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ScienceDirect
Antiviral Research 76 (2007) 21–29
Antiviral Research
www.elsevier.com/locate/antiviral

Antiviral activity of carbohydrate-binding agents against *Nidovirales* in cell culture

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Received 30 January 2007; accepted 24 April 2007

Abstract

Coronaviruses are important human and animal pathogens, the relevance of which increased due to the emergence of new human coronaviruses like SARS-CoV, HKU1 and NL63. Together with toroviruses, arteriviruses, and roniviruses the coronaviruses belong to the order *Nidovirales*. So far antivirals are hardly available to combat infections with viruses of this order. Therefore, various antiviral strategies to counter nidoviral infections are under evaluation. Lectins, which bind to N-linked oligosaccharide elements of enveloped viruses, can be considered as a conceptionally new class of virus inhibitors. These agents were recently evaluated for their antiviral activity towards a variety of enveloped viruses and were shown in most cases to inhibit virus infection at low concentrations. However, limited knowledge is available for their efficacy towards nidoviruses. In this article the application of the plant lectins *Hippeastrum* hybrid agglutinin (HHA), *Galanthus nivalis* agglutinin (GNA), *Cymbidium* sp. agglutinin (CA) and *Urtica dioica* agglutinin (UDA) as well as non-plant derived pradimicin-A (PRM-A) and cyanovirin-N (CV-N) as potential antiviral agents was evaluated. Three antiviral tests were compared based on different evaluation principles: cell viability (MTT-based colorimetric assay), number of infected cells (immunoperoxidase assay) and amount of viral protein expression (luciferase-based assay). The presence of carbohydrate-binding agents strongly inhibited coronaviruses (transmissible gastroenteritis virus, infectious bronchitis virus, feline coronaviruses serotypes I and II, mouse hepatitis virus), arteriviruses (equine arteritis virus and porcine respiratory and reproductive syndrome virus) and torovirus (equine Berne virus). Remarkably, serotype II feline coronaviruses and arteriviruses were not inhibited by PRM-A, in contrast to the other viruses tested.

Keywords: *Nidovirales*; Glycosylation; In vitro assay; MTT; Immunoperoxidase; Luciferase

1. Introduction

Coronaviruses are long known as important veterinary pathogens. In humans the importance has recently increased considerably with the emergence of new human coronaviruses such as SARS-CoV (Drosten et al., 2003), HKU1 and NL63 (Pyrc et al., in press). The outbreak of the coronavirus infection which causes severe acute respiratory syndrome (SARS) has proven that an infection with a member of the order of the *Nidovirales* can have serious health consequences (De Clercq, 2004). *Nidovirales* consist of a broad group of viruses with glycosylated envelopes containing linear, single-stranded RNA genomes of positive polarity. Coronaviruses belong to this order together with toroviruses, arteriviruses, and roniviruses (Gorbalenya et al., 2006).

In the past, the control or prevention of nidovirus infections by antiviral compounds were not considered as a high priority because they were not regarded serious enough to justify costly development of specific drugs. Reliance on vaccines for protection is possible for only a few of these nidoviruses and

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doi:10.1016/j.antiviral.2007.04.003

when available safety and efficacy are under debate. Therefore, it seems advisable to develop anti-nidoviral strategies that are save and effective. New anti-SARS coronavirus strategies were swiftly explored (De Clercq, 2004, 2006) and it became clear that the application of carbohydrate-binding agents (CBA) directed against the glycosylated envelope of these viruses may show promising antiviral activity (Balzarini, 2005).

Indeed, proteins that bind to N-linked glycans of enveloped viruses can be considered as a new class of virus inhibitors. Their antiviral potential was explored for retroviruses (Balzarini et al., 1991, 1992, 2004a; Hansen et al., 1989; Robinson et al., 1987; Witvrouw et al., 2005), cytomegalovirus (Balzarini et al., 1991, 1992; Grail and Norval, 1986), Ebola virus (Barrientos et al., 2003), hepatitis C virus (Helle et al., 2006), influenza A and B virus strains (O'Keefe et al., 2003) and to a limited extent for coronaviruses (Balzarini et al., 2004c; Vijgen et al., 2004; Ziolkowska et al., 2006). Importantly, the ability of lectins to bind pathogens has been explored in vivo as plant and cyanobacterium-derived lectins were recently studied as microbicides to prevent sexual transmission of HIV (Balzarini et al., 2004a; Tsai et al., 2004). Moreover, systemic application of these compounds in animals did not result in acute toxic effects (Balzarini et al., 2004a, 2005a, 2007, 2003) which justifies further exploration of this class of antivirals.

Several lectins were evaluated in this study for their anti-nidoviral activity. The plant lectins HHA (*Hippeastrum* hybrid agglutinin) and GNA (*Galanthus nivalis* agglutinin), are 50 kDa tetramers with an α(1,3) and/or α(1,6) mannose tropism. CA (*Cymbidium* sp. agglutinin) is a 25 kDa dimer with specificity for mannose sugars of which the preferred conformation is not known. UDA (*Urtica dioica* agglutinin) is among the smallest plant monomeric lectins, 8.7 kDa in size, with a *N*-acetylglucosamine specificity (Van Damme et al., 1998). Interestingly, promising non-plant derived glycan-targeting compounds such as the mannose-specific pradimicin-A (PRM-A) extracted from the actinomycete strain *Actinomadura hibisca*, showed fungi (Oki et al., 1988) and human immunodeficiency virus (HIV) binding capacities (Tanabe-Tochikura et al., 1990). Also cyanovirin-N (CV-N), a lectin derived from the procaryotic cyanobacterium *Nostoc ellipsosporum* is specific for α(1,2) mannose oligomers and shows a remarkable anti-HIV activity (Boyd et al., 1997; Dey et al., 2000).

To evaluate the antiviral efficacy of compounds against viruses several techniques may be used. Inhibition of virus production or cytopathogenicity can be assessed (Bedard et al., 1999; Pauwels et al., 1988). Alternatively reporter genes can be used to monitor the amount of infected cells and virus replication. (Olivo, 1996; Westby et al., 2005). In this study the antiviral activity of plant lectins, the non-peptidic antibiotic PRM-A and the procaryotic CV-N was determined against members of the order *Nidovirales*. Immunocytochemistry, reporter gene expression and colorimetric (MTT) assays, which measure the number of infected cells, virus replication and cell viability, respectively, were compared as screening methods for antiviral activity of lectins. Using these assays we were able to show that most of the investigated nidoviruses were sensitive to carbohydrate-binding agents.

2. Materials and methods

2.1. Test compounds

The mannose-specific plant lectins from *G. nivalis* (GNA), *Hippeastrum* hybrid (HHA), *Cymbidium* hybrid (CA), and the *N*-acetylglucosamine (GlcNAc) specific lectin from *U. dioica* (UDA) were derived and purified from these plants, as described previously (Van Damme et al., 1998). Pradimicin-A (PRM-A) was obtained from T. Oki and Y. Igarashi, Japan. Purified recombinant cyanovirin N (CV-N), a cyanobacterial protein, was produced in *Escherichia coli* as reported previously (Mori et al., 1998).

2.2. Cells, viruses

As representatives of the different genera of the order *Nidovirales* we investigated the equine arteritis virus and porcine reproductive and respiratory syndrome virus (respectively EAV and PRRSV, genus *Arterivirus*), equine torovirus (Berne virus, genus *Torovirus*) and several viruses within the genus *Coronavirus*: transmissible gastroenteritis virus (TGEV), feline coronaviruses (FCoVs) both belonging to group I, mouse hepatitis virus (MHV) belonging to group II and infectious bronchitis virus (IBV) of poultry which is a member of group III (De Vries et al., 1997). The feline coronaviruses have evolved into several sublineages in which two serotypes can be distinguished. We evaluated both feline coronaviruses serotypes I and II.

Feline FCWF cells (obtained from N.C. Pedersen, UC Davis, Davis, USA) were used for the antiviral experiments with, and propagation of, FCoV serotype II FIPV (strain 79-1146), FCoV (strain 79-1683; obtained from J. Evermann, Washington State University, USA; McKeirnan et al., 1981) and FIPV-Δ3abcFL (de Haan et al., 2005) and the FCoV serotype I FIPV Black TN406HP (Pedersen and Black, 1983; obtained from N.C. Pedersen, UC Davis, Davis, USA). Mouse LR7 cells, a L-2 murine fibroblast cell line stably expressing the MHV receptor (Rossen et al., 2001) were used for the experiments with, and propagation of, MHV (strain A59; American Type Culture Collection, ATCC) and MHV-EFLM (de Haan et al., 2003).

MHV-EFLM and FIPV-Δ3abcFL are viruses containing a firefly luciferase gene, respectively, in a MHV A59 and FIPV 79-1146 background (de Haan et al., 2005). Growth properties and infectivity are similar to the parental viruses (de Haan et al., 2003).

Porcine ST cells (obtained from M. Pensaert, UGent, Gent, Belgium) were used for the experiments with, and propagation of, TGEV (strain Purdue, Purdue University, West Lafayette, USA). Simian Vero cells (ATCC) were used for the experiments with, and propagation of, the Vero-cell adapted IBV strain Beaudette (ATCC, Alonso-Caplen et al., 1984). Berne virus (obtained from M. Weiss, Berne, University of Berne, Switzerland, Weiss et al., 1983) and equine arteritis virus (EAV) strain Bucyrus (ATCC) were grown on equine dermis (Ederm) cells (ATCC). All of the above-mentioned cells were cultured on Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml

streptomycin. Titrations and antiviral tests were performed in DMEM containing 5% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies Ltd., Paisley, United Kingdom).

Primary porcine alveolar macrophages (PAMs) were used for the experiments with, and propagation of, the prototype European PRRSV isolate Lelystad virus (provided by G. Wensvoort, Central Veterinary Institute, Lelystad, The Netherlands). PAMs were obtained as described earlier (Delputte and Nauwynck, 2004). The PAMs were cultivated in Earle's modified Eagle medium (EMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (BDH Chemicals Ltd., Poole, England), 1% nonessential amino acids 100×, Gibco BRL), 1 mM sodium pyruvate, and a mixture of antibiotics in a humidified 5% CO_2 atmosphere at 37 °C.

2.3. Antiviral assays

2.3.1. Colorimetric MTT assay for the determination of cell toxicity and antiviral activity against nidoviruses

Cell viability was evaluated as described previously (Pauwels et al., 1988) with minor modifications by adding to each well a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO, USA) in PBS at a final concentration of 0.5 mg/ml. After 2 h incubation at 37 °C and 5% CO_2 the medium containing the MTT was removed and the cells were lysed by addition of 200 μl DMSO to each well. Following 10 min incubation at room temperature on a rocking plate the optical density (OD) values at 570 nm were determined.

Antiviral activity was based on the viability of the cells that had been infected with 100 $TCID_{50}$ (50% tissue culture infective dose) of the viruses mentioned above, in the presence of various concentrations of the test compounds. The virus–drug mixture was preincubated at 37 °C and 5% CO_2 for 1 h and added to the cells after a single wash with PBS DEAE. The mixture was removed after 1 h. Cells were washed with PBS Ca^{2+}/Mg^{2+} and the test compounds were added again at the same concentrations. The MTT assay was performed 2 days (FIPV 79-1146, FCoV 79-1683) or 3 days (all other viruses) after the infection when complete CPE was visible in the cell cultures without addition of test compound. The compound concentration preventing the cytopathic effect induced by the virus by 50% was defined as the 50% effective concentration (EC_{50}). Cytotoxic activity determination was based on the viability of the cells that had been incubated at 37 °C and 5% CO_2 in the presence of various concentrations of the test compounds during 3 days. The compound concentration that decreased the viability of 50% of the cells was defined as the 50% cytotoxic concentration (CC_{50}).

2.3.2. EMA staining of porcine alveolar macrophages (PAMs) for the determination of cytotoxicity

PAMs were incubated for 24 h with various concentrations of the test compounds. Dead cells were visualized by incubating with 0.05 mg/ml ethidium monoazide bromide (EMA; Molecular Probes) before fixation with 3% paraformaldehyde (Costers et al., 2006). Stained cells were counted by fluorescence microscopy. The compound concentration that decreased the viability of 50% of the PAMs was defined as the 50% cytotoxic concentration (CC_{50}).

2.3.3. Immunoperoxidase staining (IPOX) assay

Antiviral activity measurements were based on the reduction in numbers of focus forming units (FFU) when the cell cultures were infected with nidoviruses in the presence of various concentrations of the test compound. The cell monolayer was infected at a multiplicity of infection (MOI) of 0.5. The virus–drug mixture was preincubated at 37 °C and 5% CO_2 for 1 h and added to the cells after a single wash with PBS DEAE. The mixture was removed after 1 h. Cells were washed with PBS Ca^{2+}/Mg^{2+} and the test compounds were added again at the same concentrations. At 6 h post-infection (or 16 h for FIPV Black TN406HP infected FCWF) the cells were fixed during 15 min with 4% formaldehyde, and subsequently permeabilized with 70% ethanol for 5 min. When FIPV Black TN406HP was assessed, the cells were fixed at 16 h post-infection, because serotype I coronavirus antigen expression in FCWF cells appeared at a later moment compared to serotype II FCoV strains. Immunoperoxidase (IPOX) detection of MHV-positive cells was carried out by using a rabbit polyclonal antibody against MHV (K135) (Rottier et al., 1981) in combination with a HRP swine–anti-rabbit antibody (Dako A/S, Glostrup, Denmark). An ascitic fluid sample (A40) from a cat that had succumbed to feline infectious peritonitis was used for the immunodetection of FCoV 79-1683, FIPV Black TN406HP and FIPV 79-1146 combined with a HRP goat–anti-cat (ICN Biomedicals Inc., Aurora, OH, USA). The infection and immunoperoxidase staining of PRRSV-infected PAMs were described earlier (Delputte and Nauwynck, 2004). Virus (MOI 1) was preincubated for 1 h at 37 °C with different concentrations of the test compound and added to the PAMs for 1 h. Cells were washed with medium without fetal bovine serum (FBS) to remove unbound virus. PAMs were fixed at 10 h post-infection by a 20 min treatment with methanol at −20 °C. The fixative was removed, and the plates were dried and kept at −70 °C until staining. Fixed cells were washed once with PBS, and rinsed three times with water. The endogenous peroxidase activity was blocked by incubating the cells with PBS supplemented with 1% sodium azide and 0.5% H_2O_2 for 10 min. PRRSV-infected cells were incubated for 1 h at 37 °C with MAb P3/27, directed against the PRRSV-nucleocapsid protein (Wieczorek-Krohmer et al., 1996) in combination with a HRP goat anti-mouse antibody (Dako A/S, Glostrup, Denmark).

Infected cells were counted using the light microscope, and the effective concentration at which the number of infected cells (focus forming units, FFU) was lowered by 50% (EC_{50}) compared to the mock-treated cells was calculated.

2.3.4. Luciferase-based assay

FCWF or LR7 cells were infected with FIPV-Δ3abcFL or MHV-EFLM, respectively, in the presence of various concentrations of the test compound. FCWF or LR7 cell monolayers were infected at a multiplicity of infection (MOI) of 0.5. The virus–drug mixture was preincubated at 37 °C and 5% CO_2

Table 1
Quantification of antiviral activity of CBA (plant lectins and pradimicin-A) by the colorimetric MTT assay

CBA	MHV A59	FIPV Black TN406HP (serotype I)	FIPV 79-1146 (serotype II)	FCoV 79-1683 (serotype II)	IBV Beaudette	TGEV Purdue	Berne virus torovirus	Equine arteritis virus
GNA	ND	0.008 ± 0.005	0.13 ± 0.002	0.43 ± 0.30	0.0002 ± 0.0002	0.004 ± 0.0008	0.12 ± 0.09	0.18 ± 0.07
HHA	ND	0.007 ± 0.003	0.10 ± 0.002	0.11 ± 0.03	0.0002 ± 0.0002	0.004 ± 0.002	0.07 ± 0.07	0.13 ± 0.04
UDA	ND	0.023 ± 0.012	0.24 ± 0.14	0.11 ± 0.03	0.05 ± 0.05	0.08 ± 0.07	0.25 ± 0.20	0.39 ± 0.01
CA	NT	0.13 ± 0.002	0.29 ± 0.11	NT	NT	NT	NT	NT
PRM-A	ND	2.5 ± 1.6	>120	>120	2.9 ± 2.0	4.7 ± 0.8	31 ± 24	>120

Antiviral activity of plant lectins *Galanthus nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA), *Cymbidium* sp. agglutinin (CA), *Urtica dioica* agglutinin (UDA) and pradimicin-A (PRM-A) against nidoviruses. Values represent the CBA concentrations resulting in 50% inhibition of virus infection (EC_{50} ± S.D.) and are expressed in μM. ND = could not be determined due to syncytium formation; NT = not tested.

for 1 h and added to the cells after a single wash with PBS DEAE. The mixture was removed after 1 h. Cells were washed with PBS Ca^{2+}/Mg^{2+} and the test compounds were added again at the same concentrations. At 6 h post-infection the culture media were removed and the cells were lysed using the appropriate buffer provided with the firefly luciferase assay system (Promega, Madison, WI, USA). Intracellular luciferase expression was measured according to the manufacturer's instructions, and the relative light units (RLU) were determined with a Turner Designs TD-20/20 luminometer. The effective concentration at which 50% of the luciferase expression was inhibited (EC_{50}) compared to the mock-treated cells was then calculated.

3. Results

3.1. Antiviral activity of plant lectins determined by a colorimetric MTT assay

Nidoviruses were evaluated for their sensitivity to CBA by a conventional colorimetric MTT assay (Table 1), which evaluates cell survival after 2–3 days of incubation of the virus-infected cells in the presence of different compound concentrations. The antiviral activity of the compounds is represented by the 50% effective concentration (EC_{50}). In all cases the mannose-specific lectins GNA, HHA and CA showed antiviral activity against all *Nidovirales* evaluated. The plant lectins GNA and HHA showed EC_{50} values in the higher picomolar or lower nanomolar concentration range for IBV, FIPV Black TN406HP and TGEV (Table 1). Replication of serotype II FCoV strains, Berne virus and equine arteritis virus was inhibited by the mannose-specific plant lectins at concentrations that rank in the higher nanomolar concentration range (Table 1). Also, the GlcNAc-specific UDA showed pronounced antiviral activity. Although it was 10–20-fold less effective against FIPV Black TN406HP, IBV and TGEV than the mannose-specific lectins, it proved virtually equally active against the other viruses (Table 1).

MHV A59 infection with 100 $TCID_{50}$ per well induced massive syncytium formation in the cell culture but not a complete cell lysis within the timeframe examined. Even though the formation of syncytia seemed to decrease upon the addition of lectins, this antiviral effect was not reflected in the OD values. Therefore, an exact EC_{50} value could not be determined.

In conclusion the results indicate a strong inhibitory effect of plant lectins on the infection process of nidoviruses.

3.2. Antiviral activity of plant lectins determined by immunoperoxidase and luciferase-based assays

Next, an immunoperoxidase (IPOX) method (Table 2) and a luciferase-based method (Table 3) were used to evaluate the antiviral activity of the lectins. These assays are based on the number of infected cells and expression of viral proteins, respectively.

In the IPOX assay MHV-EFLM, FIPV Black TN406HP, FCoV 79-1683, FIPV-Δ3abcFL and PRRSV Lelystad virus were used. The influence of various concentrations of plant lectins on the infection of these viruses in LR7 (for MHV), FCWF (for FCoV strains) and PAM (for PRRSV Lelystad virus) cell cultures was assessed (Tables 2 and 3). The plant lectin EC_{50} as determined by IPOX ranged from 0.04 to 0.53 μM against MHV-EFLM and 0.03–0.20 μM against FIPV-Δ3abcFL and FCoV (serotype II). The serotype I feline coronavirus FIPV Black TN406HP showed in general a markedly higher sensitivity for the compounds; the EC_{50} values varied from 0.004

Table 2
Quantification of antiviral activity of CBA (plant lectins and pradimicin-A) by the immunoperoxidase assay

CBA	MHV-EFLM	FIPV Black TN406HP (serotype I)	FIPV-Δ3abcFL (serotype II)	FCoV 79-1683 (serotype II)	PRRSV Lelystad virus
GNA	0.04 ± 0.02	0.012 ± 0.006	0.07 ± 0.04	0.2 ± 0.06	>2
HHA	0.07 ± 0.02	0.004 ± 0.002	0.03 ± 0.02	0.06 ± 0.02	>2
UDA	0.53 ± 0.02	0.02 ± 0.01	0.14 ± 0.05	0.17 ± 0.10	4.8 ± 3.2
PRM-A	10.7 ± 5.8	7.8 ± 2.0	>120	>120	>120

Antiviral activity of *G. nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA), *U. dioica* agglutinin (UDA) and pradimicin-A (PRM-A) against murine and feline coronaviruses and the arterivirus PRRSV. Values represent the CBA concentrations resulting in 50% inhibition of virus infection (EC_{50} ± S.D.) and are expressed in μM.

Table 3
Quantification of antiviral activity of CBA (plant lectins and pradimicin-A) by the luciferase-based assay

CBA	MHV-EFLM	FIPV-Δ3abcFL
GNA	0.006 ± 0.004	0.016 ± 0.006
HHA	0.004 ± 0.006	0.008 ± 0.006
UDA	0.08 ± 0.08	0.18 ± 0.11
CA	0.032 ± 0.04	0.016 ± 0.016
PRM-A	3.5 ± 3.8	>120
CV-N	0.002 ± 0.001	0.006 ± 0.005

Antiviral activity of *G. nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA), *Cymbidium* sp. agglutinin (CA), *U. dioica* agglutinin (UDA), pradimicin-A (PRM-A) and cyanovirin-N (CV-N) against MHV-EFLM and FIPV-Δ3abcFL. Values represent the CBA concentrations resulting in 50% inhibition of virus infection (EC_{50} ± S.D.) and are expressed in μM.

to 0.02 μM. The IPOX assay showed in most cases a slightly lower EC_{50} compared to the MTT assay. PRRSV Lelystad virus infection of PAMs could be reduced at 2 μM to 40% by GNA and HHA, insufficient to obtain an EC_{50} value but indicating sensitivity to the plant lectins. The EC_{50} for UDA was 4.8 μM.

The application of the luciferase-based assay indicated a similar coronavirus sensitivity for the plant lectins. The luciferase tests showed in all cases lower EC_{50} values compared to the above-mentioned IPOX test results (EC_{50} 0.004–0.18 μM) (Table 3). It should be noticed that none of the plant lectins showed appreciable cytotoxicity at 2 μM, which is at a concentration substantially higher than their antiviral activities (Table 4).

3.3. Antiviral activity of pradimicin-A against nidoviruses

The MTT assay (Table 1), IPOX assay (Table 2) and also the luciferase-based assay (Table 3) was applied to evaluate the sensitivity of the nidoviruses to the inhibitory activity of the non-peptidic low-molecular-weight antibiotic pradimicin-A (PRM-A). All assessed viruses were sensitive to PRM-A, except for FCoV serotype II strains (FCoV 79-1683, FIPV 79-1146 and FIPV-Δ3abcFL) and the arteriviruses (PRRSV and equine arteritis virus). These viruses were not significant inhibited at 120 μM PRM-A that is at a concentration that represents the maximum solubility of the compound. In contrast, the infections with MHV-EFLM and FIPV Black TN406HP (serotype I) were clearly inhibited by PRM-A in the same concentration range (Table 2). The feline coronavirus serotype I FIPV Black TN406HP and FCoVs serotype II were derived from FCWF cells, indicating that the activity/inactivity of PRM-A is virus-related, rather than cell type-related. Overall, the nidoviruses were less sensitive to the inhibitory action of PRM-A when compared to the mannose- and GlcNAc-specific plant lectins GNA, HHA and UDA. However, as evident from Table 1, PRM-A showed low-micromolar activity against those virus strains that showed low nanomolar sensitivity to GNA and HHA. No marked inhibition of PRM-A could be observed for those virus strains that showed higher nanomolar sensitivity to GNA and HHA.

3.4. Antiviral activity of cyanovirin-N against MHV-EFLM and FIPV-Δ3abcFL

The mannose-specific procaryotic cyanovirin-N (CV-N) was also evaluated for antiviral activity with the luciferase-based assay using MHV-EFLM and FIPV-Δ3abcFL. CV-N was exquisitely active against both coronaviruses (low nanomolar range; Table 3). Other nidoviruses were not assessed.

3.5. Cytotoxicity of plant lectins, pradimicin-A and cyanovirin-N

The cytotoxicity of the CBA against the different cell types is represented by the 50% cytotoxic concentration (CC_{50}; Table 4). The mannose-specific plant lectins HHA, GNA and CA were hardly cytotoxic to the cells ($CC_{50} \geq 2$ μM). UDA showed a slight cytotoxic activity with CC_{50}'s that ranged from 2 to 10 μM for the different cell types tested. Pradimicin-A was poorly cytotoxic in ST and Vero cells (CC_{50}: 120 μM). Cyanovirin-N showed cytotoxicity in vitro towards FCWF cells (CC_{50} 1.4 μM), which are used for the virus propagation and the antiviral tests with FCoVs. Microscopically the cell changes became already visible at 0.3 μM or higher concentrations of CV-N. Also the LR7 cells showed morphological changes due to exposure of 0.14 μM or higher CV-N concentrations, although no overt toxicity ($CC_{50} > 2$ μM) could be noticed on these cells by the MTT assay. Since CV-N is active at low concentrations, the therapeutic index (TI = 700) is still high. The CC_{50} of all test compounds was low for the PAMs, only PRM-A showed detectable cytotoxicity at 18 μM.

Table 4
Cytotoxicity of CBA (plant lectins, pradimicin-A and cyanovirin-N)

CBA	LR7	FCWF	Vero	ST	Ederm	PAMs
GNA	>2	>2	>2	>2	>2	>2
HHA	>2	>2	>2	>2	1.9 ± 0.3	>2
UDA	9.9 ± 4.8	2.2 ± 0.3	3.4 ± 1.3	6.1 ± 1.0	9.5 ± 3.1	>12
CA	>4	3.3 ± 0.8	NT	NT	NT	NT
PRM-A	>120	>120	120 ± 32.4	121.2 ± 27.6	>120	18.1 ± 6.4
CV-N	>2	1.4 ± 0.5	NT	NT	NT	NT

Cytotoxicity of *G. nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA), *Cymbidium* sp. agglutinin (CA), *U. dioica* agglutinin (UDA), pradimicin-A (PRM-A) and cyanovirin-N (CV-N) on cells used to propagate the different nidovirus strains. The cytotoxicity of CBA towards PAMs was determined using the EMA staining; cytotoxicity towards all other cell types was determined using a MTT assay. Values represent CBA concentration resulting in 50% cytotoxicity (CC_{50} ± S.D.) and are expressed in μM. NT = not tested.

3.6. Bioinformatic analysis of envelop glycoproteins of nidoviruses

Based on the mechanism of antiviral activity (i.e. envelope glycan binding by CBA) we wanted to examine whether there is a correlation between the number of *N*-glycosylation sites and the EC_{50} of the CBA. Therefore, we made a prediction of the number of *N*-glycosylation sites using the NetNGlyc server (see web reference: NetNglyc, inpress). No clear relation could be found between the number of predicted glycosylation sites and the EC_{50} concentrations for the various CBA.

4. Discussion

As nidoviruses are covered with many *N*-glycosylated sites on their envelope proteins, these glycans pose an interesting target for the development of new and targeted antivirals. Therefore, we investigated the antiviral activity of glycan-targeting compounds, including mannose and GlcNAc-binding plant lectins, pradimicin-A (PRM-A), that is a mannose-binding non-peptidic antibiotic formerly evaluated for antifungal activity and cyanovirin-N (CV-N), an α(1,2) mannose-specific procaryotic lectin. Glycosylation of proteins is not unique for viruses and glycans are also present on vertebrate host cell glycoproteins. The sugar content of the viral envelope fully depends on the host cell glycosylation machinery. Although, the characteristics of *N*-glycosylation of host cell and virus glycoproteins are in principle similar, it should be kept in mind that still striking differences in the nature of the glycans can be found between viral (i.e. HIV, HCV) envelope glycoproteins and host cellular glycoproteins. Both HIV and HCV glycoproteins contain a high amount of high-mannose type glycans. Such glycan types are much less abundantly present, or even absent, in many mammalian glycoproteins. Still, antiviral agents specifically targeting glycosylated proteins can have side effects when administered systemically. This potential problem was, however, not observed in vivo for a number of carbohydrate-binding agents (CBA) (Balzarini et al., 2004a, 2005a, 2007, 2003). In these tests no short-term toxicity was shown, encouraging the research on the application of CBA as anti-nidoviral agents.

To assess the antiviral activity of CBA three methods were applied, all with a different evaluation principle. Cell viability after virus infection as indicated by color formation in an MTT-based assay is classically used to evaluate the inhibition of viral infection by antivirals. In this assay multiple replication steps will occur as the infection process is initiated at a low multiplicity of infection. To establish a faster and more accurate method for antiviral examination against coronaviruses, two alternative methods were explored. Both IPOX and luciferase-based assays represent the evaluation of the initial infection processes: attachment and entry followed by protein production. The tests are terminated before virus release from infected cells. Therefore, in contrast to the MTT assay, no multiple replication cycles occur. This might explain the differences in EC_{50} values obtained using IPOX and luciferase-based assays compared to the MTT assay. Nevertheless, all assays were indicative for rather similar antiviral activity of CBA towards nidoviruses. In that respect the assays were interchangeable. Therefore, the possibility to use the luciferase-based evaluation method for high throughput screening may facilitate the research of antivirals directed against coronaviruses. Both IPOX and luciferase assays were used as an alternative to screen the ability of CBA to inhibit MHV A59. Since the MTT assay could not be used due to the syncytium formation following infection. Mitochondria in giant cells reduced MTT to a similar level as non-infected control cells.

In the MTT assay the efficacy of GNA and HHA against nidoviruses was very high ($EC_{50} < 0.01$–0.18 μM). These results are in the same ranges as found for GNA and HHA in their efficacy against HIV-1, HIV-2 and SIV (Balzarini et al., 2004a) and the SARS coronavirus (Balzarini et al., 2004c). For all viruses evaluated the host cells in the antiviral assays were derived from the target species of the virus. However, we used a simian Vero cell adapted virus strain for evaluation of IBV, as a chicken-based cell system was not available. This non-natural host will generate a simian-like glycosylation pattern on the IBV envelope proteins. This might explain the very low EC_{50} found for the plant lectins GNA and HHA. We showed also inhibitory effects for CA towards FIPV and MHV. In earlier studies this compound formerly demonstrated a high antiviral activity against retroviruses (EC_{50}: 0.003 μM) but less towards cytomegalovirus infections (EC_{50}: 0.35 μM) (Balzarini et al., 1992). Cytomegalovirus, SIV and HIV-1 infections could all be very efficiently inhibited by UDA (Balzarini et al., 1992). We were able to show for UDA also a high antiviral efficacy against all evaluated *Nidovirales* except PRRSV. The low antiviral effect of CBA towards PRRSV might be explained by the fact that sialic acids structures are mainly involved in the attachment of PRRSV to PAMs whereas high-mannose glycans are reported to be less important (Delputte and Nauwynck, 2004).

Pradimicin-A, a mannose-binding non-peptidic antibiotic showed antiviral activity against MHV, FIPV serotype I, TGEV and IBV. The efficacy against Bernevirus infections was low. The EC_{50} values were in line with the anti-HIV-1 EC_{50} of PRM-A ranges found earlier: ≥4.2 μM in HIV-infected MT-4 and CEM cytopathogenicity systems and ≥15 μM in a giant cell formation assay (Balzarini et al., 2007; Tanabe et al., 1988). Despite the fact that PRM-A has a specificity for D-mannose structures (Fujikawa et al., 1998), it was not able to inhibit the FCoV serotype II strains FIPV 79-1146 and FCoV 79-1683 or the arteriviruses PRRSV and EAV, even at the highest concentrations evaluated. It is somewhat puzzling that PRM-A is inactive against FCoV serotype II strains and arteriviruses, since it is suggested to have an α(1,2)-mannose configuration tropism (Iagrashi and Oki, 2004) similar to CV-N. Additionally, we included CV-N in our studies for comparative reasons. Cyanovirin-N was shown to have an inhibitory effect towards HIV-1 and SIV (Balzarini et al., 2006; Boyd et al., 1997) and was highly active against both MHV and FIPV serotype II infections as determined by the luciferase-based assay (lower nanomolar range). Therefore, we conclude that glycans containing these structures are present on the FIPV serotype II and MHV viruses propagated in our culturing systems. The discrepancy in PRM-A antiviral activity towards FCoV serotypes I and II might be due

to the different receptor usage influencing the entire infection process (Smith and Helenius, 2004). Serotype II FCoV utilize fAPN (Tresnan et al., 1996) as primary receptor whereas for serotype I coronaviruses the attachment to the host cell is relying on a different molecule, currently unknown (Hohdatsu et al., 1998). Alternatively, since serotype I coronaviruses are also less sensitive to HHA and GNA, PRM-A may be active against these viruses as well, but those drug active concentrations cannot be reached due to insolubility of the compound.

It was examined whether the differences in CBA EC_{50} values would be reflected in the amount of predicted *N*-glycosylated sites present on the viral envelope. In our evaluation no correlation between the number of *N*-glycosylated sites and EC_{50} was found. It is assumed that not only the actual number of glycosylated sites will be of influence, but also the glycan types present (i.e. high-mannose-type versus complex/hybrid-type) and the protein abundance in the viral envelope may be even more important.

In plants, lectins are mainly involved in recognition processes either within or outside the plant by binding to carbohydrates (Van Damme et al., 1998). In the natural situation they may also play an important role in the defense mechanism of the plant (Vierheilig et al., 1996). Similar molecules are participating in the innate immune system of vertebrates, a specifically binding the glycosylated proteins of pathogens (Hart et al., 2002). CBA binding mannose (GNA, HHA, CA, CV-N or PRM-A) or GlcNAc (UDA) are not expected to bind to selected glycosylated sites in a specific manner. As mannose and GlcNAc molecules are present throughout the glycosylated proteins CBA may concomitantly bind at several glycans on the virus envelope. However, it should be mentioned that CBA usually show poor recognition of monosaccharides but their affinity and selectivity is much higher for specific carbohydrate oligomer configurations. These properties may also explain the different potencies of CBA against different viruses, even within the same virus family. The lectins used in our test inhibit infection but do not seem to have virucidal properties as adsorption to the host cell of the virus in the presence of the compound still lead to a productive infection upon removal of the lectin (unpublished results).

For HIV the envelope sugars are suggested to be involved in the retroviral mechanism to escape host immunity (Blay et al., 2006; Reitter et al., 1998; Rudd et al., 2001; Wei et al., 2003). The glycosylation on coronavirus envelope proteins is suggested to serve a similar purpose (Ansari et al., 2006; Chakraborti et al., 2005). One can speculate that by targeting this protective shield with carbohydrate-binding agents viruses are forced to generate mutants with deleted or altered glycosylation sites as already demonstrated to occur for HIV in the presence of several types of CBA (i.e. plant lectins, PRM-A, CV-N) (Balzarini et al., 2004b, 2005b, 2006, 2007). As a consequence the immunogenic epitopes will be exposed to the immune system (Balzarini, 2005) resulting in triggering of the production of neutralizing antibodies or a cellular immune response against the uncovered immunogenic epitopes. Therapy of chronic virus infections can benefit from this strategy. Whether this also applies to nidoviral infections is uncertain, although some of these viruses remain in the animal body for a prolonged time period (Glaser et al., 1997; Herrewegh et al., 1997; Naqi et al., 2003).

In summary, with this study we have shown that CBA have antiviral activity towards nidoviruses. As members of the *Nidovirales* order usually induce diseases with an acute or prolonged character, a systemic application as therapeutic agent seems appropriate. The low in vivo toxicity and high in vitro efficacy is encouraging to continue the exploration of these compounds as antivirals.

Acknowledgments

The research was supported by the René Descartes Prize-2001 of the European Commission and the Centers of Excellence (EF-05/15) of the K.U. Leuven.

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