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Inhibition of Hepatitis C Virus by the Cyanobacterial Protein MVL: mechanistic differences between the high-mannose specific lectins MVL, CV-N, and GNA

Alla Kachko^{#1}, Sandra Loesgen^{#2}, Syed Shahzad-ul-Hussan^{2,3}, Wendy Tan¹, Iryna Zubkova¹, Kazuyo Takeda⁴, Frances Wells¹, Steven Rubin⁵, Carole A. Bewley², and Marian E. Major^{1,*}

¹Laboratory of Hepatitis Viruses, Division of Viral Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892 USA

²Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda Maryland 20892, USA

³Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda Maryland 20892, USA

⁴Microscopy and Imaging Core Facility, Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda, MD 20892. USA

⁵Laboratory of Method Development, Division of Viral Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892. USA

These authors contributed equally to this work.

Abstract

Plant or microbial lectins are known to exhibit potent antiviral activities against viruses with glycosylated surface proteins, yet the mechanism(s) by which these carbohydrate-binding proteins exert their antiviral activities is not fully understood. Hepatitis C virus (HCV) is known to possess glycosylated envelope proteins (gpE1E2), and to be potently inhibited by lectins. Here, we tested in detail the antiviral properties of the newly discovered *Microcystis viridis* lectin (MVL) along with cyanovirin N (CV-N) and *Galanthus nivalis* agglutinin (GNA) against cell culture HCV, as well as their binding properties towards viral particles, target cells, and recombinant HCV glycoproteins. Using infectivity assays, CV-N, MVL and GNA inhibited HCV with IC₅₀ values of 0.6 nM, 30.4 nM, and 11.1 nM, respectively. Biolayer interferometry analysis demonstrated a higher affinity of GNA to immobilized recombinant HCV glycoproteins compared to CV-N and MVL. Complementary studies, including FACS analysis, confocal microscopy and pre and post virus binding assays, showed a complex mechanism of inhibition for CV-N and MVL that includes both viral and cell association; while GNA functions by binding directly to the viral particle. Combinations of GNA with CV-N or MVL in HCV infection studies revealed synergistic inhibitory effects, which can be explained by different glycan recognition profiles of the mainly high-mannoside specific lectins, and supports the hypothesis that these lectins inhibit through different and complex modes of action. Our findings provide important insights into the mechanisms by which lectins inhibit HCV infection. Overall, the data suggest MVL and CV-N

*Corresponding Author: Marian.Major@fda.hhs.gov. Tel: 301-827-1881..

Supporting Information

Figure showing glycan array profiles. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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have the potential for toxicity due to interactions with cellular proteins while GNA may be a better therapeutic agent due to specificity for the HCV gpE1E2.

Keywords

hepatitis C; antiviral lectin; HCV entry inhibitor; cyanovirin-N (CV-N); HCVcc; biolayer interferometry

Introduction

Hepatitis C virus (HCV), which can lead to serious liver disease, is the most common chronic blood borne infection in the United States, with around 3 million adults infected. HCV establishes persistent infection, which is characterized by variable viremia and escape from immune responses through antigenic variation.¹ Cellular entry by the virus involves several host cell proteins including CD81,² scavenger receptor B1 (SR-B1),³ claudin1 (CLDN1),⁴ and occludin (OCLN)⁵ (reviewed by Meredith⁶) among other newly identified factors.^{7,8} Membrane associated C-type lectins, DC-SIGN and DC-SIGNR,^{9,10} as well as cell surface heparan sulfate proteoglycans mediate HCV-cell interactions stressing the physiological significance of carbohydrate recognition during infection.¹¹ Following receptor binding, virions are internalized via clathrin-mediated endocytosis,¹²⁻¹⁴ where the lower pH triggers fusion of viral and endosome membranes, depositing the nucleocapsid into the cytoplasm.¹⁵

The HCV envelope glycoproteins E1E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal hydrophobic anchor that act during the entry and fusion steps of the virus life cycle.¹⁶ Sequence analyses of E1 and E2 have identified 5 and 11 potential N-glycosylation sites, which are strongly conserved among different genotypes.^{16,17} The glycan portion of the E1E2 heterodimer comprises up to one third of its total molecular weight and effectively reduces the immunogenicity of envelope proteins.^{18,19} It has been shown that several of these glycans play an important role in HCV assembly and infectivity, and can mask access of neutralizing antibodies to epitopes presented on the surface of the viral particle.^{18,20,22} Thus, regions of gpE1E2 that are critical to the entry step of the HCV life cycle are highly conserved and can be an attractive target for antiviral drug therapy.

To date, we are still lacking a 3-dimensional structure of the HCV envelope protein, as well as a comprehensive chemical study of its glycan content. Carbohydrate binding agents are useful tools to probe the presence and functionality of carbohydrates on pathogens. Recently, it was reported that the mannose-specific cyanobacterial lectins cyanovirin-N (CV-N),²³⁻²⁵ microvirin (MVN),^{26,27} and *Microcystis viridis* lectin MVL,^{28,29} as well as the plant-derived lectin GNA³⁰ and algal lectin griffithsin^{31,32} efficiently neutralize human immunodeficiency virus (HIV) infection and prevent viral entry into host cells. Due to the presence of high-mannose glycans on HCV a similar approach has been used for investigating inhibitory activity of the lectins CV-N,³³ GNA,³⁰ and griffithsin³⁴ against HCV pseudoparticles (HCVpp) and HCV cell culture (HCVcc) virus. It was shown that these lectins inhibit HCV at μ M to nM concentrations and prevent HCV infection at early entry steps.

Among the potent anti-HIV lectins, the cyanobacterial lectin MVL has not been studied for its effect on HCV infection. MVL was identified from the fresh water bloom-forming cyanobacterium *Microcystis viridis* NIES-102.³⁵ Structural and biophysical studies showed that this novel 13 KDa protein contains two carbohydrate binding sites per monomer, exists

as a monodisperse dimer in solution, and lacks sequence homology to existing protein families.²⁸

Despite possessing similar or overlapping carbohydrate recognition profiles, not all lectins are able to inhibit HIV.^{36,37} An outstanding question in this field concerns the structural and functional requirements for potentially inhibiting enveloped viral entry via carbohydrate-mediated interactions. Here we sought to define some of these factors for HCV antiviral activity by performing complementary inhibition and binding studies with a carefully chosen group of lectins, including MVL, CV-N and GNA. Recent advances in glycan array technology and analysis have enabled the detailed description of the binding specificity of these lectins.^{38,39} Additionally, the number of binding sites, or valency, and the oligomeric states have been thoroughly characterized through 3-dimensional structures and biochemical and biophysical studies (Fig. 1A). In particular, MVL is known to bind with sub-micromolar affinities oligomannosides that contain the chitobiose core, exemplified by Man₃GlcNAc₂ and Man₆GlcNAc₂,^{28,29} while CV-N binds with high affinity to the Man α 1,2Man termini of Man₈GlcNAc₂ (Man-8) and Man₉GlcNAc₂ (Man-9)²⁴ (Fig. 1B, Supplement Figure 1). The plant lectin GNA has a different carbohydrate recognition profile, binding to mannose termini, as well as lactosamine structures that are present in hybrid-type and complex-type *N*-glycans and can be found at the termini of *O*-linked glycans (Fig. 1A, Supplement Figure 1). In addition to their carbohydrate specificities, these lectins differ in their quaternary structures and valency. CV-N contains two carbohydrate binding sites, which supports-2 site binding with the branched glycans Man-8 and Man-9; the homodimeric protein MVL possesses four binding sites in total; and GNA, which exists as a tetramer, contains 12 carbohydrate binding sites. The human anti-HIV antibody 2G12 exists as a domain-swapped dimer, and only recognizes clusters of Man α 1-2Man terminal structures (Figure 1B) with high affinity.^{40,41} 2G12 was included in this study as a reference agent.

In terms of binding mammalian glycoproteins, these binding profiles indicate that CV-N will only recognize Man-8 or Man-9, 2G12 will only bind to glycoproteins that display a cluster of Man-8 and/or Man-9 glycans, MVL may bind any oligomannoside from Man-3 to Man-9, and GNA may bind to a broader group of glycans.

By using an integrative approach involving HCV neutralization assays and binding and microscopy studies, we found that CV-N, MVL and GNA inhibit HCVcc infection through different mechanisms. CV-N and MVL appear to function by binding to both viral protein *N*-glycans and to glycans on cell surface molecules. In contrast GNA binds specifically to the glycans of the viral protein and does not appear to function by binding to cell surface proteins. These distinct differences in the mechanism of HCV inhibition by lectins provide insights into how the lectins may be useful for in vivo treatment or prevention of HCV infection.

EXPERIMENTAL METHODS

Lectin Production and Fluorescent Labeling

Recombinant CV-N and MVL were produced in *E. coli* and purified as reported previously.^{23,29} HIV mAb 2G12 was purchased from Polymun Scientific (Klosterneuburg, Austria) and GNA was purchased from Sigma-Aldrich (St. Louis, MO). All lectins and the mAb 2G12 were fluorescently labeled with AlexaFluor 546 for FACS analysis and confocal cell imaging following the manufacturer's instructions (Invitrogen, Carlsbad CA). Man₉GlcNAc₂ (Man-9) and mannobiose were purchased from QA-Bio (Palm Desert, CA) and Sigma-Aldrich (St Louis, MO), respectively. Glycan array data for each of the lectins used in this study are publicly available at the Consortium for Functional Glycomics (www.functionalglycomics.org).

HCVcc Production and Neutralization with lectins

Four different HCVcc chimeras were used in these studies, based on the JFH1 genotype 2a backbone.⁴² The J6/JFH1 construct was a kind gift from Dr. Charles Rice. The 1a, 1b (Accession number HQ110091) and 3a (Accession number JX826592) chimeras were generated as previously described.⁴³ IC₅₀ titers were determined by performing 2-fold dilutions of lectins or 2G12 (6000 ng/ml to 0.36 ng/ml) and 100 ffu of HCVcc as previously described.⁴³ Foci were counted using an automated counting system (Cellular Technology Limited) using BioSpot 5.0 software. Dose-response curves, dose-reduction and combination indices and IC₅₀ neutralization titers were determined using CalcuSyn 2.0 (www.biosoft.com).⁴⁴

For pre-binding studies, serially diluted lectins were added to Huh7.5 cells for 1 hour and washed 3 times with PBS before the addition of HCVcc. For post-binding studies, HCVcc was inoculated onto cells and incubated for 1, 2 or 3 hours at 4 °C and 37 °C. Unbound virus was removed by washing with complete DMEM three times before incubation with serial dilutions of lectins for 1 hour. Competition studies with glycan ligands were performed by adding Man₉GlcNAc₂ to lectins at the IC₅₀ concentration of each prior to adding to the neutralization assays.

Purification of Recombinant E1E2 protein

A recombinant adenovirus (rAd-E1E2T) was constructed expressing aa171-aa715 of GT 1a HCV (H77) with the carboxy terminus 6-HIS tag, using the AdEasy vector system (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Huh7.5 cells were infected with rAdE1E2T at an MOI of 10. After 24 hours the cells were harvested as previously described⁴⁵ and stored at -80 °C. The crude lysate was loaded onto a 1.5 mL His-Trap FF crude column (GE Healthcare) using an automated FPLC system (Äkta, GE Healthcare) and protein was eluted with an imidazole gradient (Tris 7.4 20 mM, NaCl 200 mM, imidazole 20 mM to 0.5 M). Fractions containing the protein were determined by western blotting, pooled and concentrated (Vivaspin 6, MWCO 3000). The integrity of the recombinant protein was tested in Western blot and ELISA with specific anti-E1 (A4) and anti-E2 (A11) MAbs (data not shown).

Biotinylation of Recombinant E1E2

His-tagged rE1E2 was biotinylated with Sulfo-NHS-LC-LC-Biotin according to the manufacturer's instructions (Thermo Scientific) and yielded 800 µL of 65 µg/mL biotinylated rE1E2.

Determination of Binding Affinities by Biolayer Interferometry

An Octet RED96 platform (ForteBio) was used to perform binding and kinetics experiments. Briefly, biotinylated rE1E2 protein was immobilized on streptavidin-coated sensor tips (ForteBio). After loading the target protein, sensors were moved to wells containing varying concentrations of the lectins and antibodies during the association phase, followed by dissociation in wells containing buffer only. All six analytes were prepared in dilution series and the binding experiments performed in duplicate. The curve from a buffer-only reference sensor (with immobilized rE1E2) was subtracted from all curves in a parallel-referencing process. Equilibrium dissociation constants (K_{DS}), and association (k_{on}) and dissociation (k_{off}) rates were determined by globally fitting the curves obtained for three different concentrations using the Octet Analysis Software (version 7.0)

FACS Analysis of Lectin Binding to Cells

Infected and uninfected cells were harvested using Accutase (Innovative Cell Technologies). Infected cells were generated following transfection of Huh7.5 cells with *in vitro* transcribed RNA representing the full length genome of HCVcc 1a/2a as previously described.⁴⁶ The cells were passaged every 3 days. After passage 5 the virus titer in the supernatant was determined to be 2.3×10^4 ffu/mL and the presence of positive cells was confirmed by fluorescence.⁴⁶ The cells were then used for analysis. For intracellular staining, 10^6 cells were permeabilized using the BD CytoPerm/Fix kit (BD Biosciences, CA) according to the manufacturer's instructions. Cells were incubated with AlexaFluor 546 labeled lectins diluted 1:100 for 60 minutes at 4°C. Cells were washed three times in PBS/1% bovine serum albumin and resuspended in 1% paraformaldehyde. Cells were analyzed using a FACSCalibur flow cytometer (at least 10^5 events were collected) and FlowJo software.

Confocal Microscopy of Lectin-Stained Cells

Nunc chamber slides were pretreated with poly-L-lysine hydrobromide (0.1 mg/mL) for 5 minutes at room temperature, washed twice with water and left to dry at room temperature for 60 minutes. Infected and uninfected Huh7.5 cells were seeded onto chamber slides 24 hours before use. For surface binding live cells were incubated with anti-E1 (A4) and anti-E2 (A11; Ab41) monoclonal antibodies (diluted 1:1000) for 60 minutes at 4°C followed by labeled lectins (diluted 1:100).^{47,48} AlexaFluor 488 goat anti mouse antibody (Molecular Probes, Oregon, USA) was used to visualize the anti-E1E2 antibody binding (30 minutes at 4°C). Cells were washed with PBS and fixed with 2% formaldehyde for 20 minutes at room temperature. For intracellular staining cells were treated with ice-cold isopropanol for 15 minutes at 4°C and stained as described above. Cells were visualized using a Zeiss Cell Observer SD confocal microscope.

Cell Viability Assays

A cytotoxicity assay was performed using the same concentrations of CV-N, MVL and GNA lectins employed during virus neutralization. The CellTiter-Glo Luminiscent Cell Viability Assay (Promega, Madison WI) was used according to the manufacturer's instructions. This method determines the number of viable cells in culture based on the quantification of ATP present, an indicator of metabolically active cells. Geneticin sulfate (G418) at a concentration of 1 mg/mL was used as a positive control to induce toxicity.

RESULTS

MVL, CV-N, and GNA Inhibit HCVcc Infection in Huh7.5 Cells

We evaluated the neutralizing activities of MVL on HCVcc chimeras displaying the envelope proteins from different HCV genotypes *in vitro*. For comparison we included a panel of known lectin inhibitors of HCV such as CVN and GNA as well as the HIV-antibody 2G12 as negative control. The viruses were incubated with increasing concentrations of MVL, CV-N, GNA and 2G12 for 1 hour and then inoculated onto Huh7.5 cells. We found dose-dependent inhibitory effects of MVL, CV-N, and GNA on virus infection irrespective of the envelope genotype displayed by the HCVcc (Figure 2). Based upon the molecular weight of each lectin the IC₅₀ values, the molar concentration of lectin needed to inhibit 50% of the virus, were calculated using Calcsyn 2.0 software (Table 1). 2G12 was non-inhibitory at the highest concentration tested (6 µg/mL). CV-N was found to be the most potent inhibitor with IC₅₀ concentrations ranging from 7 ng/mL (0.6 nM) to 21 ng/mL (1.9 nM) (Table 1). MVL and GNA were less potent; IC₅₀ values were more than 10-fold higher ranging from 325 ng/mL (14.1 nM) to 792 ng/mL (34.3 nM) and 575 ng/mL (11.1 nM) to 1325 ng/mL (25.5 nM), respectively (Table 1). As all HCVcc chimeras were

inhibited to a similar extent by the different lectins, we used the 1a/2a HCVcc chimera in all further studies.

Pretreatment with Man-9 Reverses the HCV Inhibitory Effect of the Lectins

It is known that these lectins interact with *N*-linked high-mannose oligosaccharides. To confirm that their inhibitory activities were carbohydrate-mediated, we incubated solutions of CV-N, MVL and GNA with increasing concentrations of Man₉GlcNAc₂ (Man-9) prior to testing for inhibition of HCVcc infection in vitro. Virus was treated with each lectin using the IC₅₀ previously determined against 1a/2a HCVcc (0.6 nM for CV-N, (7 ng/mL), 30.4 nM for MVL (701 ng/mL), and 11.1 nM for GNA (576 ng/mL)). Man-9 reversed the inhibitory effect of all three lectins in a dose dependent manner. The inhibition of HCVcc by CV-N and MVL was reduced from 50% to ~20% at the 5 μM dose (Figure 3). At this same concentration Man-9 had little effect on the inhibitory activity of GNA; however, when a 10-fold higher concentration of ligand was used (50 μM) we observed a similar reversal of the inhibitory effect with inhibition reduced from 50% to 10% (Figure 3). These data demonstrate that the lectins inhibit HCVcc infection through binding to oligomannose-bearing glycoproteins present either on the surface of cells or on the surface of the viral particles.

MVL Blocks Infection During the Early Steps of Virus Entry.

To better understand the mechanism of MVL inhibition of HCVcc infection we evaluated the inhibitory effects of the lectins during different stages of virus binding and entry. Huh7.5 cells were incubated with lectins for 1 hour, and the unbound lectin was removed by washing, followed by addition of HCVcc. With this pre-binding treatment of target cells, we found that MVL still resulted in potent dose-dependent neutralization of HCVcc 1a/2a infection but with a 6-fold increase in IC₅₀, from 30.4 nM (701 ng/mL) (Figure 2A, Table 1) to 183.8 nM (4228 ng/mL) (Figure 4A and Table 2). A similar result was observed for CV-N where the lectin still inhibited viral infection although the IC₅₀ increased by only 3-fold. In contrast, no inhibitory activity was observed for GNA following pretreatment of Huh7.5 cells (Figure 4A, Table 2); the dose response curve was similar to that seen for the negative control 2G12. Together these data suggest that *N*-linked oligomannosides are present on the surface of Huh7.5 cells, that MVL and CV-N can bind to the cells, and that this binding contributes to inhibition of viral entry. Further, the carbohydrate structures recognized by GNA must be absent or inaccessible on the surface of Huh7.5 cells precluding inhibition by GNA through cellular binding. Thus the data suggest that MVL and CV-N inhibit HCV through a more complicated mechanism of action, possibly involving both cellular and viral glycan interactions.

To analyze the effects of lectins on HCVcc infection post binding virus was inoculated onto Huh7.5 cells and incubated for 1 hour at 4 °C. At this temperature virus binds to the cell surface but does not initiate cell entry. After incubation unbound viral particles were removed by washing, followed by addition of lectins at 37 °C. When lectins were added after virus binding CV-N was still able to neutralize the infection (Figure 4B) though at a significantly lower potency. The IC₅₀ was calculated as 19.1 nM (210 ng/mL) (Table 2), almost a 30-fold increase over the IC₅₀ when CV-N is mixed with virus prior to cell inoculation. The IC₅₀ for MVL also increased but only by 6-fold to 200.6 nM (4614 ng/mL) (Figure 4B and Table 2). Surprisingly, GNA was found to be equally potent even when added an hour after virus binding as compared to the standard assay, with an IC₅₀ of 8.1 nM (421 ng/mL). Thus GNA is equally effective regardless of whether it is added directly to the virus prior to infection (IC₅₀ 11.1 nM (577 ng/mL), Table 1), or post viral attachment to target cells but it is ineffective when added to target cells prior to virus inoculation.

To study the effects of lectins during the post-entry step, virus was incubated with cells for 1 and 2 hours at 37 °C, followed by washing and addition of increasing concentrations of lectins. We found that the inhibitory effects of CV-N, MVL and GNA were significantly reduced when added to cells during or after virus entry (Figure 4C and 4D, Table 2). When lectins were added to virus and cells after 1 hour incubation a weak dose dependent effect could still be seen for MVL and GNA, but only CV-N retained activity sufficient to neutralize 50% of the virus albeit with a near 300-fold increase in IC_{50} (184.5 nM (2029 ng/mL), Figure 4C and Table 2). When the lectins were added 2 hours post viral infection at 37 °C no inhibitory effects were observed for any of the lectins at the concentrations used (Figure 4D).

MVL and CV-N Bind to the Surface of Uninfected Cells

The finding that CV-N and MVL are able to inhibit HCVcc when incubated and then removed from target cells prior to addition of virus suggested that these lectins interact with external cellular glycans, whereas GNA preferentially interacts with glycans on the surface of the viral particles. To confirm this result we performed flow cytometry staining of uninfected cells using lectins directly labeled with AlexaFluor 546. We found that of the four agents tested CV-N followed by MVL show the highest levels of binding to the surface of uninfected cells (Figure 5A). Binding of 2G12 to the surface of uninfected cells was not detected, while GNA was found to have minimal levels of binding. Confocal microscopy confirmed this differential binding of lectins to the surface of cells. We observed clear binding of MVL and CV-N to the surface of uninfected cells but only a very weak signal when GNA was used (Figure 5C). The observed binding for CV-N and MVL was reversible when AlexaFluor 546-labeled lectin binding was challenged with increasing amounts of Man-9 (Supplemental Figure 2). We found that CV-N- and MVL-binding to cells was hindered by Man-9 in a dose dependent manner while no effect was observed for Man-9 on the low level of GNA binding to uninfected cells confirming that the CV-N and MVL binding is glycan mediated. When the same analysis was performed using infected cells we saw a significantly stronger signal using GNA on the surface of cells (Figure 5B and 5D).

GNA Binds HCV rE1E2 with Higher Affinity than CV-N or MVL

From neutralization experiments, FACS analysis and confocal microscopy we concluded that MVL and CV-N bind primarily to glycans on the surface of cells during inhibition of HCV infection. To determine whether these lectins can also function through interactions with HCV envelope proteins, CV-N, MVL and GNA were tested for binding to recombinant E1E2 (rE1E2) purified from Huh7.5 cells. This was accomplished by using bio-layer interferometry where association (k_{on}) and dissociation (k_{off}) rates are measured directly allowing for determination of binding affinities (K_D) for the antibodies and lectins. The curves obtained for each lectin binding to immobilized rE1E2 at three different concentrations of ligand as a function of time are shown in Figure 6.

K_D values (Table 3) were obtained through global fitting of the binding data for each lectin or antibody at three concentrations using the Octet Analysis Software. We observed concentration dependent binding to immobilized rE1E2 by all four of the lectins and two antibodies. Of the lectins tested GNA has the highest affinity for rE1E2 with a K_D of 1.0×10^{-5} M, followed by CV-N and MVL (Table 3). The K_D values obtained for binding of the lectins to rE1E2 ($\sim 10 \mu M$) were found to be much higher than the amounts needed to inhibit viral infection (Tables 1 and 2). However, affinity binding studies and neutralization studies are two very different systems and values cannot be directly correlated. For example, the antibody 2G12 binds rE1E2 with high affinity although it does not neutralize the virus. The anti-E1 monoclonal antibody used as a positive control (IgG) also does not neutralize HCV in cell culture despite having the highest affinity for rE1E2 in this study (K_D of 3.6×10^{-7}

M, octet curve not shown). Thus it is not unexpected that a monoclonal antibody can bind recombinant envelope proteins or envelope proteins expressed in infected cells yet fail to neutralize the virus.^{49,50}

The association of these agents with HCV E1E2 was further studied through confocal microscopy using HCVcc infected cells. From our initial confocal studies we observed a higher level of GNA binding to the surface of cells infected with HCVcc (Figure 5). The confocal image showed a punctate staining for GNA suggesting that the lectin was located within clefts on the cell surface (Figure 5D). When infected cells were co-stained with monoclonal antibodies to HCV E1E2 together with the labeled lectins we observed co-localization of CV-N, MVL and GNA on the cell surface (Figure 7A) and in the cytoplasm (Figure 7B) with the envelope glycoproteins. Although we cannot rule out changes in cellular glycan expression profiles due to HCV infection the increased binding and punctate pattern observed for GNA on intact cells appears to be due at least in part to binding of the lectin to envelope proteins expressed on the surface of the infected cells. No binding of antiE1E2 antibodies was observed in uninfected cells (Figure 7C). Additionally, the surface and intracellular confocal microscopy data demonstrate that CV-N and MVL do indeed bind HCV E1E2 providing further evidence that these lectins function through a dual mechanism of inhibition by binding both cellular and viral glycans.

GNA Inhibits HCVcc Synergistically with CV-N and MVL

We have hypothesized that GNA inhibits HCVcc infection by a different mechanism than that of CV-N or MVL. If this is the case we would expect the lectins to act either synergistically or additively if combined in a neutralization assay with HCVcc. To test this, we performed HCVcc neutralization assays where virus was treated with individual lectins or a constant ratio combination of two lectins at 2-fold serial dilutions beginning with concentrations eight times their IC₅₀ values, as previously calculated (Table 1). We found that when CV-N was combined with GNA the inhibition of HCVcc was enhanced for both lectins (Figure 8A), with a 3.4 fold and 4.2 fold increase in the dose reduction indexes (DRI) for CV-N and GNA, respectively. A combination index (CI) can be calculated for drug combinations according to the method of Chou *et al.*,⁴⁴ which can provide indications of synergistic (CI <0.85), antagonistic (CI >1.1) or additive (CI = 0.85-1.1) effects. Using CalcuSyn software the calculated CI for CV-N and GNA was found to be 0.65 (Figure 8D) indicating a synergistic effect on HCVcc neutralization. Using the same approach we found that when MVL is combined with GNA the levels of HCVcc inhibition increase (Figure 8B) and the observed CI value of 0.66 (Figure 8D) again indicates synergistic effects between these two drugs. In contrast, CV-N and MVL were found to interact in an antagonistic/additive manner. When combinations of CV-N and MVL were added to HCVcc, inhibition was reduced compared to CV-N alone (Figure 8C), while inhibition increased relative to MVL alone. The combination index of 1.1 (Figure 8D) suggests CV-N enhances MVL function in an additive manner but that there may be antagonism of CV-N function by MVL. These studies confirmed our hypothesis regarding the different mechanisms of neutralization and carbohydrate specificities for GNA and CV-N. The antagonistic interaction between MVL and CV-N reflects their competition for binding to high-mannose type N-linked carbohydrates.

Lectins Enhance HCVcc Inhibition When Combined with an HCVcc Neutralizing Antibody

If lectins are functioning through binding to glycans on the surface of cells or the HCV envelope proteins we may also expect them to act synergistically or additively with neutralizing antibodies that target other regions of the HCV E1E2. To test this we performed neutralization experiments where virus was treated with lectins either alone or in combination with the monoclonal antibody mAb41 that has been shown to neutralize

HCVcc in vitro and targets the region on E2 comprising amino acids 427-446.⁴⁸ Starting with concentrations that were 8 times higher than their IC₅₀ values, combinations of inhibitors were added as 2-fold serial dilutions. We found that in all cases the combination of lectins with mAb41 resulted in enhanced HCVcc inhibition (Figure 9). Combining CV-N and mAb41 had a synergistic effect (CI=0.7) whereas GNA (CI=0.86) and MVL (CI=1.0) were found to have an additive effect when combined with mAb41.

GNA Binds to and is Inhibited by Sialyl Lactosamine-containing Glycans

GNA is considered to be a mannose specific lectin. However, recently deposited data from glycan array profiling studies showed that in addition to oligomannosides, GNA also binds processed *N*-linked glycans including the hybrid-type and complex-type.⁵¹ This recognition profile indicates that the Man₃GlcNAc₂ core that is present in all three types of *N*-linked glycans represents a general binding epitope for GNA, but also suggests that GNA can tolerate the complex-type branches present on hybrid- and complex-type glycans. This is in contrast to CV-N and MVL that have been shown not to bind to hybrid- and complex-type glycans.^{24,28} To determine whether GNA binding to other types of glycans may account for its differential mode of action, we treated GNA with three carbohydrates that comprise the branches of hybrid and complex type glycans. As seen in Figure 10, treatment with α 2-6 sialyl lactosamine inhibited the effect of GNA on HCVcc in a dose dependent manner, as did Man-9, which was expected. Pretreatment with *N*-acetyl lactosamine had no effect, while pretreatment with α 2-3 sialyl lactosamine mildly competed with GNA. When the same concentrations of α 2-6 and α 2-3 sialyl lactosamine were used to treat MVL and CV-N we observed no competition with the lectins in an HCVcc neutralization assay (data not shown). These results are consistent with GNA having a unique mode of action compared to MVL and CV-N, and might account for GNA's selectivity for HCV glycoproteins over cellular proteins.

Discussion

During recent years, it has been clearly established that certain lectins have antiviral activity against HCV and/or HIV.^{27,30,33} Whereas lectin induced immune-mediated selection pressure of the HIV glycan shield leads to removal of glycosylation sites⁵² this does not appear to be the case for HCV where the glycosylation sites on the E1E2 are highly conserved.^{52,53} The HCV glycan shield does not seem to be evolving which makes it an attractive target for therapeutic interventions.¹⁹ Previous studies with CV-N and HCV concluded that CV-N exerts its inhibitory action primarily through interactions with high-mannose glycans present on the surface of enveloped viruses.³³ However, there is the potential for high amounts of *N*-glycans on the surface of target cells, which play an important role in cell survival and proliferation.⁵⁴

Here we sought to determine the effect of MVL on HCVcc given its strong anti-HIV activity, and to compare its mode of action with the other mannose binding lectins CV-N and GNA. Our studies indeed reveal MVL to be a new inhibitor of HCV infection, and using standard infectivity assays we show that all three lectins inhibit HCVcc infection. However, when studied in more detail, we found that these lectins display properties that suggest more than one mode of inhibition. The oligomannose specific lectins MVL and CV-N were found to bind to target Huh7.5 cells, and binding to these cells alone (followed by washing) resulted in inhibition of HCVcc infection. This was not the case for GNA, which showed no inhibition when added to target cells. Flow cytometry and confocal microscopy confirmed these infectivity results; fluorescently labeled CV-N and to a lesser degree MVL were observed to bind uninfected target cells, while no binding was observed for GNA. However, when the same set of FACS and microscopy experiments were performed on HCV-infected

cells, binding by GNA was then observed (Figure 5). These results strongly suggest that CV-N and MVL target uninfected Huh7.5 cells that display at least some high mannose-containing glycoproteins from their surface, and importantly, that GNA is not binding to the same epitopes. It is important to note that our studies are not in conflict with previous reports showing that CV-N exerts its anti-HCV activity primarily through binding to HCV particles.³³ In fact, using rE1E2 and biolayer interferometry, in addition to confocal staining of infected cells, we confirm that these lectins are able to bind recombinant HCV E1E2 though with significantly reduced levels of affinity than those seen against the infectious virus. Using biolayer interferometry we found that GNA had a higher affinity for the HCV glycoproteins than CV-N and MVL despite being less active against the virus. This is consistent with our other findings that CV-N and MVL function by binding to cellular proteins as well as the HCV envelope. The affinity binding studies were performed with purified rE1E2 proteins and these preparations would most likely have been lacking cellular glycans recognized by CV-N or MVL. Our full dose response curves with MVL and CV-N pre and post virus binding show nanomolar inhibition constants toward HCVcc (Figure 4) and further confirm that binding to cell surface glycans must contribute to the inhibition observed. This is also consistent with previous studies that showed inhibition of HCVpp entry following pretreatment of Huh7.5 cells with CV-N.³³ The inhibition in this case was performed with only a single concentration of CV-N (100 ng/mL) and resulted in 25% reduction in signal whereas our dose response studies showed 50% inhibition using 24 ng/mL of CV-N. It is difficult to directly compare values obtained with single concentration studies with those from dose response studies and these differences could be explained by the use of different reagents and systems. However, the overall finding that pretreating Huh7.5 cells with CV-N can still lead to inhibition of HCV entry is confirmed.

In contrast we found that GNA, which also inhibits HCV at nanomolar concentrations, functions by binding exclusively to the viral particle, which is supported by FACS analysis, synchronized infectivity assays, and confocal staining. The activities of MVL, CV-N, and GNA were each inhibited by treatment with Man-9 (Figure 3). Several fold higher levels of Man-9 were required to reverse the inhibitory effects of the lectins. This could indicate that Man₉GlcNAc₂ is not the optimal ligand, particularly for GNA; that higher concentrations of soluble glycan are required to compete with high avidity, multivalent interactions that may be occurring between the lectins and E1E2 glycoprotein; or that the glycans being targeted by CV-N or MVL on the cell surface are highly abundant. Overall this data suggests that binding to mannose-containing glycoproteins accounts for at least part of their mode of action. GNA was unique among these lectins as its inhibitory effect could also be competed by treatment with α 2-6 sialolactosamine, present in hybrid- and complex-type N-linked oligosaccharides, that are not recognized by MVL and CV-N. These findings suggest that GNA's specificity for HCV envelope protein might be derived through binding glycans other than oligomannosides. This difference in specificity between CV-N and GNA is supported by studies of HIV escape mutants where HCV mutants that were resistant to GNA were not cross-resistant to CV-N.⁵⁵ The fact that GNA acts synergistically with the strictly high-mannose type lectins CV-N and MVL suggests that there are key glycosylation sites present on HCVcc E1E2 that possess complex- or hybrid-type glycans that are recognized by GNA but not CV-N or MVL. Recently, Bräutigam *et al.* reported that E2 contains numerous O-linked glycosylation sites, comprising primarily Core 1 and Core 2 mucin-type structures. These may represent still additional sites for GNA's extended binding profile.⁵⁶

Overall, the processing of the HCV glycoproteins may be more complex than previously thought and our data indicate that the proteins most likely bear both high-mannose type and complex type sugar moieties on the asparagine residues. Our findings that GNA seems to be highly specific for HCV and that it retains its inhibitory effect post virus binding also

suggest that the glycans recognized by GNA are present on a region or regions of E1E2 that remain accessible post binding and are sites of vulnerability for E1E2-directed inhibitors.

Inhibitors of virus entry such as lectins are attractive alternatives or additions to antiviral therapy for HCV. We have shown that different lectins can inhibit virus entry through various mechanisms some of which involve high levels of interaction with cellular proteins with potential toxic side effects. Indeed toxicity studies performed by us and other groups on cells treated with increasing doses of lectins indicated a dose dependent toxic effect by CV-N while no such effects were observed with GNA.^{25,57} However, we also show that other lectins can function almost uniquely through the viral envelope glycoproteins. Further studies on glycan recognition profiles and specific development of lectins similar to GNA could provide a new family of viral entry inhibitors that exhibit minimal cellular toxicity while providing enhanced treatment and protection against HCV infections and HCV/HIV co- infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

CV-N	Cyanovirin-N
MVL	<i>Microcystis viridis</i> lectin
GNA	<i>Galanthus nivalis</i> agglutinin
ELISA	enzyme-linked immunosorbent assay
HCV	hepatitis C virus
HCVcc	hepatitis C virus cell culture
HCVpp	hepatitis C virus pseudo-typed particles
gpE1E2	glycoprotein E1E2
Man-9	Man ₉ GlcNAc ₂
Man-8	Man ₈ GlcNAc ₂
2G12	anti-HIV mAB
PBS	phosphate buffered saline
FACS	fluorescence-activated cell sorting
FPLC	fast protein liquid purification
K_D	Equilibrium dissociation constant
k_{on}	association rate
k_{off}	dissociation rate
Huh7.5	human hepatoma cell line clone of Huh7

IgG immunoglobulin G

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Figure 1. High resolution structures of each of the lectins used in these studies

(**A**) Lectins in complex with their carbohydrate ligand(s). Protein Data Bank accession codes are shown in parentheses. Protein backbones are shown as blue ribbons, and carbohydrate ligands as green sticks with oxygen atoms colored red (**B**). Structure of Man₉GlcNAc₂-Asn (Man-9). The minimal epitopes recognized by each lectin or the monoclonal antibody 2G12 are highlighted by shaded spheres. CV-N, 2G12: yellow, GNA: pink, MVL: blue. Glycan specificity was taken from glycan array data (Supplement Figure 1).

Figure 2. Dose-dependent effects of lectins on inhibition of HCVcc

Huh7.5 cells were infected with HCVcc expressing the structural proteins of genotypes (A) 1a/2a, (B) 1b/2a, (C) J6/JFH1, and (D) 3a/2a, following incubation of the virus with increasing amounts of the lectins CV-N, MVL, GNA, or 2G12. The percent inhibition was calculated and a dose-effect plot was created by CalcuSyn 2.0 software. Experiments were performed in duplicate and repeated in three independent experiments.

Figure 3. Soluble glycans can compete for lectin binding

Inhibition of HCVcc 1a/2a infection following incubation with lectins at their IC₅₀ concentrations, and in the presence of increasing amounts of Man-9. Error bars represent standard error of the mean. Experiments were carried out in duplicate and repeated in three independent experiments. Dotted line denotes 50% inhibition.

Figure 4. Dose-dependent inhibition of HCVcc infection at early binding and entry steps
 A) Huh 7.5 cells were treated with lectins (6000 ng/ml to 46.8 ng/ml) for 1 hour prior to infection with HCVcc 1a/2a. B) Addition of lectins to cells after binding of HCVcc 1a/2a at 4°C for 1 hour C) Addition of lectins to cells after infection with HCVcc 1a/2a at 37 °C for 1 hour, and (D) for 2 hours. Dose-response plots were prepared using CalcuSyn 2.0 software. Experiments were performed in duplicate and repeated in three independent experiments.

Figure 5. FACS and confocal analysis of AlexaFluor-546-labeled lectins bound to infected and uninfected cells

A) Uninfected Huh 7.5 cells surface labeled with lectins and analyzed by flow cytometry. Unstained cells are shown in black, cells incubated with labeled lectins are shown in grey.
 B) Huh 7.5 cells infected with HCVcc 1a/2a and surface labeled with lectins. Confocal microscopy of (C) uninfected and (D) infected cells after surface labeling with lectins.

Figure 6. Binding analysis of immobilized HCV glycoprotein rE1E2 to antibodies and lectins by bio-layer interferometry

Each plot shows the response in nm as a function of time for three different concentrations of lectin and antibody. Blue, orange and turquoise curves correspond respectively to 5.0, 2.5 and 1.25 μM 2G12; and to 10.0, 5.0 and 2.5 μM GNA, CV-N, and MVL. The calculated best-fit curves are shown as red lines.

Figure 7. Confocal Analysis of hepatoma cells with labeled lectins

A) Huh 7.5 cells infected with HCVcc 1a/2a surface labeled with lectins and specific anti-E1E2 antibodies. B) Huh 7.5 cells infected with HCVcc 1a/2a and labeled intracellularly with lectins and specific anti-E1E2 antibodies. C) Uninfected Huh7.5 cells, stained with DAPI and anti-E1E2 as controls.

Figure 8. Dose-response effect of combined MVL, CV-N, and GNA lectins on HCVcc infection
Dose-dependent inhibition by (A) CV-N, GNA and CVN & GNA; (B) MVL, GNA and MVL & GNA; and (C) CV-N, MVL and CV-N & MVL. HCVcc assays were carried out with lectin solutions starting at concentrations 8 times their respective IC₅₀ values, with 2-fold serial dilutions ending at 1/32 of their respective IC₅₀ values. The observed shift (black arrow) shows the combined effect of the lectins. (D) Combination index (CI) for each lectin mixture. The CI values are plotted as the mean of value $\pm$ SD. CI values less than 0.85 indicate synergism, 0.85-1.1 additive effects, and greater than 1.1 antagonism. Data are representative of three independent experiments each carried out in duplicate. The dose-effect plot and combination indices were calculated with CalcuSyn 2.0 (www.biosoft.com).⁴⁴

Figure 9. Dose-response effect of combined lectins and neutralizing monoclonal antibody on HCVcc infection

Dose-dependent inhibition by (A) CV-N, mAb41 and CVN & mAb41; (B) MVL, mAb41 and MVL & mAb41; and (C) GNA, mAb41 and GNA & mAb41. Assays were performed as described for Figure 8. (D) Combination index (CI) for each lectin/mAb41 mixture. The CI was plotted as the mean of two independent experiments each carried out in duplicate. Error bars represent SD. CI values less than 0.85 indicate synergism, 0.85-1.1 additive, and greater than 1.1 antagonism. The dose-effect plot and combination indices were calculated with CalcuSyn 2.0 (www.biosoft.com).⁴⁴

Figure 10. Sialylated glycans can compete for GNA binding

Inhibition of HCVcc 1a/2a infection following incubation of GNA at IC_{50} concentrations with increasing amounts of carbohydrates is shown. $\alpha 2-6$ sialyl lactosamine competes for GNA binding. Bars represent standard error of the mean. Experiments were carried out in duplicate and repeated in three independent experiments. Horizontal dotted line represents 50% inhibition.

Table 1

Lectin IC₅₀ values for inhibition of chimeric HCVcc. IC₅₀ values were calculated using CalcuSyn 2.0 software, based on dose-effect plots shown in Figure 2.

Genotype	IC ₅₀ ± SD (nM)		
	CV-N (monomer, 11 kDa)	MVL (dimer, 23 kDa)	GNA (tetramer, 52 kDa)
1a/2a	0.6 ± 0.1	30.4 ± 2.2	11.1 ± 1.5
1b/2a	1.9 ± 0.9	14.1 ± 2.1	25.5 ± 2.2
2a/2a	0.4 ± 0.1	23.6 ± 3.0	12.5 ± 2.9
3a/2a	1.4 ± 0.8	34.3 ± 2.6	20.6 ± 2.0

Table 2

IC₅₀ values for inhibition of 1a/2a chimeric HCVcc by lectins pre and post virus binding. IC₅₀ values were calculated using CalcuSyn 2.0 software, based on dose-effect plots shown in Figure 4.

	IC ₅₀ ± SD (nM)		
	CV-N	MVL	GNA
Pre virus binding	2.2 ± 1.3	183.8 ± 6.3	<50% *
Post virus binding, 4 °C	19.1 ± 3.0	200.6 ± 6.0	8.1 ± 2.0
Post virus binding, 37 °C, 60 min	184.5 ± 5.7	<50% *	<50% *
Post virus binding, 37 °C, 120 min	<50% *	<50% *	<50% *

* Indicates a loss of inhibition at or above 50% at the highest concentration tested.

Table 3

Equilibrium dissociation constants (K_D s) for antibodies and lectins binding to recombinant E1E2. K_D values were determined by globally fitting the curves for k_{on} and k_{off} measured at three concentrations (Figure 6) with the Octet Analysis Software.

Binding agent	K_D [M]
IgG	3.6×10^{-7}
2G12	1.0×10^{-5}
GNA	1.0×10^{-5}
CV-N	1.3×10^{-5}
MVL	1.8×10^{-4}