

## The carbohydrate-binding plant lectins and the non-peptidic antibiotic pradimicin A target the glycans of the coronavirus envelope glycoproteins

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**Objectives:** Many enveloped viruses carry carbohydrate-containing proteins on their surface. These glycoproteins are key to the infection process as they are mediators of the receptor binding and membrane fusion of the virion with the host cell. Therefore, they are attractive therapeutic targets for the development of novel antiviral therapies. Recently, carbohydrate-binding agents (CBA) were shown to possess antiviral activity towards coronaviruses. The current study further elucidates the inhibitory mode of action of CBA.

**Methods:** Different strains of two coronaviruses, mouse hepatitis virus and feline infectious peritonitis virus, were exposed to CBA: the plant lectins *Galanthus nivalis* agglutinin, *Hippeastrum* hybrid agglutinin and *Urtica dioica* agglutinin (UDA) and the non-peptidic mannose-binding antibiotic pradimicin A.

**Results and conclusions:** Our results indicate that CBA target the two glycosylated envelope glycoproteins, the spike (S) and membrane (M) protein, of mouse hepatitis virus and feline infectious peritonitis virus. Furthermore, CBA did not inhibit virus–cell attachment, but rather affected virus entry at a post-binding stage. The sensitivity of coronaviruses towards CBA was shown to be dependent on the processing of the N-linked carbohydrates. Inhibition of mannosidases in host cells rendered the progeny viruses more sensitive to the mannose-binding agents and even to the *N*-acetylglucosamine-binding UDA. In addition, inhibition of coronaviruses was shown to be dependent on the cell-type used to grow the virus stocks. All together, these results show that CBA exhibit promising capabilities to inhibit coronavirus infections.

Keywords: *Coronaviridae*, glycosylation, lectins

### Introduction

Development of intervention strategies for coronavirus infections has been boosted after the severe acute respiratory syndrome (SARS) coronavirus epidemic. Successes have been recorded but the demand for antiviral chemotherapeutics that are safe and active in low concentrations perpetuates the search for new compounds. The use of interferons<sup>1</sup> and human monoclonal antibodies<sup>2,3</sup> is under research in case of a re-emergence of the SARS coronavirus. Further, coronavirus entry, including fusion,

proteases and viral RNA were already envisaged as antiviral targets.<sup>4,5</sup> The heavily glycosylated coronavirus envelope constitutes an appealing target for therapeutic intervention. Because the sugar content of glycoproteins is critical for the effective replication of the virus, viral protein glycosylation plays an important role in the course of virus infection, replication and virus–host interactions.<sup>6–8</sup>

Compounds that specifically bind to or alter carbohydrate structures on these exterior glycoproteins were recently evaluated for their properties as antiviral agents.<sup>9,10</sup> It has been

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demonstrated that a variety of carbohydrate-binding agents (CBA) attach to N-glycosylated molecules and possess antiviral activity.<sup>11,12</sup> Moreover, it seems that the genetic barrier to evade CBA inhibition by altering the N-glycosylation pattern on viral envelope glycoproteins is high, hence resistance to many CBA is not easily acquired.<sup>13–16</sup>

An interesting group of CBA are the plant lectins.<sup>17</sup> *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum* hybrid agglutinin (HHA) are tetrameric  $\alpha(1,3)$  and/or  $\alpha(1,6)$  mannose-binding proteins that were previously found to be active towards human, simian and feline retroviruses, cytomegalovirus<sup>9,18,19</sup> and members of the *Nidovirales* order.<sup>20</sup> *Urtica dioica* agglutinin (UDA)<sup>13,18</sup> is an *N*-acetylglucosamine (GlcNAc)-binding lectin which also displayed pronounced antiviral properties. Derived from the stinging nettle root, it is among the smallest monomeric plant lectins known.<sup>21</sup> Mannose-binding lectins derived from prokaryotic origin, such as cyanovirin N (CV-N) or pradimicin A (PRM-A), are currently under investigation for their retro-, and SARS-coronavirus inhibiting properties.<sup>11,12,22–24</sup> PRM-A is an actinomycete (*Actinomadura hibisca*)-derived D-mannose-binding agent<sup>25</sup> described as a 'lectin-mimic antibiotic'.<sup>26</sup> It was shown to be active against fungi, yeast,<sup>25</sup> HIV-1<sup>27,28</sup> and several viruses from the *Nidovirales* order.<sup>20</sup> Strikingly, PRM-A demonstrated antiviral activity against serotype I but not serotype II feline coronaviruses (FCoV).<sup>20</sup> The exact PRM-A tropism is currently not known, but it is suggested that  $\alpha(1,2)$ -mannose configurations on the N-glycans are important for recognition by PRM-A.<sup>29</sup>

Coronaviruses are enveloped, plus-strand RNA viruses that invariably contain at least four structural proteins: the membrane (M), envelope (E), spike (S) and nucleocapsid (N) protein. The N protein wraps the genomic RNA into a nucleocapsid and is not exposed on the outside of the virus particle. The S, M and E proteins, of which the former two are glycosylated, are anchored in the envelope. The M protein, which contains a short ectodomain, is the most abundant envelope glycoprotein, and usually contains one glycan tree. The heavily glycosylated S protein, which mediates virus–cell attachment and fusion, forms large trimers that protrude from the virion surface. Two different coronaviruses were used to study the mode of action of CBA. Feline infectious peritonitis virus (FIPV) strain 79-1146 causes a progressive systemic infection in cats. Mouse hepatitis virus (MHV) strain A59 induces neuropathy and liver inflammation in mice. The interaction of CBA with the different virus envelope glycoproteins was evaluated. Furthermore, it was determined which step of the virus entry process was affected by the CBA. Finally, the influence of glycan maturation and cell-type specificity of glycosylation on inhibition by CBA was assessed. The results facilitate future research on coronavirus glycosylation and anti-coronavirus therapy.

## Materials and methods

### Test compounds

The mannose-specific plant lectins from GNA, HHA and the *N*-acetylglucosamine (GlcNAc)-specific UDA were derived and purified as described previously<sup>17</sup> and kindly provided by E. Van Damme (Ghent, Belgium). PRM-A was obtained from T. Oki and

Y. Igarashi (Bristol–Myers Research Institute, Ltd, Tokyo Research Center Japan).

### Cells and viruses

*Felis catus* whole fetus (FCWF) cells (obtained from N. C. Pedersen, Davis, CA, USA) were used for experiments with, and propagation of, feline infectious peritonitis virus (FIPV strain 79-1146) and FIPV- $\Delta$ 3abcFL. FIPV 79-1146 is a serotype II feline coronavirus. FIPV- $\Delta$ 3abcFL contains a firefly luciferase gene in a FIPV 79-1146 background.<sup>30,31</sup> Mouse LR7 cells, an L-2 murine fibroblast cell line stably expressing the murine hepatitis virus receptor<sup>32</sup>, were used for the experiments with, and propagation of, MHV (strain A59) and the M gene MHV mutants Alb138, Alb244, Alb248 and MHV-EFLM. M gene MHV mutants designated Alb138, Alb244, Alb248 contained, respectively, an O-glycosylated, a N-glycosylated or an unglycosylated M protein at the amino-terminal ectodomain.<sup>33</sup> The Alb138 O-glycosylated mutant is biologically identical to MHV A59. MHV-EFLM contains a firefly luciferase gene in a MHV A59 background.<sup>30,31</sup> All mentioned cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 mg/L streptomycin. Titrations and tests were performed on the same medium containing only 5% FBS. MHV-EFLM<sub>HeLa</sub> is an MHV-EFLM strain propagated on HeLa cells stably expressing murine carcinoembryonic antigen cell adhesion molecule 1a (mCEACAM1a) (M. H. Verheije, unpublished data). For the production of FIPV- $\Delta$ 3abcFL<sub>HeLa</sub> HeLa cells stably expressing the feline coronavirus receptor, feline aminopeptidase N (fAPN) was used.<sup>34</sup> Both cell lines were maintained on DMEM containing 10% FBS, 100 IU/mL penicillin, 100 mg/L streptomycin and 0.5 mg/mL G418 (Life Technologies, Ltd, Paisley, UK).

### The influence of CBA on syncytium formation

For the syncytium formation experiment, LR7 cells were infected with MHV A59 and FCWF cells with FIPV 79-1146 both at a multiplicity of infection (m.o.i.) of 5. After a 1 h infection period, the cells were washed three times with PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$ . Subsequently the cells were incubated in the presence of 50 mg/L GNA, HHA or PRM-A or 6.25 mg/L UDA. Uninfected and infected cells without CBA addition were used as controls. After an additional 6 h incubation period at 37°C and 5%  $\text{CO}_2$ , the cells were fixed at –20°C for 10 min using 95% methanol and 5% acetic acid. The staining procedure was identical as described for the immunoperoxidase (IPOX) assay.

### Luciferase-based assay

FCWF or LR7 cells were infected with FIPV- $\Delta$ 3abcFL or MHV-EFLM, respectively, in the presence of various concentrations of the test compounds. FCWF or LR7 cell monolayers were infected at an m.o.i. of 0.5. The virus–drug mixture was preincubated at 37°C and 5%  $\text{CO}_2$  for 1 h and added to the cells after a single wash with DEAE PBS. The mixture was removed after 1 h. Cells were washed with PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$  and new test compounds in DMEM supplemented with 5% FBS were added in the same concentration. At 6 h post-infection (p.i.), the culture medium was 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

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effective concentration at which 50% of the luciferase expression was inhibited compared with the mock-treated cells ( $EC_{50}$ ) was calculated. The  $EC_{90}$  was the concentration of antiviral compound capable of reducing by 90% the luciferase expression in comparison with that of mock-treated cells.

*IPOX assay*

Antiviral activity measurements were based on the reduction of focus forming units when infected in the presence of various concentrations of the test compound. The cell monolayer was infected at an m.o.i. of 0.5. The virus–drug mixture was preincubated at 37°C and 5% CO<sub>2</sub> for 1 h and added to the cells after a single wash with DEAE PBS. The mixture was removed after 1 h. Cells were washed with PBS Ca<sup>2+</sup>/Mg<sup>2+</sup> and new test compounds in DMEM supplemented with 5% FBS were added. At 6 h p.i., the cells were fixed for 15 min with 4% formaldehyde and subsequently permeabilized with 70% ethanol for 5 min. IPOX detection of MHV A59 or the M gene MHV mutant (Alb138, Alb244, Alb248) positive cells was carried out by using a rabbit polyclonal antibody against MHV (K135)<sup>35</sup> in combination with a horseradish peroxidase (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 FIPV 79-1146 combined with an HRP-conjugated goat-anti-cat antibody (ICN Biomedicals Inc., Aurora, OH, USA). Focus forming units were counted by using the light microscope, and the effective concentration at which 50% of the infection was inhibited compared with the mock-treated cells ( $EC_{50}$ ) was calculated.

*Virus–cell entry assay*

The efficacies of 50 mg/L of GNA, HHA, UDA or PRM-A in inhibiting virus infection when present at different stages of the infection process were determined using MHV-EFLM. Monolayers of LR7 cells were grown in 96-well plates with DMEM containing 5% FBS, 100 IU/mL penicillin and 100 mg/L streptomycin. MHV-EFLM was preincubated with or without CBA on melting ice for 1 h. LR7 cells were also preincubated on melting ice for 15 min, washed with ice-cold DEAE PBS and inoculated with MHV-EFLM at an m.o.i. of 0.5 in the presence or absence of the antiviral compound, at 4°C. One hour post-infection, the cells were washed three times with ice-cold PBS Ca<sup>2+</sup>/Mg<sup>2+</sup>. To each well, 200 µL of pre-warmed (37°C) medium was added in the presence or absence of antiviral compound. At 6 h p.i., cells were lysed and the virus infection was scored using the luciferase assay.

*Antiviral activity of CBA against virus propagated in 1-deoxymannojirimycin (dMM)-treated cells*

A monolayer of LR7 cells and FCWF cells was infected with MHV-EFLM or FIPV-Δ3abcFL, respectively, at an m.o.i. of 0.5 following a prior wash with DEAE PBS. One hour after the onset of infection, the inoculum was removed, the cells were washed three times with PBS Ca<sup>2+</sup>/Mg<sup>2+</sup> and further incubated in DMEM containing 10% FBS, 100 IU/mL penicillin and 100 mg/L streptomycin and 1 mM dMM (Sigma Chemical Co., St Louis, MO, USA). At 9 h p.i., the medium was harvested and stored at –80°C. Virus derived from dMM-treated cells was designated MHV-EFLM<sub>dMM</sub> or FIPV-Δ3abcFL<sub>dMM</sub>. As a control, these viruses were also grown under the same conditions without the addition of dMM. The antiviral activity of CBA against the virus stocks derived from dMM- and mock-treated cells was compared. An antiviral assay was

performed in which the obtained viruses were used to infect the host cells at an m.o.i. of 0.5 with various amounts of GNA, HHA, UDA and PRM-A, ranging from 20 ng/mL to 100 mg/L. At 6 h p.i., cells were lysed and the virus infection was scored using the luciferase assay.

*Statistical analyses*

Statistical analyses were performed using the Student's *t*-test.

**Results***CBA prevention of syncytium formation*

Coronaviruses contain two glycosylated envelope proteins (M and S), which both may be targeted by CBA during virus entry. In order to discriminate between CBA binding to either the M or S protein, the influence of CBA on syncytium formation was studied. The expression of coronavirus S proteins on the cell surface is solely responsible for cell–cell fusion and formation of multinucleated giant cells (syncytia). The M protein does not play a role in this process. LR7 and FCWF cells infected with MHV A59 or FIPV 79-1146, respectively, were incubated in the presence or absence of CBA. Syncytia were abundant in the infected cells without CBA treatment, whereas syncytium formation was markedly reduced to a low level when CBA were present, although syncytia were not completely absent. Representative pictures are shown in Figure 1. UDA and HHA were the most potent syncytium-inhibiting agents. We conclude that the syncytium formation is significantly reduced in the presence of CBA, most likely due to binding of these compounds to the coronavirus S glycoproteins.

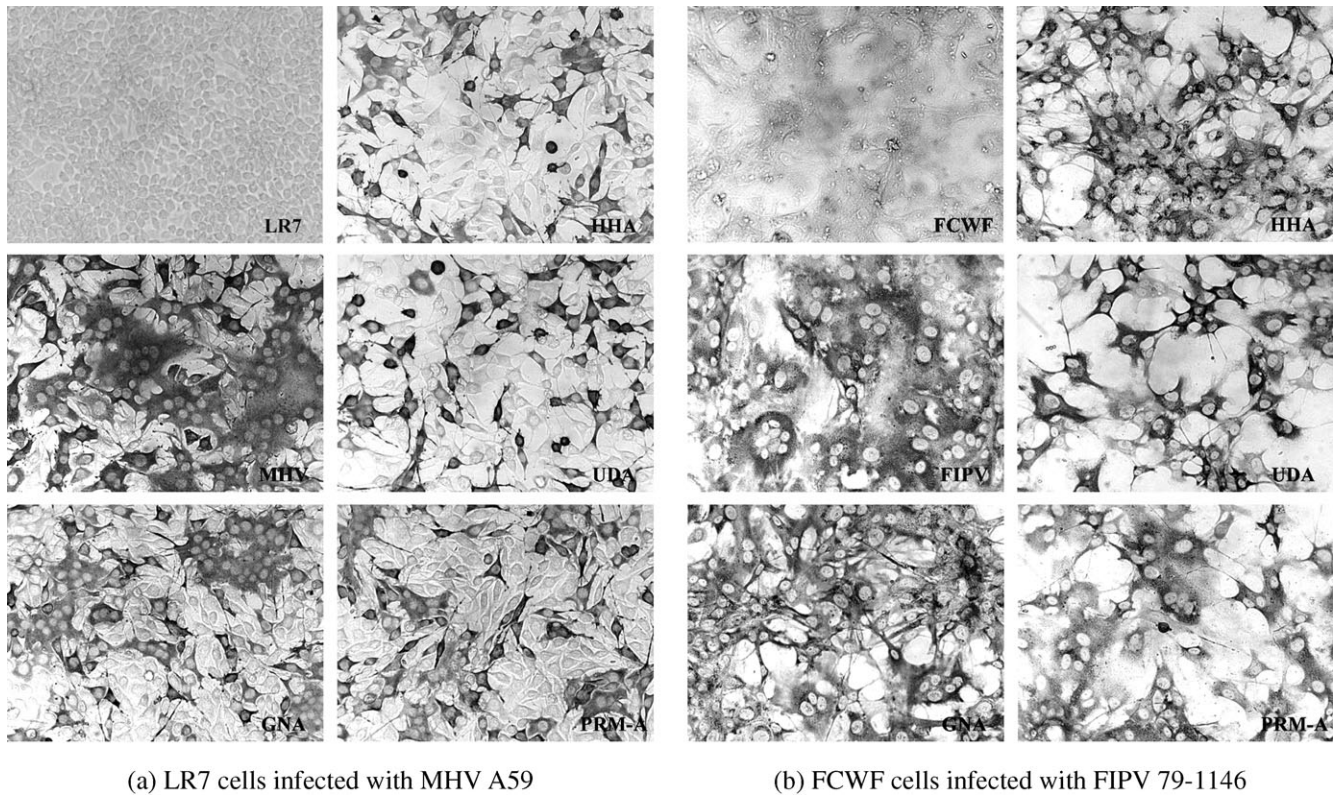
*Influence of M glycosylation on CBA efficacy*

Next, the targeting of the envelope glycoprotein M by CBA was examined. To this end, we used mutants of MHV, which express M proteins with either O-linked sugars (Alb138), N-linked sugars (Alb244) or have no sugars attached (Alb248).<sup>33</sup> Wild-type MHV A59 M contains an O-glycosylation site. The three different MHV variants were evaluated for their sensitivity to GNA, HHA, UDA and PRM-A. The  $EC_{50}$  values of the compounds were determined by IPOX (Table 1). Recombinant virus Alb248 (O–N+) showed the highest sensitivity to the CBA ( $EC_{50}$  0.4–1.2 mg/L for the plant lectins and 2.0 mg/L for PRM-A) among all mutant virus strains. The obtained Alb248 (O–N+)  $EC_{50}$  values were significantly different ( $P < 0.01$ ) from the Alb244 (O–N–)  $EC_{50}$  values ( $EC_{50}$  2.8–4.1 mg/L). The CBA  $EC_{50}$  values of Alb138 (O+N–) ( $EC_{50}$  1.8–2.7 mg/L) did not differ from the  $EC_{50}$  values obtained for Alb244 ( $P > 0.05$ ). Based on these results, the N-glycosylation site in the M glycoprotein can be regarded as a target for CBA. Thus, besides the S protein the M protein may be an additional antiviral target for the plant lectins and PRM-A.

*Fusion interception by CBA*

In order to distinguish between the attachment and the fusion stage of the two-step entry process, an assay separating these two stages was performed. In this assay, virus inoculation was





**Figure 1.** Immunoperoxidase staining of LR7 cells (a) and FCWF cells (b) infected with, respectively, MHV A59 and FIPV 79-1146. Upper left panels of (a) and (b) (designated LR7 and FCWF) are non-infected controls. Panels indicated with either MHV or FIPV are infected but not treated with CBA. One hour after infection, CBA were added (50 mg/L GNA, HHA and PRM-A; 6.25 mg/L UDA) for 6 h as indicated in the panels.

performed at a temperature of 4°C, allowing attachment of the S glycoprotein to the receptor, but not fusion since the temperature-sensitive conformational changes in the fusion protein required for membrane fusion are arrested at this temperature. The fusion process can start at an incubation temperature of 37°C. When cells and virus were inoculated at 4°C and subsequently incubated at 37°C (+/+), both steps in the presence of antiviral compounds, the number of MHV-EFLM infected cells (expressed as RLU) significantly reduced (Figure 2). The presence of CBA during the fusion but not the attachment stage only reduced the infection when UDA and PRM-A were used. The presence of HHA, UDA or PRM-A only during the binding stage (+/-) did not inhibit but surprisingly enhanced MHV-EFLM infection, an effect most pronounced for UDA.

**Table 1.** Influence of M glycosylation on the sensitivity of the virus to CBA measured through EC<sub>50</sub> values (mg/L)<sup>a</sup>

Glycosylation		Mutant	GNA	HHA	UDA	PRM-A
M protein						
O-N+	Alb248	0.4 ± 0.3*	1.2 ± 0.4*	0.7 ± 0.2*	2.0 ± 0.5*	
O-N-	Alb244	3.7 ± 4.1	2.9 ± 0.6	2.8 ± 2.2	4.1 ± 1.8	
O+N-	Alb138	1.8 ± 0.5	1.8 ± 0.5	2.7 ± 1.3	5.7 ± 0.3	

<sup>a</sup>The EC<sub>50</sub> (± SD) was determined using the immunoperoxidase assay.

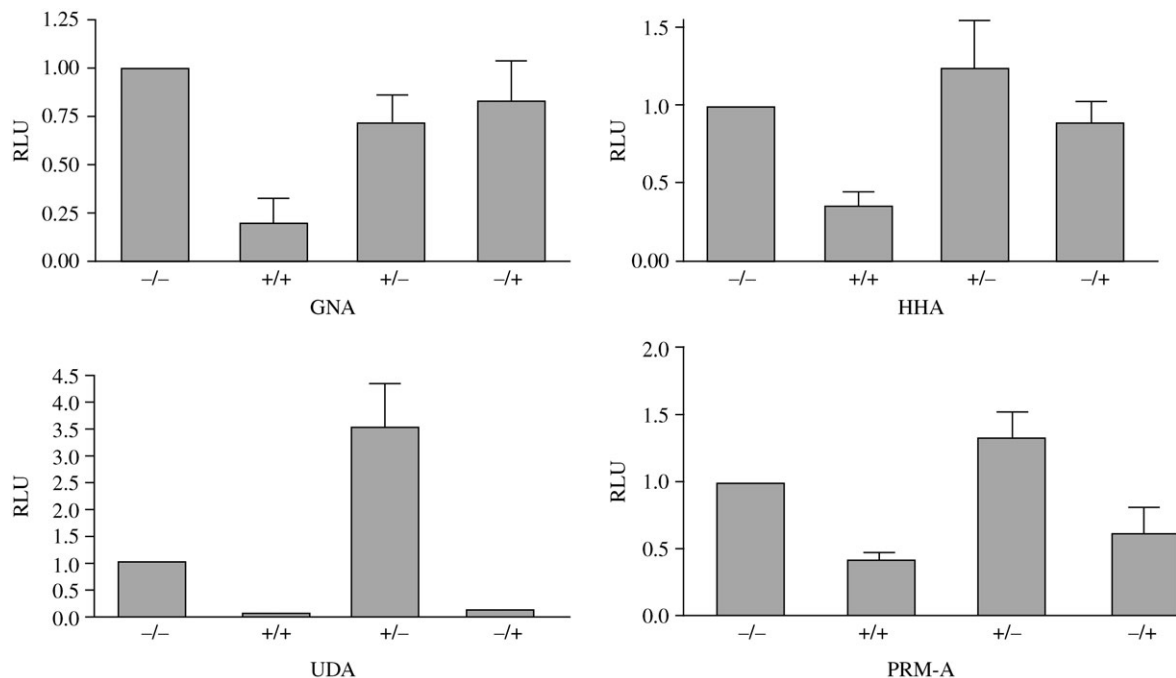
\*The Alb248 (O-N+) EC<sub>50</sub> values were significantly different from the Alb244 (O-N-) EC<sub>50</sub> values ( $P < 0.01$ ).

Similar procedures performed with GNA (+/-) led in some but not all cases to a slight enhancement of infection (not shown). Summarizing, in order to inhibit virus infection, the mannose-binding plant lectins GNA and HHA must be present during the entire infection process. UDA and PRM-A primarily inhibit at the post-receptor-binding stage.

#### *Effect of CBA on MHV-EFLM and FIPV-Δ3abcFL derived from dMM-treated cells*

The N-linked glycans attached to the viral proteins undergo extensive processing by cellular enzymes. The maturation stage of the N-linked glycans is likely to influence the inhibitory capacity of CBA. To study this in more detail, viruses were grown on cells treated with dMM. Since dMM inhibits mannosidase activity in the Golgi complex, its addition to host cells results in progeny virions carrying envelope proteins containing high-mannose-type glycans. The influence of high-mannose-containing N-glycans on viral glycoproteins was evaluated using MHV-EFLM and FIPV-Δ3abcFL both derived from their host cells treated with dMM (designated MHV-EFLM<sub>dMM</sub> and FIPV-Δ3abcFL<sub>dMM</sub>). MHV-EFLM<sub>dMM</sub> showed in all cases a much higher sensitivity to CBA inhibition compared with virus derived from non-dMM-treated LR7 cells (Figure 3). In previous studies, very limited PRM-A antiviral activity towards FIPV-Δ3abcFL was detected at 120 μM.<sup>20</sup> Interestingly, FIPV-Δ3abcFL<sub>dMM</sub> was clearly sensitive to PRM-A. Moreover, the GlcNAc-binding lectin UDA showed a higher inhibitory potency

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**Figure 2.** Influence of CBA during receptor binding and viral fusion. Antiviral activity of CBA (plant lectins GNA, HHA and UDA and the non-peptidic antibiotic PRM-A) during separate phases of the infection process of MHV-EFLM on LR7 cells. The experiments were evaluated using the luciferase assay. In all graphs, the first bar represents the RLU production detected in the absence of CBA (-/-) during both incubation phases (normalized to 1). Note the differences on the y-axis. The second bar indicates the RLU production in the presence of CBA during both incubation periods (+/+). The third bar represents the RLU production when CBA were present only during the 4°C but absent during the 37°C incubation period (+/-). The last, fourth bar, shows the RLU production when CBA was present only during the 37°C period and not during the 4°C incubation period (-/+). The bars represent the average value of three separate tests; the error bars represent the standard deviation normalized to the (-/-) experiment.

towards MHV-EFLM<sub>dMM</sub> and FIPV-Δ3abcFL<sub>dMM</sub> infection of cell cultures compared with non-dMM-treated viruses. This gain in antiviral activity of CBA to virus derived from dMM-treated compared with non-dMM-treated (WT) cells was represented in the EC<sub>50</sub> and EC<sub>90</sub> values (Figure 3). Our results indicate that the CBA activity is determined by the amount of high-mannose-type glycans present on the viral glycoproteins.

#### *Effect of pradimicin A on FIPV-Δ3abcFL and MHV-EFLM depends on the nature of the host cell*

Differences between FIPV and MHV with respect to PRM-A sensitivity might be attributed to the different host cells used for virus propagation. To analyse this, FIPV-Δ3abcFL<sub>HeLa</sub> and MHV-EFLM<sub>HeLa</sub> were grown on HeLa cells expressing fAPN and mCEACAM1a, respectively. The antiviral activity of PRM-A was subsequently evaluated on the HeLa-derived viruses and compared with FCWF- or LR7 cell-derived virus strains.

FCWF cell-derived FIPV-Δ3abcFL<sub>FCWF</sub> was, as expected, still refractory to PRM-A exposure but FIPV-Δ3abcFL<sub>HeLa</sub> increased in susceptibility towards PRM-A (EC<sub>50</sub> FIPV-Δ3abcFL<sub>HeLa</sub>: 4.8 mg/L; EC<sub>50</sub> FIPV-Δ3abcFL<sub>FCWF</sub>: >100 mg/L). Pradimicin A was also more effective towards MHV-EFLM<sub>HeLa</sub> compared with MHV-EFLM<sub>LR7</sub> (EC<sub>50</sub> MHV-EFLM<sub>HeLa</sub>: 0.25 mg/L; EC<sub>50</sub> MHV-EFLM<sub>LR7</sub>: 5.4 mg/L) (Figure 4).

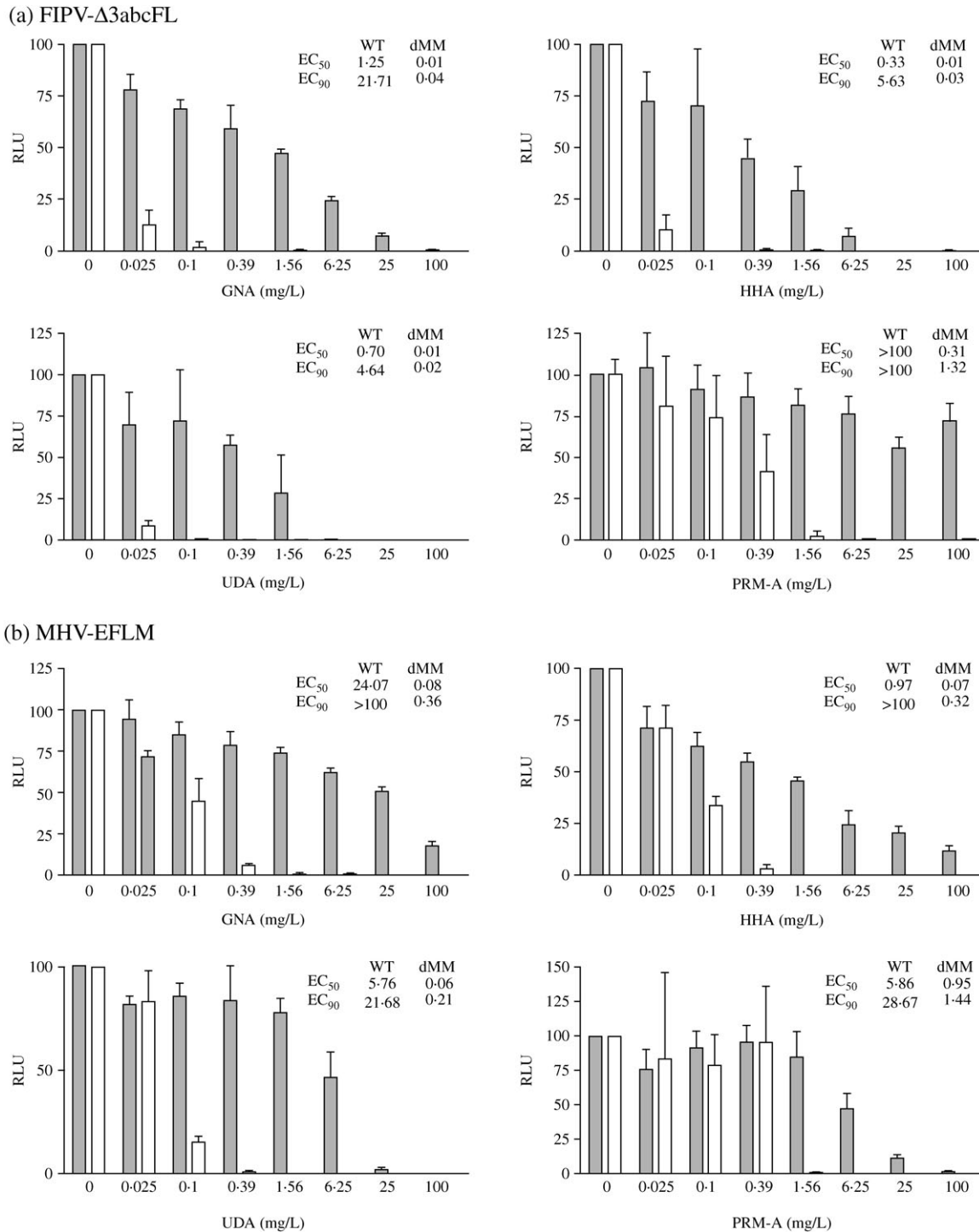
The results indicate that the host cell significantly contributes to the sensitivity of viruses to CBA. In addition, it has to be noted that PRM-A inhibited FIPV-Δ3abcFL<sub>HeLa</sub> to a lesser extent than MHV-EFLM<sub>HeLa</sub>. This indicates that the PRM-A

antiviral activity is not only affected by the host cell used to grow the virus stock, but also by the virus itself.

### Discussion

During a productive coronavirus infection, the S protein undergoes a series of conformational changes following attachment to the viral receptor molecule. This interaction eventually mediates fusion of the viral envelope with the host cell membrane.<sup>36,37</sup> CBA are likely to interfere during the virus entry process by attaching to the N-glycans of the S protein, although the exact mode of action of CBA is not clear. For HIV-1, CBA are thought to inhibit the infection via binding to gp120, resulting in steric hindrance or detrimental conformational changes.<sup>19</sup> For the SARS coronavirus, CBA were proposed to exhibit a dual mode of action: inhibiting both virus fusion and exocytosis or viral egress from the cell.<sup>38</sup> Our studies demonstrated that CBA interfere with the coronavirus entry process by targeting of the N-glycosylated M and S envelope proteins. Furthermore, the antiviral efficacy of the CBA was significantly affected by the maturation state of the N-glycans on the envelope glycoproteins.

Following coronaviral infection of FCWF cells (with FIPV 79-1146) or LR7 cells (with MHV A59) syncytia appeared. We showed that GNA, HHA, UDA and PRM-A markedly diminished the generation of these multinucleated giant cells. This effect is reminiscent of inhibition of syncytium formation by CBA observed in co-cultures of HUT-78/HIV-1 and Molt-4 cells.<sup>18,19</sup> Syncytium formation upon coronavirus infection is



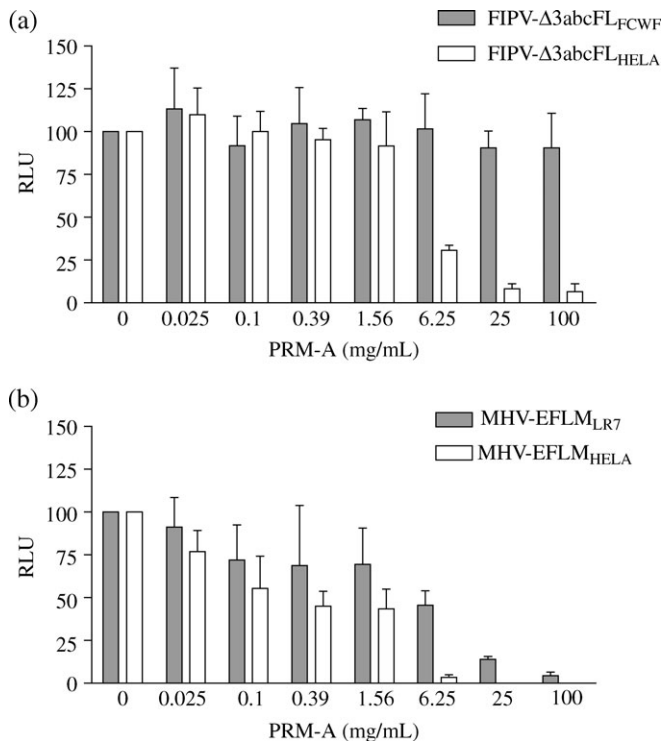
**Figure 3.** CBA efficacy against viruses derived from dMM-treated host cells. FIPV- $\Delta$ 3abcFL (a) propagated on FCWF and MHV-EFLM propagated on LR7 (b) were both evaluated for their sensitivity to the lectins GNA, HHA, UDA and to PRM-A. Bars represent the average of triplicate experiments. Whiskers represent standard deviation. White bars represent viruses derived from cells subjected to 1 mM dMM. Grey bars represent viruses derived from cells without dMM treatment (WT). Viruses were subject to treatment with different amounts of CBA (indicated at the x-axis). The RLU production as determined by a luciferase assay is depicted on the y-axis. The relative RLU production is normalized to the RLU production of non-CBA-treated (0 mg/L) viruses. Indicated in the figure are the EC<sub>50</sub> and EC<sub>90</sub> values for both WT- and dMM-virus in mg/L.

solely induced by the S protein,<sup>39</sup> indicating that this protein is a direct target of CBA. Similarly, for HIV-1, the syncytium-inducing gp120 was shown to be selectively targeted by GNA.<sup>40,41</sup>

The most abundant protein of the coronavirus envelope is the virus membrane protein (M). This protein is N-glycosylated for most coronaviruses including infectious bronchitis virus, porcine transmissible gastroenteritis virus and FIPV.<sup>42–44</sup> The MHV A59



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**Figure 4.** Host cell influences on CBA efficacy. (a) FIPV-Δ3abcFL derived from fAPN expressing human HeLa cells (FIPV-Δ3abcFL<sub>HeLa</sub>) were compared with FIPV-Δ3abcFL<sub>FCWF</sub> derived from feline FCWF cells. The RLU production of the viruses subject to different amounts of PRM-A was normalized to the mock-treated virus (0 mg/L). (b) MHV-EFLM derived from mCAECAM expressing human HeLa cells (MHV-EFLM<sub>HeLa</sub>) were compared with the same virus derived from murine LR7 cells (MHV-EFLM<sub>LR7</sub>). The RLU production of the viruses subject to different amounts of PRM-A was normalized to the mock-treated virus (0 mg/L). RLU were determined by the luciferase assay and displayed on the y-axis. PRM-A concentrations are shown on the x-axis.

M protein becomes O-glycosylated at a single site.<sup>45,46</sup> We studied the influence of M protein glycosylation on the antiviral effect of CBA using MHV recombinants. These recombinants contained either N-, or O-linked sugars or no sugars at all at the MHV M ectodomain.<sup>33</sup> A higher antiviral activity of the compounds was detected against viruses containing M protein that is N-glycosylated. Thus, besides the S protein, the M protein may also be a target for CBA.

The CBA inhibitory activity against HIV mainly results from intervention of the CBA in the viral fusion process,<sup>18,19</sup> rather than with gp120 attachment to the HIV receptor. Similar results were obtained for MHV, as virus attachment was not inhibited by all CBA tested. PRM-A and UDA were able to efficiently block virus entry when added at a post-binding step. The inhibitory activity of HHA and GNA however, only became apparent when these agents were present during the entire entry process. This result may be explained by the difference in size between the CBA. While HHA and GNA are ~50 kDa in size, PRM-A and UDA are much smaller (0.83 and 8.5 kDa, respectively). We speculate that the large CBA must bind to the S protein prior to the interaction of the S protein with the receptor. Strikingly, an enhancement of virus infection was noticed when some CBA were present during the attachment phase only.

This surprising result might be explained by a tethering effect of CBA, which might facilitate virus–cell attachment and subsequently productive infection.<sup>47</sup> Indeed, the CBA evaluated in this study are able to bind multiple carbohydrate moieties.<sup>11,12</sup>

The role of host cells in determining the sensitivity to CBA was underscored by the observed altered sensitivity of genetically identical viruses, which were propagated on different cell lines. Since glycans on virus envelope proteins are synthesized by the host cell glycosylation machinery,<sup>8</sup> these results indicate that cell-specific glycosylation may influence the ability of CBA, in this case PRM-A, to inhibit virus infection.

The influence of host cell-specific glycosylation was also demonstrated when viruses were grown on natural host cells in which the glycan maturation was interfered with by dMM. The addition of dMM leads to inhibition of the Golgi enzymes mannosidase I and II which remove mannose residues from (viral) glycoproteins. As a consequence, the viral glycoproteins contain immature N-glycans of the high mannose type.<sup>48</sup> This resulted in a much higher sensitivity of the viruses for the CBA tested. Apparently, the mannose content of the N-glycan trees determines the CBA efficacy. It should be noted that also the antiviral activity of UDA increased towards viruses carrying high mannose glycans. At a first glance, this might suggest that UDA possesses both a GlcNAc and mannose tropism. Alternatively, UDA has been shown to bind more efficiently to GlcNAc in the presence of high amounts of mannose residues.<sup>21</sup>

More detailed insight in the mode of action of CBA is imperative in the further development of antiviral therapeutic approaches using these agents. The use of CBA as microbicides to prevent HIV infection *in vivo* has already been proposed.<sup>49</sup> As coronaviruses induce diseases with a more acute onset and exploit different infection routes, the external (topical) application of these agents in order to prevent a coronavirus infection will be less appropriate. Systemic application of CBA seems required in order for CBA to function as anti-coronaviral agents. Our next goal will therefore be the establishment of a safe and systemic applicable form together with an optimized dosage regimen to impede coronavirus infections.

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**Transparency declarations**

None to declare.

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