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Aminosugar derivatives as potential anti-human immunodeficiency virus agents

(acquired immunodeficiency syndrome/glycosidase inhibitor/*N*-butyldeoxynojirimycin)

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ABSTRACT **Recent data suggest that aminosugar derivatives which inhibit glycoprotein processing have potential anti-human immunodeficiency virus (HIV) activity. These inhibitory effects may be due to disruption of cell fusion and subsequent cell–cell transmission of the acquired immunodeficiency syndrome (AIDS) virus. Free virus particles able to bind CD4-positive cells are still produced in the presence of these compounds with only partial reduction of infectivity. We now report a method to score in parallel both the degree of antiviral activity and the effect on cell division of aminosugar derivatives. We find that (*i*) the compounds 1,4-dideoxy-1,4-imino-L-arabinitol and *N*-(5-carboxymethyl-1-pentyl)-1,5-imino-L-fucitol partially inhibit the cytopathic effect (giant cell formation, etc.) of HIV and yield of infectious virus; (*ii*) the compounds *N*-methyldeoxynojirimycin and *N*-ethyldeoxynojirimycin reduce the yield of infectious HIV by an order of four and three logarithms, respectively; and (*iii*) one compound, *N*-butyldeoxynojirimycin, of the 47 compounds previously screened reduces infectious viral particles by a logarithmic order greater than five at noncytotoxic concentrations. In addition, long-term growth of infected cells in the presence of *N*-butyldeoxynojirimycin gradually decreases the proportion of infected cells, leading to eventual elimination of HIV from culture. This result suggests that replication is associated with cytolysis. The ability to break the cycle of replication and reinfection has important implications in the chemotherapy of AIDS.**

Acquired immunodeficiency syndrome (AIDS) can be caused by two distinct human immunodeficiency viruses (HIV-1 and HIV-2) (1, 2). Both viruses belong to the lentiviridae group of retroviruses and are closely related to the simian immunodeficiency viruses (2). Sequence analysis has revealed ≈40% homology between HIV-1 and HIV-2 (3).

One therapeutic strategy for the treatment of AIDS has been to develop compounds that interfere with replication of the virus. Because the envelope glycoproteins of HIV-1 and HIV-2 are heavily glycosylated, compounds that interfere with co- and posttranslational processing of glycoprotein gp120 and the transmembrane glycoprotein (gp41) may prevent viral entry into the cell. At least five groups of mutations have already been identified that affect the ability of the envelope glycoprotein to form syncytia with cells displaying the CD4 antigen (4). These data illustrate the complexity of events required for cell fusion and subsequent cell–cell transmission of virus. In addition, the mechanisms of viral penetration and uncoating are essentially unknown (5). Some or all of these mechanisms may require correct glycosylation of the glycoconjugates involved.

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The antiviral effects of castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) and deoxynojirimycin (DNJ) are thought to arise because of their ability to inhibit trimming glycosidases involved in the biosynthesis of the N-linked oligosaccharides on the envelope glycoprotein (6–8). Both the studies of Gruters *et al.* (9) and Walker *et al.* (10) found a sodium dodecyl sulfate (SDS)/PAGE mobility change of gp120 when HIV-infected cells were grown in the presence of castanospermine or DNJ. In addition, Walker *et al.* (10) reported a low cell-surface expression of envelope proteins when HIV-infected cells were grown in the presence of castanospermine. However, no difference in the level of virus-associated envelope glycoprotein was found, and virus produced in the presence of inhibitors could still bind to the CD4 receptor, although the binding constant was not quantitatively measured (10).

These early reports suggested complete inhibition of cell fusion in the presence of castanospermine and DNJ (9, 10). However, only a modest reduction in virion infectivity was found. Walker *et al.* (10) found that the infectivity of virions produced in the presence of castanospermine decreased by an order of only one logarithm, whereas Gruters *et al.* (9) found that infectivity decreased by an order of two logarithms. Both studies concluded that virus production was not impaired by the inhibitors as measured by reverse transcriptase or p24 antigen determination, data consistent with the hypothesis that these compounds disrupt a post-CD4 binding step necessary for cell fusion. The above data are also consistent with the idea that reduction in viral titers is secondary to a decreased cell-to-cell transmission of the virus (10). However, further studies concerning the mode of action of these compounds in intact cells are necessary due to the multiple effects of these sugar analogues (8).

We have found that of 47 aminosugar derivatives screened for anti-HIV activity in our study, only 5 compounds could significantly inhibit cytopathic effect (CPE) at concentrations that were not cytotoxic (11). Here we show that the compounds 1,4-dideoxy-1,4-imino-L-arabinitol (LAB) and *N*-(5-carboxymethyl-1-pentyl)-1,5-imino-L-fucitol (LFT) only partially reduced viral titers, whereas compounds *N*-methyldeoxynojirimycin (MeDNJ) and *N*-ethyldeoxynojirimycin (EtDNJ) significantly inhibited HIV replication. Moreover, only *N*-butyldeoxynojirimycin (BuDNJ) reduced the virus titer by an order of greater than five logarithms at noncytotoxic concentrations. Long-term culture of HIV-infected cells in the

Abbreviations: DNJ, deoxynojirimycin; MeDNJ, *N*-methyldeoxynojirimycin; EtDNJ, *N*-ethyldeoxynojirimycin; BuDNJ, *N*-butyldeoxynojirimycin; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; CPE, cytopathic effect; TCID, tissue culture infectious dose; LAB, 1,4-dideoxy-1,4-imino-L-arabinitol; LFT, *N*-(5-carboxymethyl-1-pentyl)-1,5-imino-L-fucitol.

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presence of BuDNJ gradually decreased the number of infected cells to a point where infectious HIV was no longer detectable. These data suggest that even with proviral DNA integration, HIV-infected cells probably undergo a lytic cycle and that long-term culture of infected cells in the presence of a drug such as BuDNJ could break the cycle of HIV replication and infection. This gives hope that compounds of this type might reduce or even eliminate HIV infection *in vivo* in patients with latent infection or overt disease.

MATERIALS AND METHODS

The T-cell line (Karpas 45) was established from a child with acute lymphoblastic leukemia (12). HIV-1 and HIV-2 were both isolated in Cambridge—HIV-1 from a British patient with AIDS (13) and HIV-2 from a West African patient with AIDS (A.K., unpublished work). Cell-free suspensions of HIV were prepared from infected cultures. The concentration of infectious particles [tissue culture infectious dose (TCID)] was estimated using an end-point titration in which the number of infectious HIV particles in each preparation was determined by the highest dilution that contained infectious HIV, as detected by syncytial formation, cytopathicity (1, 15), and HIV-antigen synthesis (16) after 10 days of culture with 10^4 T-45 cells.

The efficacy of the compounds used in this study was assessed as follows: 0.2 ml of culture medium containing 10^4 T-45 cells were transferred into each well of a 96-well flat-bottomed tissue-culture plastic plate, and the cells were allowed to settle at 37°C (see Fig. 1). After 4 hr the media were aspirated and replaced with media that contained the compound under study. After overnight incubation the media were aspirated, and 10^4 TCID of virus (HIV-1 or HIV-2) was added to each well. Incubation was continued at 37°C in 5% CO_2 for 1 hr. Thereafter, growth media containing the various compounds were added, and incubation at 37°C was continued. Control cultures were maintained as indicated in Fig. 1.

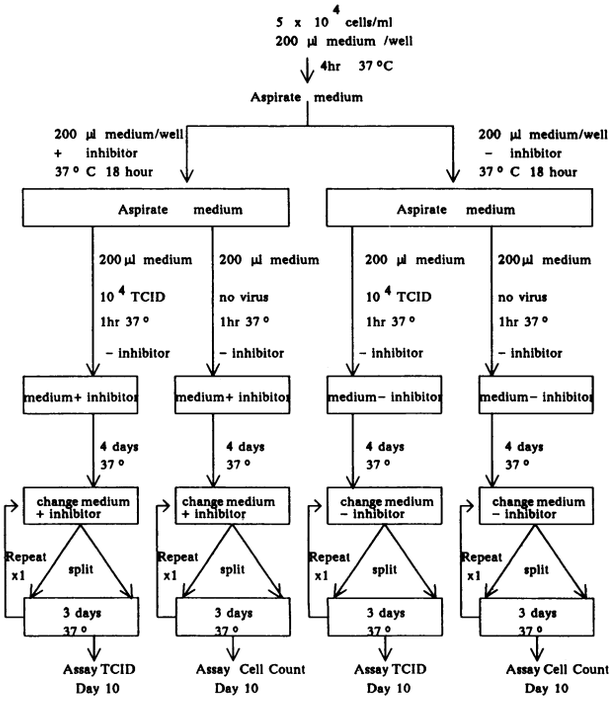

FIG. 1. Schematic outline of the strategy to assess the cytotoxic effect of the drugs on infected and noninfected T-45 cells and to assess in parallel the antiviral activity. TCID has been defined in text. Cells were cultured in RPMI 1640 medium with 10% fetal calf serum.

On the fourth day the cell suspensions from each well were split and seeded into two new wells, and 0.2 ml of fresh medium with compounds was added; this procedure was repeated on the seventh day. Cells were examined by microscopy during days 1–10 for syncytia, growth rate, and the appearance of CPE (giant cells, pyknotic nuclei, loss of refractility, etc.). CPE (100%) was defined as the absence of round, refractile, uniform cells; only giant, ballooned, pycnotic cells were present. The viability of HIV-infected cultures was determined by a rough count/estimate of cells that underwent CPE in relation to round refractile uniform (normal) cells in a given culture. Cells in wells that were incubated with compounds that appeared to inhibit or reduce HIV replication and in which there was marked cell proliferation were either transferred to larger wells (of a 24-well plate) or divided to maintain an approximately constant cell density to promote continuous cell division.

To determine whether a gradual reduction of HIV-infected cells occurred in cultures maintained with BuDNJ, aliquots of cells infected with 10^4 TICD of HIV-1 and grown in the presence of BuDNJ at 0.1 mg/ml for various durations were transferred to separate wells and grown in drug-free medium as indicated in Fig. 2. The cultures were then monitored for development of CPE, indicative of active HIV replication. The times for the appearance of an advanced CPE were recorded. To confirm that HIV-1 or HIV-2 caused the CPE, cells were fixed on glass slides to verify expression of the corresponding viral antigen by a described method (16).

Compounds were purchased, isolated, or synthesized as reported elsewhere (11).

RESULTS

The effect of various concentrations of MeDNJ, EtDNJ, castanospermine, BuDNJ, LFT, and LAB, respectively, on CPE formation are shown in Fig. 3 (for structures, see Fig. 4). The data in Fig. 3 show a bell-shaped dose dependence for

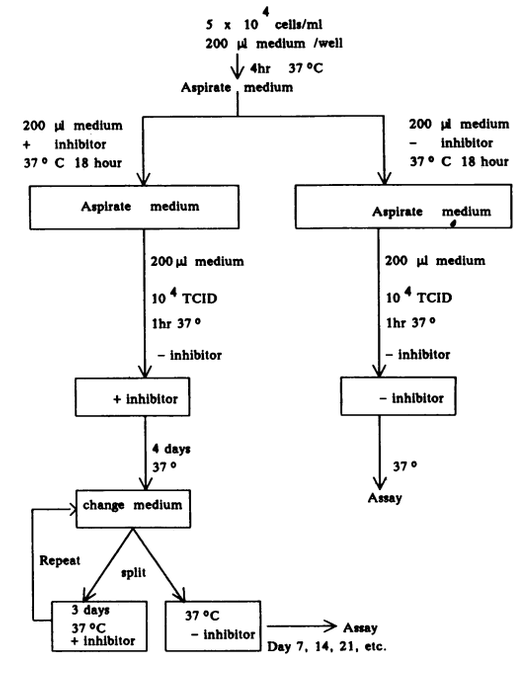

FIG. 2. Strategy to determine the relative proportion of HIV-infected cells in cultures containing BuDNJ at 0.1 mg/ml. At various times aliquots of the infected-cell suspensions were transferred to drug-free medium, and the time taken for the cells to show the CPE was recorded.

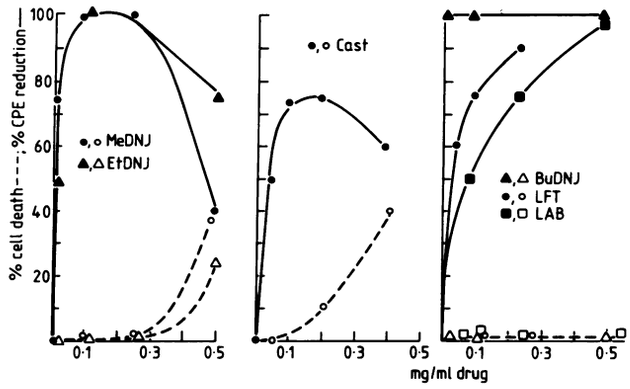

FIG. 3. Plot of the inhibition of HIV-associated CPE and drug-associated cytotoxicity versus medium concentration of various aminosugar derivatives. —, % CPE reduction; ---, % cell death. Cell viability data were determined for non-HIV infected cells. Cast, castanospermine.

MeDNJ, EtDNJ, and castanospermine. The cell-viability data shown in Fig. 3 and growth rates in Table 1 for noninfected cells grown in the presence of MeDNJ, EtDNJ, and castanospermine suggest that these compounds lack selective antiviral activity and are cytotoxic, as may be expected for inhibitors of oligosaccharide biosynthesis. The apparent loss of antiviral activity (bell-shaped dose dependence) of these drugs at high concentrations arises from the CPE of the drugs mimicking and being scored as an HIV-induced CPE. In contrast, the compounds BuDNJ, LFT, and LAB showed no cytotoxicity over the concentration range used. BuDNJ completely prevented CPE in the HIV-infected cells at all concentrations tested. The difference in behavior between BuDNJ and the other alkyl analogues of DNJ, MeDNJ, and EtDNJ, suggests that its mechanism of action differs. Similarly the α-fucosidase inhibitor LFT, which doesn't disrupt oligosaccharide biosynthesis, was not found to be cytotoxic. The properties of LAB resembled those of LFT. Activity of LAB as an α-glucosidase inhibitor has been reported (11) but has yet to be tested for activity against the processing α-glucosidases (i.e., I and II).

Quantitative data on the effect of the various compounds on noninfected cells (cytotoxicity) were obtained by comparing their growth rate to control cells not exposed to drugs (Table 1). Fig. 5 and Table 2 relate the TCID titer of culture supernatants of T-45 cells infected with HIV after 10 days in culture to drug concentration. These data show that LAB and LFT could only partially reduce the TCID, even at very high drug concentration. Similarly, DNJ, MeDNJ, and EtDNJ

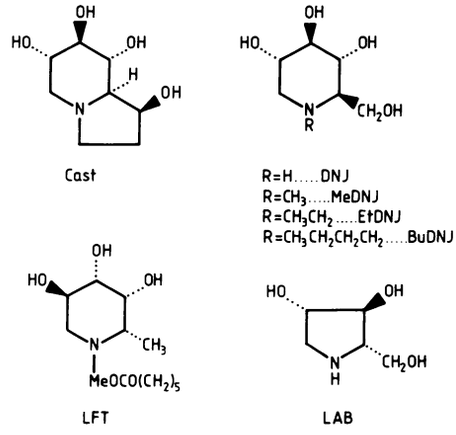

FIG. 4. Structures of drugs. Cast, castanospermine.

Table 1. Cytotoxicity and T-cell growth

Compound	Dosage, mg/ml	Estimated cell growth at virus-uninfected-day 7
No drug		1.2×10^6
DNJ	0.50	1.4×10^6
	0.25	1.4×10^6
MeDNJ	0.50	5.0×10^5
	0.25	1.0×10^6
	0.10	1.0×10^6
	0.05	1.2×10^6
	0.01	1.2×10^6
EtDNJ	0.10	1.3×10^6
	0.05	1.2×10^6
	0.01	1.2×10^6
BuDNJ	0.10	1.4×10^6
	0.05	1.2×10^6
	0.01	1.2×10^6
LAB	0.50	1.0×10^6
	0.25	1.5×10^6
	0.10	1.5×10^6
Castano-spermine	0.70	Toxic
	0.35	8.0×10^5
	0.18	8.0×10^5
	0.09	1.0×10^6
	0.02	1.2×10^6

only partially reduced the TCID, although to a greater extent than LAB and LFT. The inability of any of these drugs alone to totally reduce the TCID may be due to heterogeneity of virus production or spread. Figure 5 and Table 2 show that only BuDNJ could achieve negligible virus TCID titers at noncytotoxic concentrations. These data together with the data of Fig. 3 suggest a viral-specific activity for this compound. Table 2 also shows the results of a direct side-by-side comparison of the antiviral activity (reduction in TCID) of DNJ and its derivatives against HIV-1 and HIV-2. In addition, the MOLT-4 cell line was also used. These data show that similar antiviral activity is found against HIV-1 and HIV-2 and that this activity is not restricted to virus grown in the T-45 cell line.

To determine whether or not there was an actual reduction in HIV-infected cells with BuDNJ, aliquots of the infected cell suspension, grown for varying times with the drug, were transferred to drug-free medium as outlined in Fig. 2. The cultures were then monitored for the development of CPE and giant cell formation (indicative of active HIV replication). The increase in time taken for cells to develop CPE once drug-free medium was used (Fig. 6) suggests that prolonged exposure to BuDNJ reduced the proportion of infected cells in the cultures. Whether this result is viewed either as a reduction in the doubling time of the infected cells as compared with uninfected T-45 cells or as viral replication

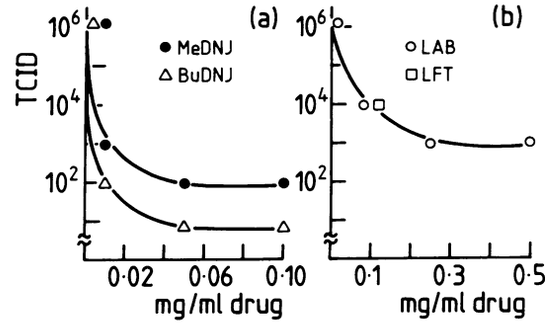

FIG. 5. Relation between TCID titer and drug concentration.

Table 2. Comparison of DNJ derivatives

Virus	Dosage, mg/ml	HIV-1, TCID	HIV-2, TCID
Control		10^6	10^6
DNJ	0.10	10^5	10^5
MeDNJ	0.10	10^2	10^2
EtDNJ	0.10	10^3	10^3
BuDNJ	0.10	<10	<10

Identical results were found when 10^4 T-45 cells or MOLT-4 cells were treated with DNJ and its three derivatives before infecting the cells with HIV-1 and separately with HIV-2 (10^4 TCID). The HIV-1 that was used to infect the T-45 cell line was first passaged in T-45 cells; likewise HIV-1 that was used to infect MOLT-4 was first passaged in MOLT-4 cells. The same procedure was done with HIV-2. TCID values were measured after day 10.

with cytolysis does not alter the conclusion that natural turnover of the cell population *in vivo* would eventually reduce the number of infected cells dramatically and possibly break the cycle of reinfection. Future experiments will be necessary to determine the relationship between CPE and the proportion of infected cells in culture.

DISCUSSION

To date no evidence has been presented that viruses encode in their genomes the enzymes required for biosynthesis of N-linked oligosaccharides (8, 17). Consequently, the host-cell glycosylation apparatus must be used to generate virions expressing species-specific and cell-type-specific oligosaccharides on the envelope glycoprotein. However, the finding that some viruses use posttranslational glycosylation (18) (i.e., not cotranslational) transfer to protein of nonglucosylated oligomannose oligosaccharides (8) or modify the kinetic properties of cellular sialyl- and galactosyltransferases (19) suggest that viruses might code for nonstructural factors. At present it is not known whether viral surface glycoproteins already embedded in the outer cell membrane before viral budding begins supply viral assembly (i.e., the biosynthesis of the ellipsoid glycoprotein knobs of HIV) (5). Consequently the distribution of oligosaccharides associated with the same envelope glycoprotein on the cell surface need not be the same as those on the virion surface. Indeed, some RNA viruses bud at intracellular sites, such as the Golgi or endoplasmic reticulum, rather than the plasma membrane. If the budded virus behaved analogously to a "secreted glycoprotein" rather than a "cell-surface" glycoprotein, then differential glycosylation would be predicted between plasma membrane gp120 and virus-associated gp120 (20).

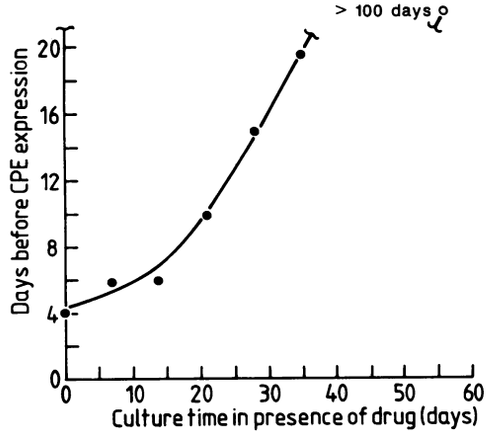

FIG. 6. Relationship between the time taken from HIV-infected cells grown with BuDNJ at 0.1 mg/ml to develop CPE once drug-free medium was used and the time of culture with BuDNJ. After 55-day culture (○) with BuDNJ no virus had yet appeared in 100 days of culture after BuDNJ removal.

The gp120 envelope protein of the HIV-1 virus contains 24 potential N-glycosylation sites, and half its molecular weight is due to attached carbohydrate (21). The envelope protein can bind directly to the CD4 surface glycoprotein of T lymphocytes, suggesting that the envelope protein is the viral-adhesion protein (22, 23). Purified gp120 is a potent inhibitor of this fusion, but nonglycosylated recombinant gp120 fragments, immunologically indistinguishable from gp120, are not (24). Matthews *et al.* (25), using cell-surface-derived gp120 (i.e., not virus), found that enzymatic deglycosylation reduced its ability to inhibit a cell–cell aggregation assay by 50-fold. The N-linked carbohydrates of CD4 are probably not involved in binding gp120, because soluble recombinant forms of CD4 lacking glycosylation sequons still powerfully inhibit HIV infection *in vitro* (26). The above data suggested that the oligosaccharides of gp120 may be important in its highly specific interaction with CD4 and be responsible for the CD4-dependent HIV-1 cell tropism.

However, the physiological relevance of these data is unclear. For example, in the study by Matthews *et al.* (25), a 50-fold reduction in the binding constant estimated at $<10^{-9}$ M for gp120 to CD4 may not be physiologically significant, considering the multivalency of the interaction. Recent data support the idea that the oligosaccharides of gp120 do not act as ligands in the gp120–CD4 interaction. In particular, both Walker *et al.* (10) and Gruters *et al.* (9) reported that a gp120 glycosylation variant, produced in the presence of castanospermine or DNJ, still binds to CD4 receptors. These data suggest that the effects of these inhibitors may arise from inhibition of the biological activity of either gp41 or gp120. This conclusion is consistent with the general literature in which oligosaccharides have been shown to be important in modifying activity of the attached protein, rather than being involved in simple ligand–receptor interactions (17). Indeed, results similar to those reported here for HIV have previously been reported for herpes simplex virus. Synthesis, transport to intracellular localization of virus budding, and proper function of the viral cell-attachment component of herpes simplex virus were found not to depend on N-linked oligosaccharides. In contrast, fusion activity of herpes simplex virus glycoproteins was abolished by the absence of N-linked glycans (8).

The recent report (27) showing that gp120 binding to the CD4 receptor is accompanied by activation of protein kinase C and phosphorylation of CD4 may be analogous to the case for the binding of the glycohormone human chorionic gonadatrophin to its receptor. Deglycosylated human chorionic gonadatrophin still binds to its receptor, but without biological activity (i.e., no activation of adenylate cyclase) (28). The reported decrease of viral infectivity produced with castanospermine or DNJ may arise from a similar mechanism.

The antiviral effects of the α-fucosidase inhibitors reported separately (11) and in particular (LFT) (Figs. 3 and 5) may result from disruption of the virion uncoating stage, which could involve lysosome action (5, 29). Metabolic labeling experiments have shown that gp120 contains little, if any, fucose. In contrast, gp41 contains fucosylated oligosaccharides (Francois Clavel, personal communication). Cell-associated gp41 has recently been shown to be essential for cell fusion (14).

Further, the action of many of these compounds may not be straightforward (8). For example, in intact cells DNJ exerts its action on both α-glucosidase I and α-glucosidase II, but DNJ preferentially inhibits α-glucosidase II. In contrast, in intact cells MeDNJ mainly inhibits the action of α-glucosidase I (8). Further, the biological effects of DNJ may not necessarily be related to the inhibition of trimming glucosidases but due in part to a general inhibition of the

synthesis of lipid-linked oligosaccharides (30) or its broad spectrum of inhibitory activity against glycosidases (31). Castanospermine is an even less selective inhibitor of glycosidases than DNJ (31). In addition, castanospermine inhibits β-glucocerebrosidase, which has been proposed to explain the toxicity of this compound, because a genetic insufficiency of the latter enzyme is a characteristic of Gaucher disease (32). The downstream effect of the glycosidase inhibitors may also show subtle differences. For example, swainsonine allows sulfation and fucosylation to occur, with the processing of some oligosaccharide chains to hybrid structures, whereas castanospermine, which blocks at an earlier point, does not allow sulfation and fucosylation (33, 34). The importance of oligosaccharide sulfation is suggested by sulfate esters on the oligosaccharide moieties of many viral envelope glycoproteins (e.g., influenza, Sendai) (35, 36).

Inhibition of syncytia formation has been previously used as an end point to quantitate HIV replication. With the drugs tested here, at least one cycle of HIV replication occurs; consequently, even a biologically defective virus could induce some syncytia formation. In addition, aminosugar derivatives could inhibit syncytia formation by binding directly to either viral or host-cell carbohydrate receptors without any effect on viral replication. Likewise, an HIV glycosylation variant could still be antigenically recognized by polyclonal antisera, and as a consequence HIV-antigen determination would probably not reveal which drug produces an optimal inhibitory effect.

Low TCID values (<10) were found when concentrations of MeDNJ, EtDNJ, and castanospermine exceeded 0.25 mg/ml, suggesting that these compounds have potent antiviral activity (data not shown). However, it is evident from the drug titrations in Fig. 3 that these low titers, at concentrations >0.25 mg/ml, result from drug cytotoxicity. In addition, we could not disassociate the cytotoxic effect of 2,5-dideoxy-2,5-D-mannitol (DMDP) from its antiviral effects (11) in contrast to an earlier report (37), suggesting the drug had potent anti-HIV activity. Such information emphasizes why determination of concentration dependency of drug cytotoxicity and viral titer must always be done in parallel, as described.

That BuDNJ reduces the proportion of cells harboring infectious HIV suggests that infected cells eventually undergo cytolysis. Moreover, if BuDNJ were found as effective *in vivo* as *in vitro*, it could possibly break the cycle of infection; namely, it could eventually "clear" the virus from an infected individual.

Finally, the production of infectious virus particles in cell culture only measures part of the viral "life cycle." Terminal glycosylation of N-linked oligosaccharides and synthesis of O-linked oligosaccharides of viral glycoproteins may be important for viral spread and pathogenesis (see ref. 8 *op cite*). More research is needed to determine the precise mechanism of action of these classes and the critical structure–activity relationships for the development of nontoxic drugs that will break the cycle of viral replication and spread in chronic infections such as AIDS.

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