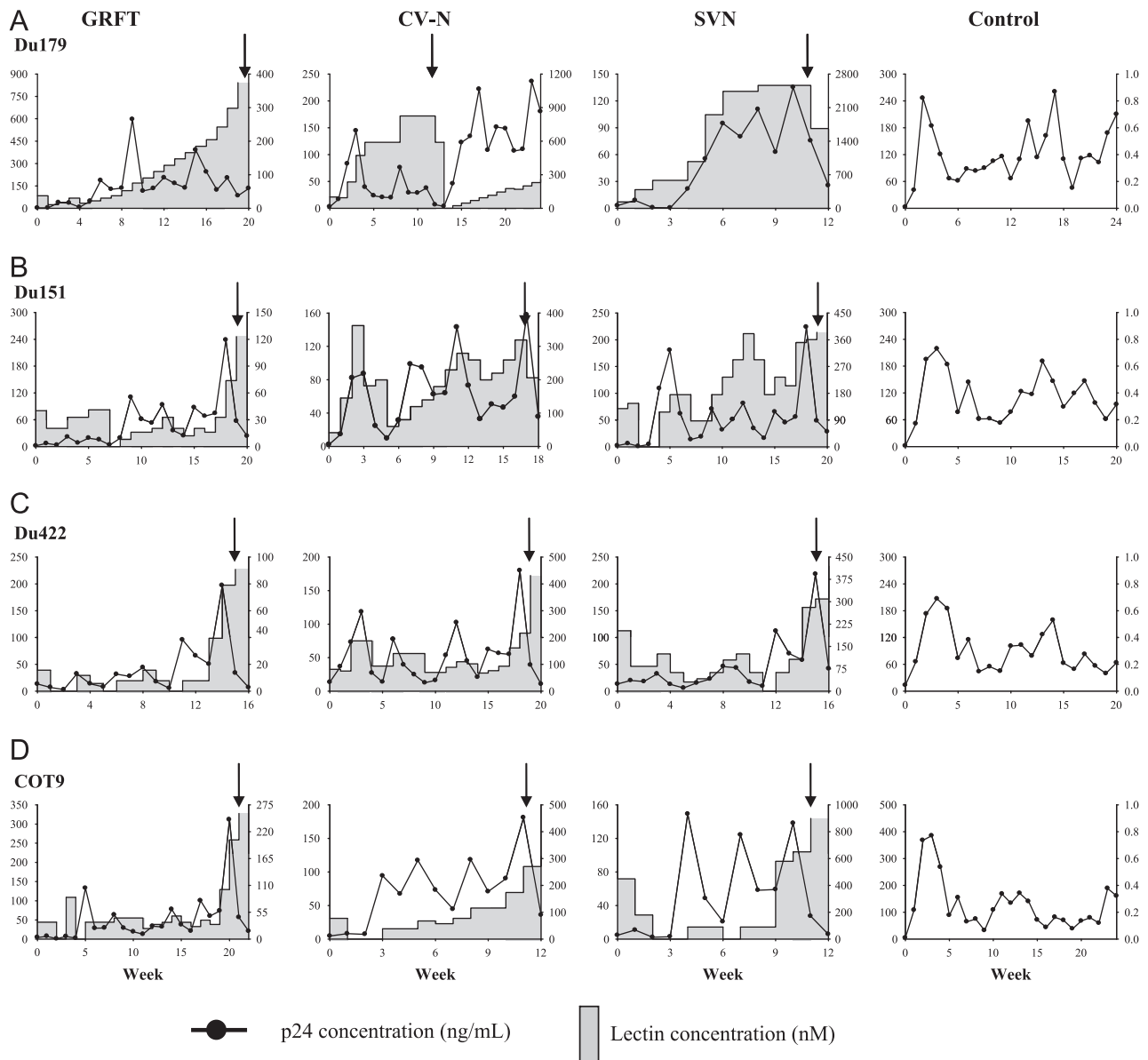


Fig. 1. In vitro generation of GRFT, CV-N and SVN resistant viruses. Primary HIV-1 subtype C isolates Du179 (A), Du151 (B), Du422 (C) and COT9 (D) were cultured in PBMC under escalating concentrations of GRFT, CV-N and SVN. The concentration of each lectin was gradually increased or reduced depending on the viral growth compared to the control cultures (containing no lectin) as determined by p24 antigen ELISA. The arrows indicate the time-point that the supernatant was collected for analysis.

suggested that in addition to directly deleting glycans, resistance to GRFT, CV-N and SVN may frequently also involve deletion of multiple amino acids that results in shifting of the position of neighboring glycans.

Single genome amplification of GRFT, CV-N and SVN resistant viruses

Since the loss of multiple glycans observed by total viral population sequencing could be the result of a mixture of viruses carrying fewer deletions, we performed single genome amplifications (SGA) of gp120. We used Du179 for this experiment since it had the highest number of glycan loss. Six to seven clones were selected for each lectin and compared to their corresponding total population sequence.

The majority of clones from GRFT cultures had the same 4 glycans deleted as the population sequence (highlighted in yellow) while the 393 glycan absent in the population sequence was deleted in only 2 of the 7 clones (Fig. 6). Furthermore, all GRFT clones had the deletion of four amino acids in V4 and most were at position 400 to 403. For CV-N, most clones had one to two

additional glycans deleted compared to the total population sequence. However, similar to the population sequence, all clones had the deletion of four amino acids in V4, although not all in the same location. With SVN both the number and glycan deletion pattern of isolated clones were almost identical to the total population sequence. But unlike this sequence, one had lost the 442 glycan while two carried deletions of amino acids in V4. Lastly, almost all GRFT, CV-N and SVN clones had GPGQ at the tip of the V3 loop which differed from the wild-type Du179 sequence with GPGK. Taken together, these data show that clones largely harbor the same mutational patterns as the total population sequence suggesting that multiple mutations on single genomes are required to develop resistance.

GRFT, CV-N and SVN resistance affects HIV-1 sensitivity to neutralizing antibodies

A previous study in subtype B showed that resistance to CV-N can affect HIV-1 sensitivity to neutralizing antibodies (Hu et al.,

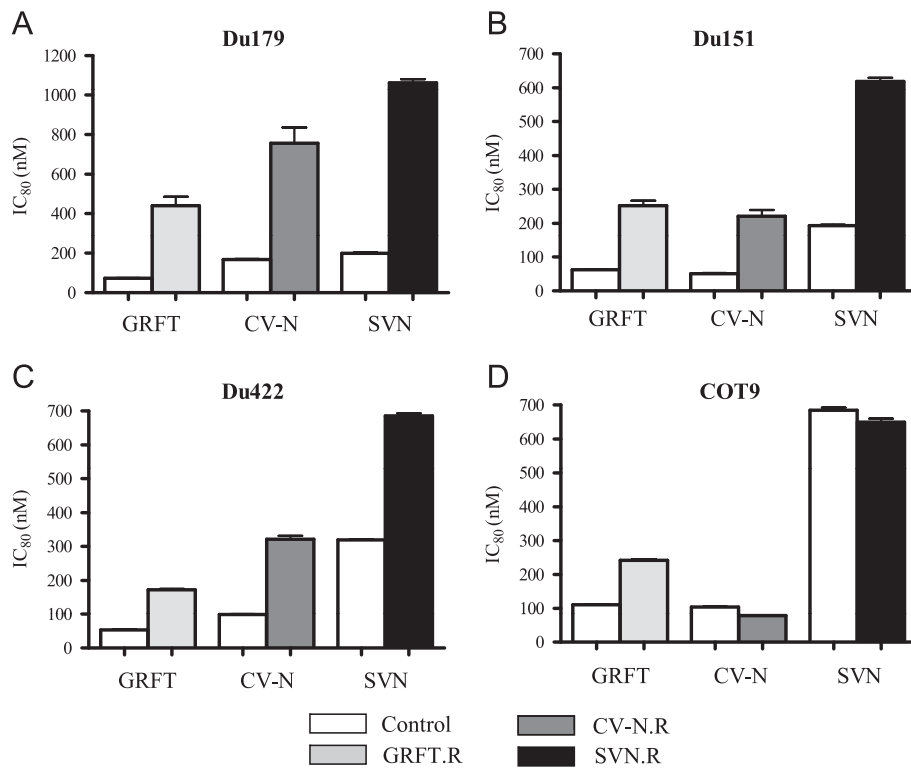


Fig. 2. Lectin-selected viruses showed a decreased sensitivity to GRFT, CV-N and SVN. Resistant primary HIV-1 subtype C isolates Du179 (A), Du151 (B), Du422 (C) and COT9 (D) were tested against GRFT, CV-N and SVN in a PBMC neutralization assay. The neutralization of HIV-1 infection was measured by p24 ELISA and the IC₈₀ of the resistant virus and the corresponding wild-type were determined by linear regression. Bars represent standard deviation of three independent experiments.

2007). Therefore, we investigated whether subtype C resistance to GRFT, CV-N and SVN impacted the sensitivity to gp120 antibodies and soluble CD4 (sCD4) in the TZM-bl neutralization assay. Five Du179 SGA-derived clones with the highest number of glycan deletions selected by each lectin, were tested together with the Du179 clone passaged without the lectins (control). All GRFT, CV-N and SVN resistant viruses showed decreased sensitivity to b12 with 4–20 fold higher antibody concentrations required for neutralization (Table 3). In contrast, all 5 clones showed an increased sensitivity to VRC01 of 2–6 fold. We also observed a moderate increase in sensitivity to sCD4, which is known to have a similar binding site to VRC01 (Zhou et al., 2010). None of the viruses showed changes in sensitivity to the PGT121 or PGT128 mAbs, which bind to the glycans at positions 301 and 332 on the gp120 outer domain (Walker et al., 2011). In conclusion, our data show that resistance to GRFT, CV-N and SVN can affect HIV-1 subtype C sensitivity to some antibodies that target gp120.

Confirmation of resistance conferring mutations by site-directed mutagenesis

To confirm if the changes seen in GRFT, CV-N and SVN selected viruses (shown in Table 2 and Fig. 5) conferred resistance to the lectins, we introduced these changes into the corresponding wild-type cloned envelopes by site-directed mutagenesis. This included all changes at glycosylation sites as well as deletions and insertions in V4. For Du179.14 a total of 6 rounds of site-directed mutagenesis were necessary to produce Du179/GRFT.R_M (where R_M indicates resistance generated by mutagenesis). We could not test the CV-N associated mutations as functional viruses could not be obtained for Du179 and Du151 and no changes were noted for Du422 and COT9. The GRFT.R_M and SVN.R_M envelope clones were tested for sensitivity to GRFT and SVN relative to their wild-type counterparts in a TZM-bl cell neutralization assay.

The effect of the combined changes in glycosylation and V4 amino acid indels associated with GRFT and SVN resistance in the four cloned envelopes resulted in a loss of sensitivity to the lectins as seen by an increase in IC₅₀ values of the mutant viruses (Table 4). For GRFT, there was a 3–4 fold decrease in sensitivity in all 4 viruses while for SVN the effect was more pronounced. In particular the insertion of 5 amino acids together with the loss of 2 glycans in Du422.1 resulted in a 16-fold increase in resistance to SVN. These data suggest that the changes we observed in the HIV-1 gp120 sequences from lectin-selected PBMC cultures were indeed resistance-conferring mutations.

Discussion

In this study we demonstrated that the continuous growth of four HIV-1 subtype C isolates under escalating concentrations of GRFT, CV-N and SVN resulted in reduced sensitivity to these lectins. This was associated with the deletion of mannose-rich glycans on gp120 and in some cases insertions or deletions of amino acids near mannose-rich glycosylation sites. These changes were observed in both the total population and clonal sequences of selected viruses and were confirmed to be resistance conferring by site-directed mutagenesis of envelope clones. This study is the first to report the mechanism of HIV-1 subtype C resistance to GRFT, CV-N and SVN and provides important insights into the binding sites of these lectins on the subtype C envelope.

The association between deletions of mannose-rich glycans on the subtype C viruses studied here and increased resistance to GRFT, CV-N and SVN is consistent with the fact that these compounds bind glycans on the viral envelope (Ziolkowska and Wlodawer, 2006). It also supports previous reports showing that glycan deletions mediate HIV-1 subtype B resistance to CV-N and GRFT (Balzarini et al., 2006; Hu et al., 2007; Huang et al., 2011;

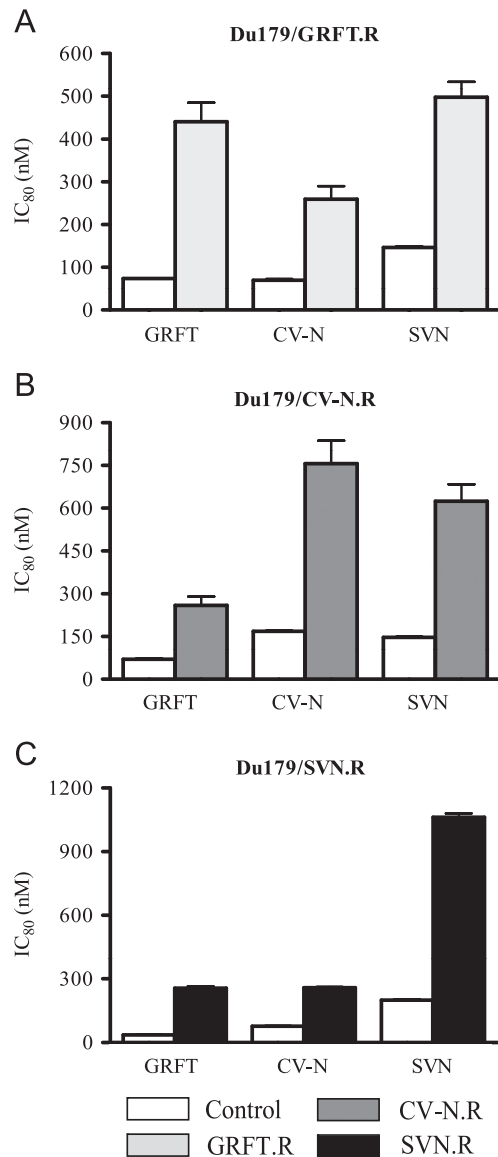


Fig. 3. Cross-resistance between GRFT, CV-N and SVN. Du179 virus selected by GRFT (A), CV-N (B) and SVN (C) were tested against all three lectins in a PBMC assay. HIV-1 neutralization was measured by p24 ELISA and the IC_{50} of the resistant virus (gray bar) and the corresponding wild-type (white bar) were determined by linear regression. Bars represent standard deviation of three independent experiments.

Witvrouw et al., 2005). Deleted glycans were located in C2, C3, V4 and C4 suggesting that the binding sites for these lectins are located in these regions, which are exposed on gp120 and hence readily accessible for binding. While the most resistant viruses generally had more glycan deletions there was no clear correlation between the number of deleted mannose-rich glycans and resistance to GRFT, CV-N and SVN. This supports our earlier study showing that the position of the glycan is also important in determining sensitivity to these lectins (Alexandre et al., 2010). The lack of correlation between the number of deleted glycans and resistance may suggest that some glycans are directly involved in lectin binding to HIV-1 while for others the involvement may be indirect i.e. they contribute to the formation of the binding site. Viruses selected by the three lectins showed cross-resistance to these compounds, also supporting our earlier finding that these lectins have overlapping binding sites on the viral envelope. Some of the affected glycans are within structural proximity of each other on gp120, which together with the symmetrical

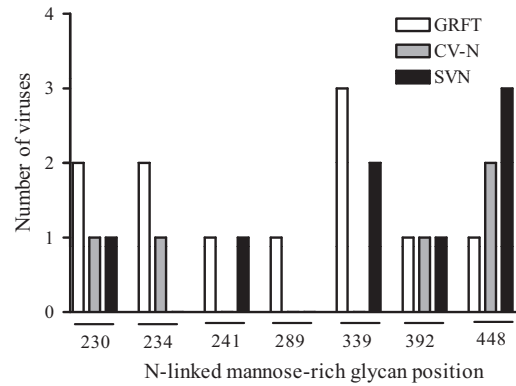


Fig. 4. Mannose-rich glycosylation sites deleted in GRFT, CV-N and SVN selected viruses. The X-axis shows the positions of mannose-rich glycans deleted in the four isolates under lectin selective pressure. The positions of glycans are numbered according to the HxB2 virus (Leonard et al., 1990) and were identified by sequence analysis. The Y-axis shows the number of resistant viruses (out of 4) that had the deletion.

arrangement of binding sites on CV-N and GRFT, suggests that cross-linking of glycans underlies the mechanism of action of lectin inhibition (Ziolkowska et al., 2006; Ziolkowska and Wlodawer, 2006). This hypothesis has previously been proposed for GRFT (Moulaei et al., 2010).

The 448 glycan was the most frequently deleted glycan suggesting that it plays an important role in GRFT, CV-N and SVN binding to subtype C gp120. This glycan together with those at positions 230 and 392 were involved in resistance to all 3 lectins and have previously been shown to be important for subtype B viruses (Balzarini et al., 2006). Despite these similarities, our data suggest that there may be subtype-specific pathways to resistance. For example, the 332 glycan was not affected in our study while it was lost in subtype B viruses that developed resistance to CV-N (Balzarini et al., 2006; Hu et al., 2007; Witvrouw et al., 2005). This being said, the differences in culture conditions in addition to the use of different viral isolates may have accounted for some of these subtype-specific effects. We previously showed that viruses naturally lacking the 234 and 295 glycosylation sites were less sensitive to GRFT, CV-N and SVN (Alexandre et al., 2010). In the current study, we observed the deletion of the 234 glycosylation site for two of the three lectins suggesting that this glycan can participate in both natural and in vitro induced resistance to these compounds. In some Du179 resistant viruses we observed the deletion of the 442 glycosylation site which is not mannose-rich on monomeric gp120 (Leonard et al., 1990). However, recent studies have indicated that glycans on envelope trimer are more resistant to mannose-trimming generating complex glycans (Bonomelli et al., 2011; Doores et al., 2010). Thus, it can be speculated that the 442 glycosylation site on the trimer contains a mannose-rich glycan and is, therefore, another potential binding site for GRFT, CV-N and SVN.

In addition to single amino acid changes that resulted in the loss of glycans, we noted 4–5 amino acid indels in V4 sequences in 3 of the 4 viruses studied. In the case of Du179 cultured in CV-N, this obliterated the 393 glycosylation site. This mechanism of resistance has also been reported by Witvrouw and colleagues who showed that HIV-1 cultured in increasing concentrations of CV-N had a 13 amino acid deletion in V4 resulting in the loss of 3 glycosylation sites (Witvrouw et al., 2005). The other indels found in our study did not involve the loss of glycans but occurred near mannose-rich glycosylation sites, which likely affected their arrangement. Further work is needed to explore the impact of these V4 changes to determine if they affect lectin sensitivity independently of glycan deletions.

Table 2

Changes in gp120 mannose-rich glycosylation patterns associated with resistance to GRFT, CV-N and SVN.

Lectin	Virus	^a Predicted mannose-rich glycosylation sites										
		230	234	241	262	289	295	332	339	386	^b 392	448
GRFT	Du179	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
	Du151	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
	Du422	Red	Red	Red	Red	Red	Red	Red	Red	Green	Red	Red
	COT9	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
CV-N	Du179	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
	Du151	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
	Du422	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
	COT9	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
SVN	Du179	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
	Du151	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
	Du422	Red	Red	Red	Red	Red	Red	Red	Red	Green	Red	Red
	COT9	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red

Red colored boxes indicate glycosylation sites that were deleted under GRFT, CV-N or SVN selective pressure.

Green colored boxes indicate glycosylation sites that were added under GRFT, CV-N or SVN selective pressure.

Grey colored boxes indicate sites that were unchanged.

Blank boxes indicate sites that were absent in the wild-type virus.

^a Mannose-rich glycosylation sites were identified from the amino acid sequence of each envelope clone (related to HxB2) based on a study using monomeric gp120 (Leonard et al., 1990).

^b Note that for Du179 and Du151 the 392 glycan is shifted to position 393 but it is placed at position 392 for simplicity.

In this study we showed that individual clones generally had the same number of deleted glycans as the total population sequence. This implies that resistance is not due to a swarm of quasispecies with different mutational profiles but that multiple mutations on each genome are needed for resistance to GRFT, CV-N and SVN. However, there was variation with some clones having slightly different glycan deletion patterns that collectively matched the population sequence. It is possible that each clone has different levels of sensitivity to the lectins that could coexist provided that they have sufficient resistance conferring mutations. It can also be speculated that the presence of strains with different levels of glycan loss is the result of differences in their mechanisms of escape. An in-depth analysis of minority variants in Du422 and COT9 may help to explain why no mutations were seen in the population sequence of these viruses despite phenotypic resistance to the lectins.

The increased sensitivity of GRFT, CV-N and SVN resistant Du179 clones to VRC01 and sCD4 suggests that lectin-escape mutations affect the exposure of the CD4 binding site (CD4bs). However, the simultaneous decrease in sensitivity to b12 that also targets this site indicates that these mutations differentially affect these compounds. This is probably due to the fact that although they all bind the CD4bs the footprint of VRC01, b12 and sCD4 on gp120 do not completely overlap (Li et al., 2011; Zhou et al., 2007, 2010). A previous study showed that HIV-1 escape from CV-N did not affect sensitivity to b12 despite the fact that this isolate became sensitive to V3 and other HIV antibodies (Hu et al., 2007). Our finding of decreased sensitivity to b12 in 5 distinct clones from a single isolate suggests that there are also virus dependent effects. We did not observe a change in sensitivity to the PGT121 and PGT128 antibodies, consistent with the fact that the 332 glycan (Walker et al., 2011) was not deleted in our resistant viruses (Table 2).

Du179 developed the highest level of resistance to these lectins. It is unlikely that this was due to differences in glycosylation as the wild-type Du179 had an identical mannose-rich glycosylation

pattern as Du151 and COT9. It is also unlikely that the pre-selection sensitivity of these viruses to GRFT, CV-N and SVN played any role in the development of resistance to the lectins given that they had similar IC₅₀ values (Table 1). However, Du179 was the only dual-tropic virus tested here (Coetzer et al., 2007; Williamson et al., 2003). We previously showed that GRFT blocks HIV infection by interfering with co-receptor binding (Alexandre et al., 2011). Thus, the ability of Du179 to enter cells via both CCR5 and CXCR4 may have provided additional opportunities to escape the inhibitory effects of these lectins. Indeed, our data suggest that lectin selection did impact on viral tropism as the GPGK at the tip of the V3 loop in Du179 was replaced with GPGQ that is characteristic of R5 viruses (Cilliers et al., 2003; Coetzer et al., 2011). Thus, testing a larger number of dual tropic viruses and comparing them to single tropic viruses may reveal to what extent the R5X4 property affects the rate at which HIV-1 develops resistance to these lectins.

The level of resistance to the lectins observed in our study was 2–12 fold, which was not as high as reported in some other studies (Balzarini et al., 2006; Witvrouw et al., 2005). The reason for this may be due to our use of PBMC that do not support HIV-1 replication to the same extent as cell lines used by other investigators. Also our viruses were primary isolates that may have different replication capacity or pathways to resistance compared to lab-adapted viruses and molecular clones used in previous studies (Balzarini et al., 2006; Hu et al., 2007; Witvrouw et al., 2005). Partial resistance to CV-N was also reported by Balzarini and colleagues and was shown to be associated with fewer glycan deletions. Defining these early events in the development of resistance will help to understand the evolution of complete lectin resistance.

GRFT, CV-N and SVN are among leading CBAs that are being studied for use in HIV-1 prevention. However, until now much of what was known about HIV-1 resistance to these lectins is the result of studies conducted with subtype B viruses that have a different glycosylation pattern compared to subtype C (Zhang et al., 2004). Thus the current study makes an important

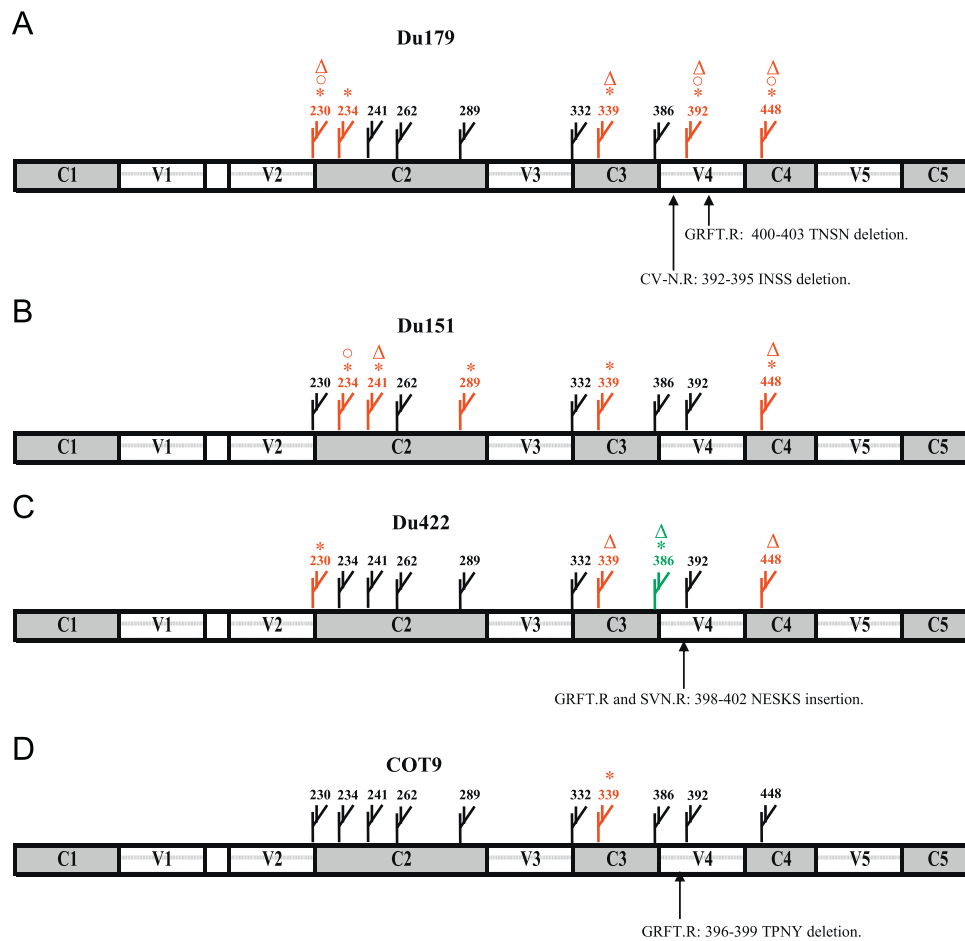


Fig. 5. Changes in gp120 associated with lectin selection. Position of mannose-rich glycans and amino acid sequences for Du179 (A), Du151 (B), Du422 (C) and COT9 (D) following selection by GRFT, CV-N and SVN. Glycan deletions are shown in red and the addition in green. Symbols indicate changes in response to GRFT (*), CV-N (○) and SVN (Δ). Deletions and insertions of amino acid sequences in V4 are shown.

contribution to our understanding of the mechanism of resistance to GRFT, CV-N and SVN in HIV-1 subtype C viruses, the main cause of HIV infections around the world. The extensive loss of glycans and amino acid sequence changes required for resistance to these three lectins poses a high genetic barrier distinct from the single glycan deletion required to confer resistance to the 2G12 monoclonal antibody. Thus lectins have a broader reactivity and would be expected to target a variety of wild-type viruses compared to antibodies that are more specific; and attempts are being made to test these compounds in humans. Taken together, our study supports further research in the use of GRFT, CV-N and SVN to prevent the spread of HIV-1.

Materials and methods

Viruses, cell lines, lectins and antibodies

The R5 infectious HIV-1 subtype C viruses Du151 and Du422 were isolated from acute infections while the R5X4 Du179 was isolated from a chronic infection in South Africa (Williamson et al., 2003); COT9 is a R5 isolate from a chronically infected pediatric patient (Choge et al., 2006). These four primary isolates were chosen because they are well characterized. Furthermore, Du151, Du422 and Du179 were previously selected as HIV-1 vaccine strains since they represented the HIV-1 subtype C epidemic (Williamson et al., 2003). The pSG3Δenv plasmid was provided

by Dr. Beatrice Hahn. The TZM-bl cell line was from the NIH Reference and Reagent Program (Cat No 8129) and the 293T cell line was obtained from the American Type Culture Collection. These two cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS). Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA. Recombinant GRFT, CV-N and SVN were purified from *E. coli* at the National Cancer Institute, MD, USA (Bokesch et al., 2003; Boyd et al., 1997; Mori et al., 2005). The PGT and b12 antibodies were kindly provided by Burton and Koff of the International AIDS Vaccine Initiative. VRC01 was obtained from the Vaccine Research Center (Bethesda, MD) while the soluble CD4 was a generous gift from Progenics Pharmaceuticals, Inc. (Tarrytown, NY).

Selection of GRFT, CV-N and SVN resistant viruses

One thousand TCID₅₀ of each HIV-1 subtype C infectious isolate were grown under escalating concentrations of GRFT, CV-N and SVN. Viruses were cultured in 2 mL of 4×10^6 peripheral blood mononuclear cells (PBMC), depleted of CD8⁺ T cells by means of RosetteSep CD8 depletion cocktail (StemCell Technologies, Vancouver, Canada). The starting concentrations of the lectins were the IC₅₀ (50% inhibitory concentration) for each virus. Cultures without lectins were included as experimental controls. All cultures were maintained in RPMI 1640 containing 20% FBS and IL-2 (0.05 μg/mL). Viruses were passaged every 7 days by transferring 500 μL of the previous culture into fresh CD8 depleted PBMC. The



Fig. 6. Amino acid sequence of isolated GRFT, CV-N and SVN resistant Du179 clones. GRFT (A), CV-N (B) and SVN (C) resistant Du179 clones gp120 sequence isolated by single genome amplification were aligned with their respective population sequences. The gray shading shows the presence of a potential mannose-rich glycosylation site, yellow shading indicates the site deletion while the red box shows the tip of the V3 loop (McCaffrey et al., 2004). Note that the glycan at position 393 is labeled as 392 in the text for comparison with other viruses.

Table 3
Sensitivity of Du179 lectin-resistant clones to neutralizing antibodies and sCD4.

Envelope	Entry inhibitors ^a IC ₅₀ (μg/mL) ^b (fold change)				
	b12	VRC01	PGT121	PGT128	sCD4
Control	0.7	0.60	0.065	0.18	4.0
GRFT clone 1	12.5 (18↓)	0.14 (4↑)	0.043	0.13	1.2 (3↑)
GRFT clone 7	5.8 (8↓)	0.10 (6↑)	0.049	0.20	1.8 (2↑)
CV-N clone 1	6.4 (9↓)	0.11 (5↑)	0.055	0.21	2.2 (2↑)
CV-N clone 2	13.9 (20↓)	0.15 (4↑)	0.057	0.07	1.5 (3↑)
SVN clone 2	2.6 (4↓)	0.31(2↑)	0.061	0.16	3.3

^a Concentration needed to inhibit HIV-1 infection by 50%.

^b Increases and decreases in sensitivity shown with arrows; only ≥2 fold changes are shown.

concentration of GRFT, CV-N and SVN was increased whenever the p24 antigen level in the lectin containing cultures was similar or higher than the control cultures without lectin. When p24 levels

dropped the lectin concentration was reduced. After every passage 500 μL aliquots of culture supernatants were stored at –70 °C for genotyping and neutralization assays.

HIV-1 neutralization assay in peripheral blood mononuclear cells

The PBMC neutralization assay was performed as described (Bures et al., 2000). Briefly, a three-fold dilution series of GRFT, CV-N, and SVN in 40 μL of RPMI 1640 containing 20% FBS and IL-2 (growth medium) was prepared in triplicate in a U-bottom 96-well plate. Five hundred TCID₅₀ of the HIV-1 isolate in 15 μL of growth medium was added to each well and the plate was incubated at 37 °C for 1 h. This was followed by the addition of 5 × 10⁵ cells/well/100 μL of phytohemagglutinin/IL-2 stimulated PBMC (PHA-PBMCs). After an overnight incubation, cells were washed 3 times with RPMI 1640 containing 20% FBS and resuspended in 155 μL of fresh growth medium. The culture supernatant was collected twice daily and replaced with an equal amount of fresh growth medium. The p24 antigen concentration in the virus control wells

Table 4
Change in IC₅₀ of mutant viruses compared to the corresponding wild type.

Pseudovirus ^b	IC ₅₀ (nM) (fold change) ^a	
	GRFT	SVN
Du179.14 (WT)	3.6 ± 0.6	9.8 ± 1.6
Du179/GRFT.R _M (N230T/T236M/N339D/N393S/N448K/400–403 aa deletion)	13.7 ± 0.6 ^b (14)	
Du179/SVN.R _M (N230D/N339K/N393D/N448I)		46.0 ± 1.6 (15)
Du151.2 (WT)	3.3 ± 0.2	14.0 ± 0.7
Du151/GRFT.R _M (N234S/S243G/S291Y)	10.5 ± 0.4 (13)	
Du151/SVN.R _M (N241K/N448D)		106.4 ± 39.2 (18)
Du422.1 (WT)	0.8 ± 0.3	7.4 ± 1.2
Du422/GRFT.R _M (N230T/D386N/ 398–402 aa insertion)	3.4 ± 0.8 (14)	
Du422/SVN.R _M (N339K/N448T/398–402 aa insertion)		117.5 ± 46.4 (16)
COT9.6 (WT)	2.7 ± 0.9	21.6 ± 7.5
COT9/GRFT.R _M (T341I/396–399 aa deletion)	9.8 ± 3.7 (13)	

The N339I mutation was not introduced in Du151.2 since this envelope clone lacked the glycan at position 339.

^a The concentration needed to inhibit HIV-1 infection by 50%. Fold change of IC₅₀ compared to WT is shown in brackets.

^b R_M indicates that resistance was generated by mutagenesis. The aa changes that were introduced by mutagenesis are shown in brackets.

was measured by ELISA using the Vironostika HIV-1 Antigen Microelisa System (Biomérieux, Beseind, the Netherlands). Levels of p24 in the lectin cultures were measured at the time-point corresponding to the early part of the linear growth period of the virus control (Zhou and Montefiori, 1997). The 80% inhibitory concentrations (IC₈₀) were calculated by plotting the lectin concentration versus the percentage inhibition in a linear regression using GraphPad Prism 4.0 and the transformation $Y = b + mX$.

HIV-1 envelope amplification and sequencing

HIV-1 RNA was extracted from frozen culture supernatants and reverse transcribed to cDNA using the Superscript III Reverse Transcriptase according to the manufacturer's instructions (Invitrogen, CA). For both the single genome amplification (SGA) and the total population amplification, the envelope gene PCR was carried out as described by Salazar-Gonzalez et al. (2008). The PCR products were gel purified using the Qiagen Gel Purification Kit according to the manufacturer's instruction (Hilden, Germany), sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and resolved on an automated genetic analyzer. Changes in the envelopes sequences were identified using Sequencher v.4.5 (Genecodes, Ann Arbor, MI), Clustal X (ver. 1.83) and Bioedit (ver. 5.0.9).

Generation of mutants env-pseudotyped virus stock

Glycosylation sites and amino acid deletions and insertions associated with GRFT, CV-N and SVN resistance were introduced in HIV-1 envelope clones using the QuikChange Site Directed Mutagenesis Kit (Stratagene, LaJolla, CA). Primers were designed to insert or remove potential glycosylation sites and indels in a stepwise fashion and were confirmed by sequencing as described above. HIV-1 pseudoviruses were generated by co-transfection of the Env and pSG3Δenv plasmids (Wei et al., 2003) into 293T cells using the Fugene transfection reagent (Roche Applied Science, Indianapolis, IN). This was followed by the quantification of the TCID₅₀ of each virus stock by infecting TZM-bl cells with serial 5-fold dilutions of the supernatant in quadruplicate in the presence of DEAE dextran (37.5 μg/mL) (Sigma-Aldrich, St. Louis, MO). After 48 h of culture, HIV-1 infection was measured using the Bright Glo™ Reagent (Promega, Madison, WI), according to the manufacturer's instructions. Luminescence was quantified in a Wallac 1420 Victor Multilabel Counter (Perkin-Elmer, Norwalk, CT) and

the TCID₅₀ was calculated as described elsewhere (Johnson and Byington, 1990).

Single cycle neutralization assay (TZM-bl assay)

The pseudovirus neutralization assay was carried out as described previously (Montefiori, 2004). Briefly, three-fold dilution series of GRFT, CV-N and SVN in 100 μL of DMEM with 10% FBS (growth medium) were prepared in a 96-well plate in duplicate. This was followed by the addition of 200 TCID₅₀ of pseudovirus in 50 μL of growth medium and the mixture was incubated for 1 h at 37 °C. Then 100 μL of TZM-bl cells at a concentration of 1×10^5 cells/mL in 10% FBS DMEM containing 37.5 μg/mL of DEAE dextran was added to each well and the plate was placed at 37 °C for 48 h. HIV-1 infection was evaluated by measuring the activity of firefly luciferase. Titers were calculated as the inhibitory concentration that causes 50% reduction (IC₅₀) of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after the subtraction of the background (wells without both the virus and the inhibitor).

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