The α(1,2)-mannosidase I inhibitor 1-deoxymannojirimycin potentiates the antiviral activity of carbohydrate-binding agents against wild-type and mutant HIV-1 strains containing glycan deletions in gp120

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Abstract Exposure of carbohydrate-binding agents (CBAs) (i.e. the mannose-specific plant lectins *Hippeastrum hybrid* agglutinin and *Galanthus nivalis* agglutinin) to HIV-1 progressively select for mutant HIV-1 strains that contain N-glycan deletions in their envelope gp120. This results in resistance of the mutant virus strains to the CBAs. Exposure of such mutant virus strains to the α(1,2)-mannosidase I inhibitor 1-deoxymannojirimycin (DMJ) results in an enhanced suppression of mutant virus-induced cytopathicity in CEM cell cultures. Moreover, when combined with CBAs at concentrations that showed poor if any suppression of mutant virus replication as single drugs, a synergistic antiviral activity of DMJ was observed. These observations argue for a combined exposure of CBAs and glycosylation inhibitors such as DMJ to HIV to afford a more pronounced suppression of virus replication, prior to, or during, CBA resistance development.

1. Introduction

The majority of enveloped viruses contains multiple glycans on their envelope proteins. In some cases (i.e. human immunodeficiency virus, HIV) [1], hepatitis C virus (HCV) [2], coronaviruses (CoV) [3], influenza virus (INF) [4]), the envelope is extensively glycosylated. The gp120 envelope of HIV is among the most heavily glycosylated proteins known [5]. Protein glycosylation may serve multiple functions, including proper folding of the nascent peptide, avoiding peptide precipitation due to the presence of lipophylic amino acid domains in the protein, protection against breakdown by proteases, increasing molecular diversity, and last but not least, in some cases, escape of immune surveillance [6]. After the glycan building block $(GlcNAc)_2Man_9Glc_3$ has been added to asparagines of the native peptide that are part of a N-glycosylation motif (NXS/T), the N-glycans are processed by α-glucosidases to remove the terminal three glucoses in the endoplasmatic reticulum (ER). Then, ER and Golgi class I α1,2-mannosidases specifically hydrolyze α1,2-mannose residues, and catalyse the trimming of the high-mannose chains involving four α1,2-linked mannose residues, and this process generates $Man_5GlcNAc_2$. Subsequent action of GlcNAc transferase I initiates complex chain formation and yields the substrate for Golgi α-mannosidase II which trims the terminal α1,3- and α1,6-mannose residues [7]. Further processing events in the Golgi apparatus eventually lead to glycans that consist of a wide variety of carbohydrates and combinations thereof [7–10]. Since mammalian viruses use the host cell glycosylation machinery for glycan synthesis and modification of the glycans that need to be incorporated in their envelope glycoproteins, it has been suggested that it is possible to target the viral envelope glycoproteins by inhibiting certain host-cell glucosidases at low levels that do not affect host-cell viability [5]. The altered glycan structures on the viral envelope proteins may then result in decreased viral infectivity (fitness), virus assembly and/or virus particle release [5]. HIV infectivity has indeed shown to be suppressed in cell culture when the virus was propagated in the presence of the α-glucosidase inhibitor NB-DNJ [11]. The latter drug has been evaluated in phase II clinical trials as an anti-HIV therapeutic [12]. For hepatitis B virus (HBV), it was demonstrated that NN-DNJ (and also to a minor extent NB-DNJ) disrupted the proper folding and efficient release of the viral envelope molecules. It was shown that NB-DNJ could reduce virus levels in a dose-dependent manner [13]. Since the E1 and E2 transmembrane glycoproteins of HCV are important for host cell entry [14], and since proper folding is calnexin-dependent [15], glucosidase inhibitors may also be expected to affect HCV entry and infectivity.

Recently, we have shown that carbohydrate-binding agents (CBA) are able to force HIV-1 to delete part of the glycans on its gp120 envelope in an attempt to escape drug pressure [16–19]. Such mutant virus strains display different degrees of phenotypic (in)sensitivity to the CBA's antiviral activity depending the number and the nature of the glycans that were deleted in gp120. In this study, we wanted to investigate whether the concomitant combination of CBAs and the glycosylation inhibitor 1-deoxymannojirimycin (DMJ) against

wild-type and mutant (glycan-deleted) gp120-containing HIV-1 strains could afford a superior antiviral activity than when added as single drugs. DMJ was used because it selectively inhibits α1,2-mannosidase I resulting in the accumulation of high-mannose glycans on the viral envelope glycoprotein. We found a significantly increased sensitivity of the mutant virus strains to the inhibition by DMJ, and a marked potentiation of the antiviral efficacy of CBAs when co-administered with DMJ, both for wild-type and mutant virus strains.

2. Materials and methods

2.1. Test compounds
The mannose-specific plant lectins from *Galanthus nivalis* (GNA) and *Hippeastrum hybrid* (HHA) were derived and purified from these plants, as described before [20,21]. DMJ was obtained from Sigma–Aldrich (St. Louis, MO) and from Calbiochem (VWR International, Haasrode, Belgium).

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

2.3. Viruses
HIV-1(III_B) was provided by Dr. R.C. Gallo and Dr. M. Popovic (at that time at the National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD). The mutant virus strains were obtained and characterized as described before [22].

2.4. Antiretrovirus assays
The methodology of the anti-HIV assays has been described previously [16,17]. Briefly, CEM cells (4.5×10^5 cells per ml) were suspended in fresh culture medium and infected with HIV-1 at 100 CCID_{50} per ml of cell suspension. Then, 100 μl of the infected cell suspension were transferred to microplate wells, mixed with 100 μl of the appropriate dilutions of the test compounds, and further incubated at 37 °C. After 4–5 days, giant cell formation was recorded microscopically in the CEM cell cultures. The 50% effective concentration (EC_{50}) corresponds to the compound concentrations required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures. In the drug combination experiments, DMJ was added to the cell cultures prior to the addition of the CBA and virus infection of the drug-exposed cells. The proper control experiments in which only one of the drugs or none of the drugs were present, were carried out under similar experimental conditions. Data of representative experiment were shown in the figures.

3. Results

3.1. Antiviral effect of the glycosylation inhibitor DMJ and the CBAs HHA and GNA against wild-type and mutant HIV-1 strains
The antiviral activity of the α(1,2)-mannosidase I inhibitor DMJ and the mannose-specific plant lectins HHA and GNA was investigated against wild-type HIV-1(III_B) and three mutant HIV-1(III_B) strains that contain a variety of 7–8 glycan deletions in their envelope gp120 (Table 1). DMJ did not suppress HIV-1(III_B)-induced cytopathicity in CEM cell cultures at a concentration as high as 500 μM. However, when DMJ was evaluated for its antiviral activity against the mutant virus strains, it had gained, as such, measurable antiviral efficacy. DMJ was inhibitory at an EC_{50} that ranged between 90 and 155 μM against the mutant virus strains. Thus, the cytopathic activity of the mutant virus strains was invariably suppressed by DMJ (Table 2). In contrast, the CBAs HHA and GNA that showed EC_{50} values as low as 0.28 and 0.16 μg/ml against wild-type HIV-1(III_B), respectively, markedly lost their pronounced suppressive activity against the three mutant virus strains (EC_{50}: 58–500 μg/ml) (Table 2). Thus, the deleted glycans in HIV-1 gp120 clearly compromised the antiviral activity of the CBAs.

3.2. Antiviral effect of CBAs in combination with DMJ against wild-type HIV-1 in CEM cell cultures
The effect of 250 and 100 μM DMJ on the inhibitory activity of the CBAs HHA and GNA against wild-type HIV-1 replication in CEM cell cultures was investigated (Fig. 1). As already mentioned above, DMJ was not inhibitory against HIV-1-induced cytopathicity at the concentrations used (250 and 100 μM). In contrast, HHA (Fig. 1A) and GNA (Fig. 1B) as single drugs completely prevented HIV-1-induced CPE in the CEM cell cultures at concentrations as low as 0.8 μg/ml. At 0.16 μg/ml, HHA and GNA were ~25% and 50% inhibitory, respectively. At 0.032 μg/ml, no residual inhibitory effect of the CBAs was observed. Interestingly, co-administration of DMJ to HHA and GNA markedly potentiated the antiviral

Table 1
Glycan deletions present in the gp120 envelope of mutant HIV-1 strains[a]

Position of the N-glycan amino acid deletion	Nature of the glycan[b]	Mutant virus strain		
		HIV-1/GNA500(CS)	HIV-1/HHA500(SN)	HIV-1/HHA500(CS)
88	C	±	+	−
197	C	−	+	−
230	M	+	−	+
234	M	+	+	+
276	C	−	−	±
289	M	±	+	−
295	M	−	+	+
301	C	+	−	+
332	M	−	+	+
339	M	+	+	+
386	M	−	+	−
392	M	+	−	+

[a]Glycan deletions at the N-glycosylation sites in gp120 (indicated as +) as determined in ref. 22. The "−" notation refers to the presence of the (glycan containing) wild-type sequence. The "±" notation refers to the presence of a mixture of the wild-type and mutated sequence in the virus isolate.
[b]C: complex-type glycan, M: high-mannose type glycan [19].

Table 2
Antiviral activity of 1-deoxymannojirimycin (DMJ) and the CBAs HHA and GNA against wild-type and mutant HIV-1 strains

Compound	EC$_{50}$ (µg/ml)[a]			
	HIV-1/WT	HIV-1/GNA500(CS)[b]	HIV-1/HHA500(CS)[b]	HIV-1/HHA500(SN)[b]
DMJ[c]	>500	90 ± 60	155 ± 141	103 ± 80
HHA	0.31 ± 0.13	67 ± 31	125 ± 35	127 ± 59
GNA	0.45 ± 0.26	103 ± 45	62 ± 36	153 ± 46

[a]50% Effective concentration or compound concentration required to inhibit virus-induced cytopathicity in CEM cell cultures by 50%.
[b]Mutant HIV-1 strains containing a variety of glycan deletions in gp120 as shown in Table 1.
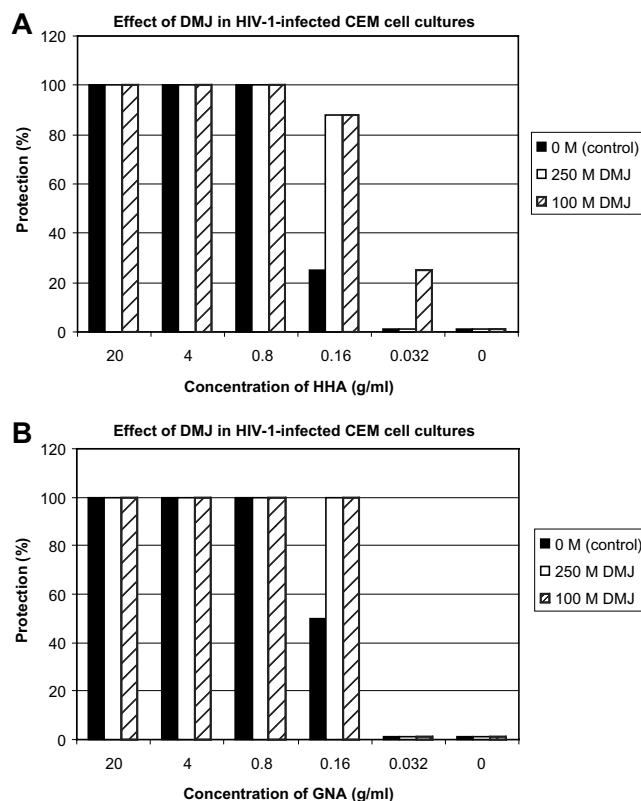[c]Data expressed in µM.

Fig. 1. Effect of 1-deoxymannojirimycin on the antiviral effect of HHA and GNA against HIV-1-infected CEM cell cultures.

efficacy of these CBAs. The inhibitory activity of 0.16 µg/ml HHA against HIV-1 increased from 25% to 90% in the presence of DMJ, and from 50% to 100% upon co-administration of DMJ with 0.16 µg/ml GNA. At lower HHA and GNA concentrations, no pronounced antiviral activity was noticed for the CBAs, neither in the absence, nor in the presence of DMJ (Fig. 1).

3.3. Antiviral effect of CBAs in combination with DMJ against mutant HIV-1 strains in CEM cell cultures

Three different HIV-1 strains that were shown to contain several N-glycan deletions in their gp120 envelope (Table 1) were exposed to GNA and HHA in the presence of a variety of DMJ concentrations. When 50, 20 and 8 µM DMJ was combined with GNA (Fig. 2A–C), DMJ acted synergistically in combination with these CBAs. For example, 4 and 20 µg/ml GNA that showed poor, if any, antiviral efficacy against HIV-1/GNA-500(CS) (Fig. 2, panel A) and HIV-1/HHA-500(CS) (Fig. 2, panel B) became 100% protective against the virus-in-
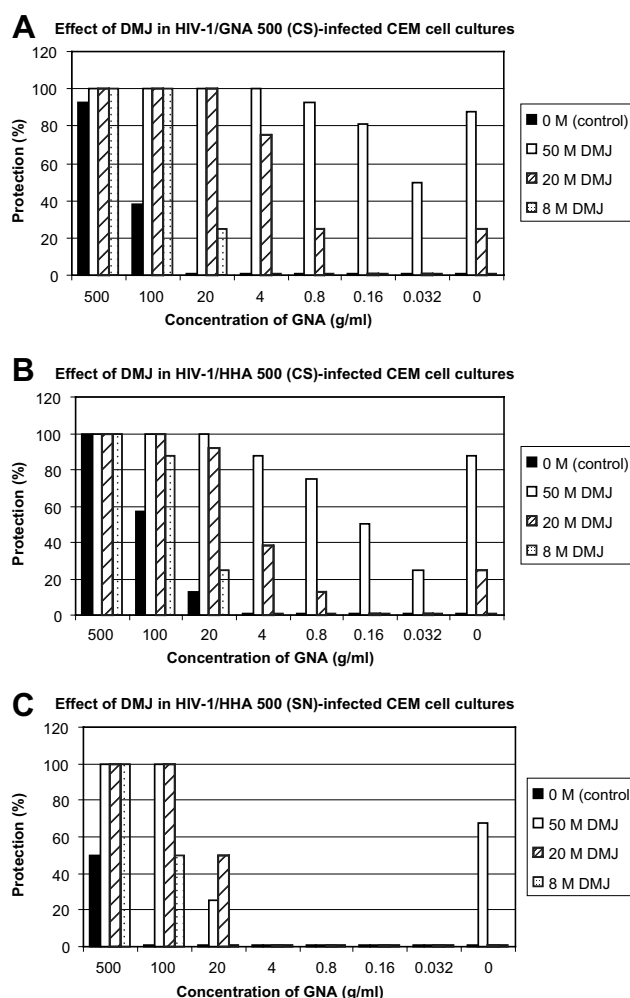

Fig. 2. Effect of 1-deoxymannojirimycin on the antiviral effect of GNA against mutant drug-resistant HIV-1 strains in CEM cell cultures.

duced cytopathic effect in the presence of 50 µM DMJ, and 40–100% protective in the presence of 20 µM DMJ. Such a synergistic effect was also seen for DMJ against HIV-1/HHA-500(SN) when GNA was administered at the higher concentration range (20–500 µM) (Fig. 2, panel C). A similar synergistic activity of DMJ was noted against the mutant virus strains when combined with HHA (Fig. 3, panels A, B and C).

Surprisingly, when the lower GNA and HHA concentrations (0.032–0.8 µM) were combined with DMJ, rather an antagonistic activity was observed. This phenomenon was consistently seen for both HHA and GNA, in the presence of the different DMJ concentrations (Figs. 2 and 3).
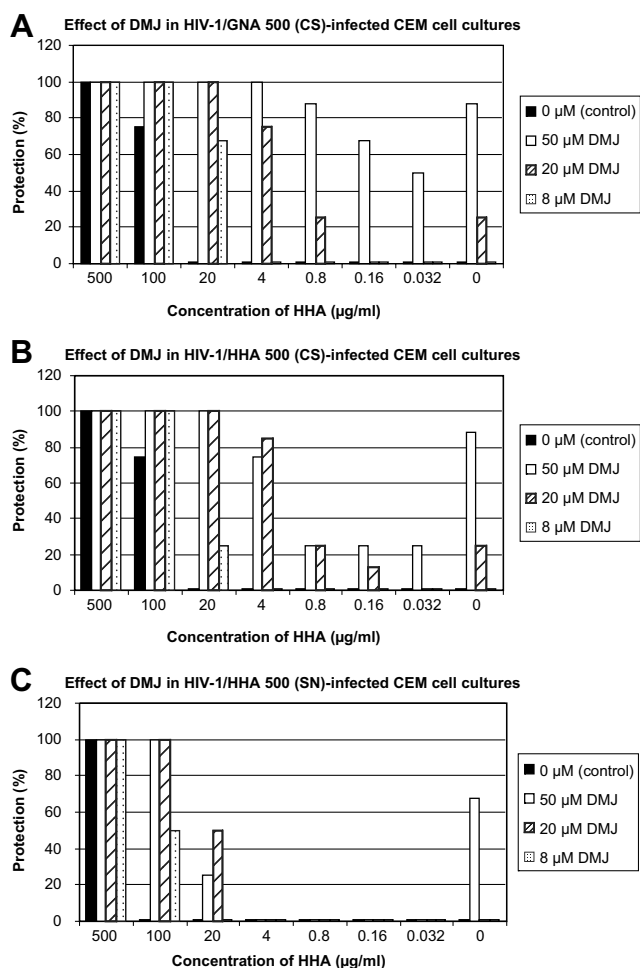
A

Effect of DMJ in HIV-1/GNA 500 (CS)-infected CEM cell cultures

B

Effect of DMJ in HIV-1/HHA 500 (CS)-infected CEM cell cultures

C

Effect of DMJ in HIV-1/HHA 500 (SN)-infected CEM cell cultures

Fig. 3. Effect of 1-deoxymannojirimycin on the antiviral effect of HHA against mutant drug-resistant HIV-1 strains in CEM cell cultures.

3.4. Cytostatic and antimetabolic activity of DMJ and CBA combinations in CEM cell cultures

Neither the CBAs (500 μM) nor DMJ (250 μM) proved inhibitory against CEM cell proliferation whether administered to the cell cultures as single drugs or combined (data not shown). The drug combinations also had no inhibitory effect on cell metabolism since radiolabeled thymidine, uridine and leucine incorporation into CEM cell DNA, RNA or proteins was not measurably affected.

4. Discussion

The glycosylation inhibitor DMJ targets the ER and Golgi $\alpha(1,2)$-mannosidase I that trims the $\alpha1,2$-mannose(s) from the $Man_9(GlcNAc)_2$ glycan after the ER α-glucosidases I and II have removed the three terminal glucose units from the N-glycan $Glc_3Man_9(GlcNAc)_2$ block [7,8]. As a result, the amount of high-mannose type glycan structures on the glycoprotein markedly increases in the presence of DMJ since further trimming/processing of the high-mannose glycans to hybrid- or complex-type glycans has been largely prevented by the DMJ-mediated blockade of the $\alpha1,2$-mannosidases I. Since the CBAs GNA and HHA are known to specifically bind to $\alpha(1,3)$-

and/or $\alpha(1,6)$-mannose oligomer structures [23], it could be reasoned that a higher amount of high-mannose type glycans on gp120 may allow these CBAs to concomitantly bind to a higher amount of glycans on the HIV-1 envelope. Consequently, they may exert a more pronounced antiviral activity in DMJ-treated virus-infected cells. We observed indeed a potentiation of the anti-HIV-1 activity of HHA and GNA in the presence of DMJ concentrations that exerted themselves no antiviral activity when used as a single drug. Thus, concomitant administration of glycosylation inhibitors (such as DMJ) and CBAs in HIV-1-infected cell cultures may further potentiate the antiviral activity of the mannose-specific CBAs.

Interestingly, whereas wild-type virus infection and replication efficiently proceed in the presence of high (i.e. 250 and 100 μM) DMJ concentrations (EC_{50} > 500 μM), the mutant HIV-1 strains that contain multiple deletions of N-glycans in gp120 gained sensitivity to the inhibitory activity of the $\alpha1,2$-mannosidase I inhibitor DMJ in the CEM cell cultures. Whereas DMJ was not effective at all at 500 μM against parent wild-type virus it could indeed inhibit mutant virus infection at an EC_{50} that ranged between 90 and 150 μM, that is at an at least more than 5–10-fold lower DMJ concentration. These mutant virus strains showed 7 or 8 glycan deletions at putative N-glycosylation sites in gp120 [22], and the deletions preferentially occurred at high-mannose type glycan sites (Table 1). Such glycan deletions in the mutant HIV-1 strains resulted in a marked phenotypic resistance to the HHA and GNA CBAs. It could be assumed that DMJ converts at least part of the remaining glycans of the mutant gp120 into high-mannose-type glycan structures, making them more vulnerable to interaction with the CBAs. Consequently, an increased antiviral activity would then be expected upon co-administration with DMJ, a phenomenon that we indeed observed to occur in the HIV-infected cultures. Interestingly, a similar phenomenon has been observed to occur when Pradimicin A, a high mannose-type glycan-binding antibiotic, was exposed to mammalian U937 cells that had been pretreated with DMJ [24]. Under these experimental conditions, the cells express high levels of high mannose-type oligosaccharides and become sensitive to PRM-A (resulting in apoptosis induction). No such apoptosis induction was observed in PRM-A-exposed cell cultures that were not pretreated with DMJ [24,25]. Thus, the combined use of CBAs and the $\alpha1,2$-mannosidase-inhibitor DMJ enabled partial restoration of the phenotypic sensitivity of the mutant HIV-1 strains against the CBAs. Our results argue for combined administration of CBAs and DMJ to wild-type virus, because phenotypic resistance development may be expected to slow down when DMJ is present during CBA treatment of HIV-1. The slight but consistently observed antagonistic activity that has been observed for DMJ when combined with the lowest CBA concentrations is rather puzzling and the molecular basis of this phenomenon is yet unclear.

There is, in general, a concern for the therapeutic application of inhibitors that target cellular enzymes such as the $\alpha1,2$-mannosidase I inhibitor DMJ. Indeed, inhibition of cellular glycosylation enzymes in virus-infected cells may not only compromise proper viral glycopeptide formation, but may also have deleterious effects on glycoproteins of non-infected cells. However, therapy with drugs, in casu glycosylation inhibitors, should not necessary aim to ablate enzyme activity but should rather be used to modulate enzyme activities involved in glycosylation [26]. Since the envelope gp120 glycoprotein of HIV is

among the highest glycosylated glycoproteins currently known and has a high (functional) requirement of high-mannose type glycans, it may be assumed that a moderate attenuation of α1,2-mannosidase I activity may have a more pronounced deleterious impact on the synthesis of the viral envelope glycoprotein than on the cellular glycoproteins, allowing for a certain degree of selectivity of such inhibitors. Proper in vivo experiments should reveal the therapeutic efficacy and feasibility of such drugs.

In conclusion, the α(1,2)-mannosidase I inhibitor DMJ was found to potentiate the inhibitory activity of CBAs against wild-type HIV-1. Administration of DMJ to cell cultures infected with mutant HIV-1 strains that contain N-glycan deletions in the gp120 envelope render the mutant virus susceptible to the inhibitory activity of DMJ. Moreover, DMJ can partially reverse the phenotypic resistance of CBAs to the mutant virus strains. These three phenomena may argue for further investigation of glycosidase inhibitors such as, but not limited to, DMJ to be used in combination with CBAs with the aim to further potentiate the antiviral activity of the CBAs and to delay resistance development that may develop under CBA drug pressure.

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