Evaluation of antivirals against corona- and lentiviruses in cell cultures

Frank van der Meer

Meer, Franciscus Johannes Ubald Maria van der
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Evaluation of antivirals against corona- and lentiviruses in cell cultures

De evaluatie van antivirale agentia gericht tegen corona- en lentivirussen in cel cultures

(met een samenvatting in het Nederlands)

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Franciscus Johannes Ubald Maria van der Meer

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Promotor : Prof. dr. P.J.M. Rottier

Co-promotor : Dr. H.F. Egberink

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“But *now*- but now that I know, now that I fully realize! O what a flowery track lies spread before me, henceforth! What dust-clouds shall spring up behind me as I speed on my reckless way!”

Toad

Contents

Chapter 1

General introduction

Virus infections constitute a continuous health threat. As illustrated by the recent emergence of new - the SARS coronavirus - and the re-emergence of known viruses - influenza, ebola - this threat is certainly not yet decreasing. Rather, many conditions and considerations indicate that viruses will continue to pose problems (66, 67, 84, 90) and that vigilance and preparedness will continue to be needed.
Prevention and intervention are the two main strategies to counter viral infections. Prevention is achieved by means of vaccination and a considerable number of viruses can indeed be controlled by available vaccines, particularly against viruses causing acute infections (106). Yet, for numerous viruses no effective vaccines exist, due for instance to the nature of the virus (e.g. antigenic variation of influenza virus and HIV) or the infection (persistence of HIV and herpesviruses). Intervention options to treat ongoing viral infections are very limited. Just a few antiviral agents are currently on the market, of which the anti-influenza virus, anti-herpesvirus and anti-HIV compounds obviously have had the greatest impact. These examples clearly demonstrate the potential power of antiviral strategies in complementing vaccination approaches.
Hence, the focus of this thesis is on the development of antivirals. As targets of our intervention studies we selected lenti- and coronaviruses because these viruses are the central focus in our laboratory already for decades. In addition to extending earlier investigations of nucleoside derivatives for their effects on lentivirus infection we explored the therapeutic possibilities of a new group of antiviral compounds that target the glycan structures present on the glycoproteins of these enveloped viruses. I start this introduction with a brief account on virus resistance development, which is followed by a summary about lentiviruses and coronaviruses and their life cycle, after which I briefly describe the process of eukaryotic protein glycosylation. Finally, I present the aims and scope of the thesis.

1. Virus resistance development, a major problem in antiviral therapy

About 20 years before the identification of human immunodeficiency virus (HIV), Horwitz and co-workers (46) synthesized 3'-azido-3'-deoxythymidine (AZT = zidovudine, a nucleoside reverse transcriptase inhibitor [NRTI]). Although shown to inhibit murine lentivirus infection (69), interest in this compound suddenly increased after the emergence of HIV (65). It was recognized that treatment with this drug is clinically beneficial in patients with advanced disease but only temporary, because the virus appeared to develop resistance to the compound already during the clinical trials (56). The genetic changes (mutations) responsible for this resistance were found to map to the sequence encoding the reverse transcriptase (RT) (57) (paragraph 2.1). It proved to be very difficult to elucidate

the resistance mechanism. The mutants exhibited an increased rate of reverse transcription of chain-terminated cDNA (1, 64). This example illustrates the swiftness and ingenuity by which lentiviruses are able to escape from the applied drugs.
Subsequent evaluations of additional compounds revealed that in almost every case resistant variants eventually appeared in the patient or that they could be generated *in vitro* by passaging the virus in the presence of the drug. Important in these studies was the time (i.e. the number of passages) needed for the virus to develop resistance. This time is determined by the genomic location where the relevant changes occur and by the necessary number of mutations. When an HIV quasi-species[a] is exposed to an antiviral agent or a combination of such agents, the viruses compete among themselves for dominance while a selection occurs for the best-fit mutants under the given circumstances (35). A great diversity of variants is generated during the replication process, due to the high mutation rate of RNA viruses. For HIV-1, one out of every 10^3 -10^4 nucleotides will be mutated, implying 1 to 10 bases per genomic reverse transcription (68). Mutations generated under antiviral pressure usually result in changes in the targeted protein. To compensate for the loss of fitness, in many cases viruses will emerge that carry additional amino acid changes in the protein.
Development of resistance is an observation so common that it can often be used practically to distinguish whether an antiviral effect is indeed a direct consequence of the interference of the drug with viral infection or merely a result of cytotoxicity of the agent (45). If no escape mutants arise, the drug is likely to exert its effect indirectly by affecting the physiology of the cell.

2. The virions

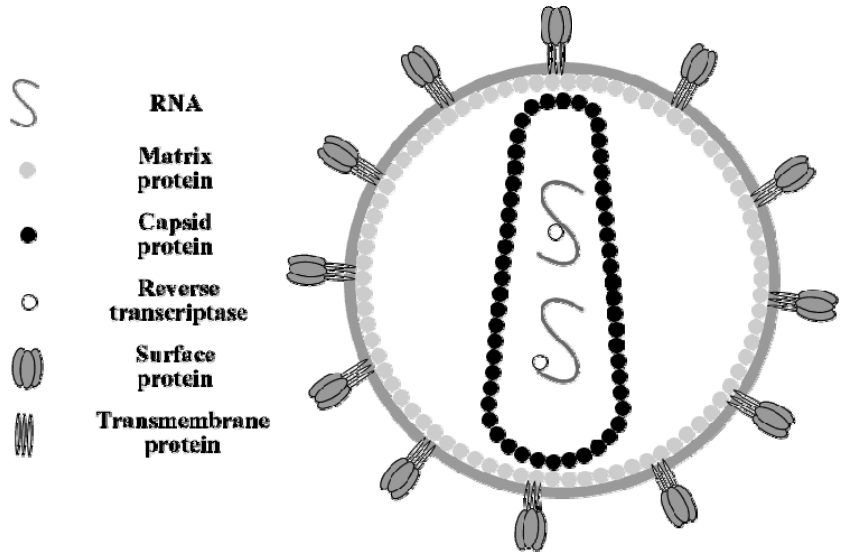
2.1 *Retroviridae*

The family *Retroviridae* is subdivided in seven genera: alpha-, beta-, gamma-, delta-, epsilon retrovirus, lentivirus and spumavirus (47). HIV, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) have all been assigned to the lentivirus genus. Virions are spherical, enveloped and contain glycoprotein surface projections. The ENV gene encodes the surface (SU) and transmembrane (TM) proteins. These glycosylated proteins together with a host derived lipid bilayer form the viral outer envelope. In the cone-shaped capsid two linear, positive sense, single stranded RNA copies are packaged. The non-glycosylated structural proteins contributing to the interior of the virion are the

[a] The genetic heterogeneity of the viral population within an infected individual.

matrix (MA), capsid (CA) and nucleocapsid (NC) proteins, all encoded by the gag gene. Non-structural proteins like the protease (PR), reverse transcriptase (RT) and integrase (IN) are encoded by the POL gene and have enzymatic functions in the retrovirus replication process. In FIV a protein called dUTPase (DU), the function of which is still under debate, is additionally present. The name '*Retroviridae*' is derived from the ability of these viruses to reverse the normal genetic transcription of DNA into RNA. By using the reverse transcriptase enzyme (3, 92) DNA copies of the viral genomic RNA are generated and subsequently incorporated as proviral DNA in the genome of the host.

Figure 1. A schematic representation of the lentivirus virion, based on HIV



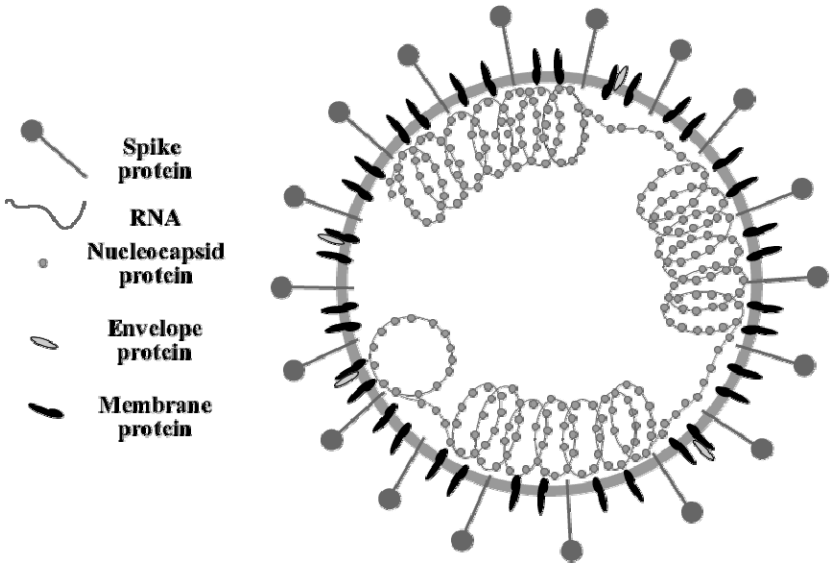
Shortly after the discovery of HIV the simian version of a lentivirus (SIV) was recognized (27, 105). Although not entirely similar in its pathogenesis and genome organization, this virus resembles HIV to such an extent that it is used as a model for HIV. It took until 1987 before feline immunodeficiency virus (FIV) was isolated from several cats displaying an immunodeficiency-like syndrome (70). FIV is presently recognized by many researchers as an important feline model for HIV as well (13, 37, 102).

2.2 *Coronaviridae*

Coronaviridae constitute, together with the *Arteriviridae* and *Roniviridae* family, the *Nidovirales* order (47). The name 'coronavirus' is derived from these viruses' crown like appearance under the electron microscope. The *Coronaviridae* are a group of enveloped, single stranded positive-sense RNA viruses subdivided in three separate genera or groups based on serological cross reactivity and genetic relatedness (20). The coronavirus virion contains four essential structural proteins: the membrane (M), the envelope (E), the spike (S), and the nucleocapsid (N) protein. The N protein wraps the genomic RNA into a nucleocapsid and is not exposed on the outside of the virus particle. A lipid membrane with the S, M, and E proteins forms the envelope.

Trimers of the heavily glycosylated S protein protrude from the virion membrane. The attachment of the S protein to the coronavirus receptor is the first step of the viral entry process. Moreover, the S protein is responsible for cell-cell fusion (31).

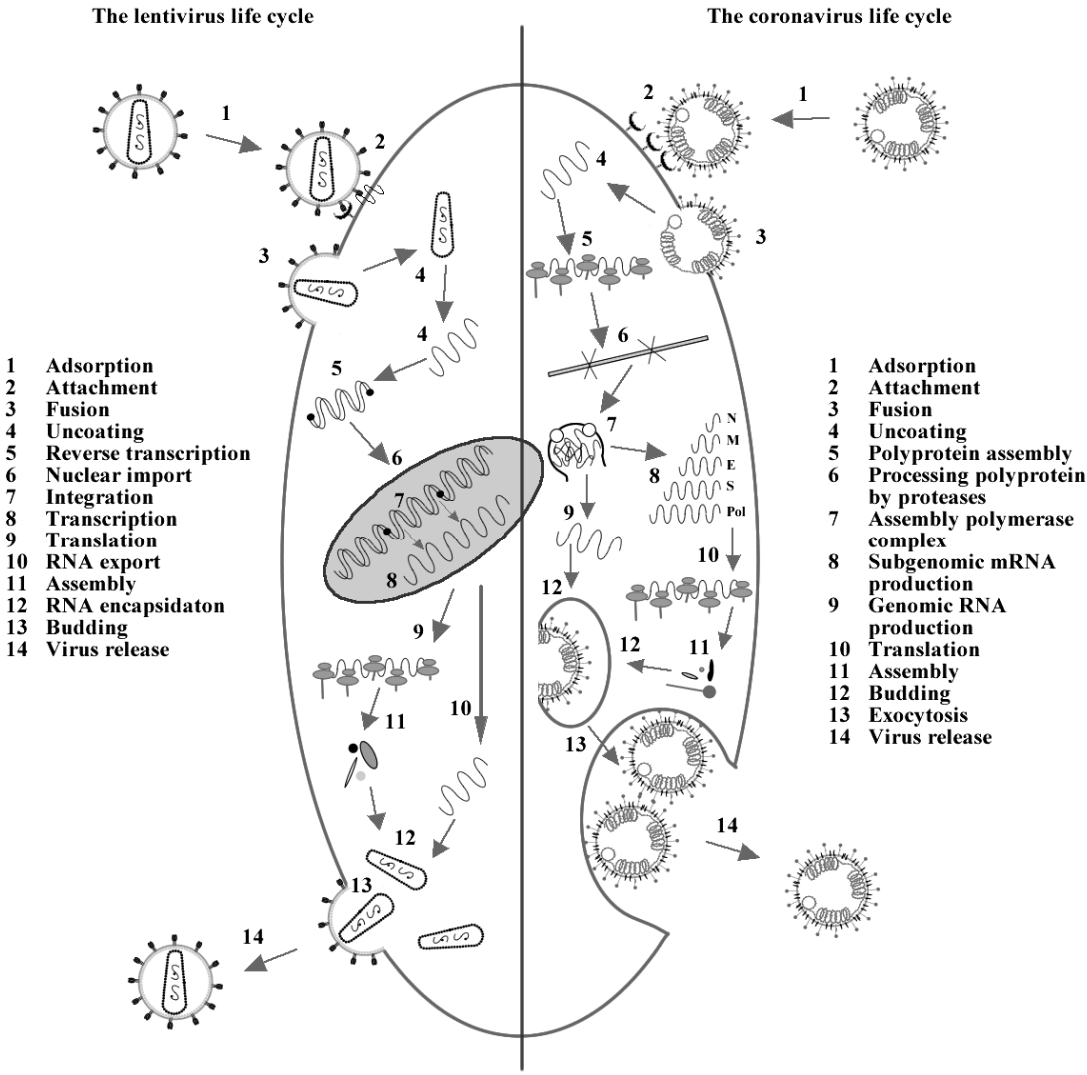
Figure 2. A schematic representation of a coronavirus virion



Coronaviruses have been isolated from humans and from a variety of animal species. NL-63, 229E, OC43, HKU1 and SARS-coronavirus (36, 41, 54, 55, 71, 75) are all human coronaviruses causing respiratory tract infections, though with varying degrees and modes of pathogenicity. The mouse hepatitis virus (MHV) (2,

21) is associated with neurological and liver disorders and currently regarded as the coronavirus type model. Feline infectious peritonitis virus (FIPV) (99) causes a lethal inflammation of the intestinal and pleural cavity in cats. Both these animal viruses are used as coronavirus model systems.

Figure 3. Lentivirus (left) and coronavirus (right) life cycle



3. The virus life cycle: targets for antivirals

The antiviral agents currently available for clinical use interfere with only a few phases during virus replication. There are, obviously, numerous possibilities left to be explored. Many of those options for new antiviral chemotherapeutics can, at least in theory, be indicated in the coronavirus and lentivirus life cycles. The division of these cycles in different stages may sometimes be an oversimplification or even be unjustified, as virus infection is a continuous process with, once started, many phases occurring simultaneously. Yet, blocking one of these phases effectively will inhibit the production of progeny virus.

3.1 The lentivirus life cycle

Adsorption: reaching the host cell [stage 1]

The lentivirus is introduced into its host via sexual transmission, blood contact or maternal infections (intra-uterine or by breast feeding). Bite accidents are an important additional route of introduction for FIV (104). Preventing the lentivirus from reaching the cells of the mucosal surface can block the infection. This blockade is possible with so called microbicides (74, 82, 87). These substances are not applied systemically but topically, and can inactivate virus particles by binding to them. This may allow the use of compounds known or expected to be toxic when applied systemically, as exemplified by the carbohydrate binding compounds (6, 17). These compounds can inhibit infection by enveloped viruses like corona- and lentiviruses by binding to sugar moieties exposed at the virus surface (4, 5, 10, 16, 48, 49, 98) and were shown effective in a monkey vaginal transmission model (94).

When pathogens eventually reach the mucosal cell layer or gain access to the submucosa, they are encountered by dendritical cells (DCs) (14). These antigen-presenting cells bind and process the particles, and present the processed antigens on MHC Class II molecules to T helper cells (CD4+) in lymph nodes (11, 12). Contact between DC and resting T cells is essential to initiate a primary immune response. Lentivirus uptake by the DC is mediated by DC-specific C-type lectins (43). Also FIV can bind to human DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) (33). A feline version of this molecule has currently not yet been identified. Lentiviruses hijack DCs for their transport towards its target cells (T-cells) and so escape immune surveillance (96). DCs loaded with HIV are able to stimulate resting T cells thereby enhancing HIV infection (19, 86). This specific HIV-DC interaction could be inhibited *in vitro* when oligosaccharide binding proteins were added (9, 95) or by using compounds that mimic the natural organization of high mannose structures (88). Peptides were able to block HIV transfer from DCs to T cells (97).

Attachment, fusion and uncoating [stages 2 to 4]
Binding [*stage 2*] to the CD4 (HIV) (26, 51) or CD134 (FIV) (32, 83) molecule and subsequent interaction with the chemokine co-receptors CCR5 (HIV (34)) and CXCR4 (FIV (103) and HIV (39)) can be regarded as the first step in the entry process. Inhibition of attachment to CD4 was studied shortly after the HIV virus was discovered. The recognition of the CD4 molecule as a receptor for HIV initiated the development of competitor molecules able to interfere with the virus attachment process, the first one of which was the peptide analogue designated 'peptide T' (72). Blocking the chemokine co-receptors is still under research. Through a series of conformational changes in the ENV protein the virus envelope fuses [*stage 3*] with the cell membrane thereby releasing the viral genetic material into the cell's cytoplasm. These conformational changes can be blocked by compounds attaching to the participating gp120 (SU) or gp 41 (TM) subunits (100, 101). Also carbohydrate binding agents can interact with the viral glycoproteins during the fusion process (7, 8), thereby preventing virus entry. These and other entry inhibitors are and will be of interest (18, 23, 50, 59, 73, 80). Little has been reported about inhibitors of viral uncoating [*stage 4*] (29). Secreted phospholipases A_2 (38) were considered recently, as were compounds targeting the zinc finger motives in the NC protein. The NC attaches firmly to the HIV genome. Compounds targeting the uncoating process are probably not only interfering during uncoating but also during virus assembly processes [*stages 11-12*].

From reverse transcription to chromosomal integration of provirus [stages 5 to 7]
The genomic HIV-1 RNA is reverse transcribed in the cytosol by the RT enzyme [*stage 5*]. The resulting proviral double stranded DNA remains in a nucleoprotein complex, the preintegration complex (PIC), which additionally contains all the necessary functions required to incorporate the provirus into the host genome, including the integrase enzyme (IN).
Several RT inhibitors are commercially available, acting in different ways. Nucleoside analogue RT inhibitors (NRTI) must be metabolically activated by phosphorylation within cells. The resulting 5′ triphosphates terminate the polymerisation reaction; no DNA copy is hence derived. Nucleoside analogues (NRTI) are converted into nucleotide analogues. When nucleotide analogues (NtRTI) are administered only 2 conversion steps are needed, in stead of 3 which are required for NRTI. A third group are the nonnucleoside RT inhibitors (NNRTI) (30). These inhibitors comprise a diversity of compounds and do not require metabolic activation. The currently licensed NNRTIs all interact with an allosteric pocket, a non-substrate binding site of the HIV-1 RT. A new enzymatic RT target involves the associated RNase H activity, an essential lentiviral enzyme that digests RNA only in an RNA/DNA hybrid duplex (93).

Before its integration in the host genome [*stage 7*] the provirus has to be transported into the nucleus [*stage 6*]. This transport gained much attention during research on HIV derived vectors. These vectors were developed for gene therapy. IN binding molecules are inhibiting the enzymatic activity; it is, however, not clear whether certain cellular proteins will be inhibited as well, hence caution is needed (25, 91).

From transcription to protein assembly [stages 8 to 11]
Another approach toward blocking HIV infection is by inhibition of several non-structural proteins, enzymes involved in transcription [*stages 8 and 10*], translation [*stage 9*] and virion assembly [*stage 11*]. In view of their importance and specificity, enzyme inhibition or substrate competition might offer very attractive inhibition possibilities (28). It is of note that, except for the rev-responsive element (RRE), FIV enzymes with functions equivalent to those of HIV have not been clearly identified.

Protein maturation, budding and release of new virions [stages 12 to 14]
Disruption of enzymes [*stage 11*] involved in protein glycosylation processes in the endoplasmic reticulum (ER) and/or Golgi apparatus have been successfully evaluated in the past (42). Further development, however, has not been pursued. When HIV buds from the cell [*stage 13*], precursor proteins are concomitantly cleaved by proteases to generate infectious viral particles [*stage 14*]. The prevention of GAG and GAG-POL precursor polyprotein cleavage into the structural proteins MA, CA, NC and the non-structural proteins PR, RT and IN constitute an attractive antiviral target (40). Inhibition of this cleavage process either by specific inhibitors (62, 63) or by mutation of the active site aspartic acid residue (53) leads to the accumulation of non-infectious, immature virus particles. The activity pattern of INF-α also includes disruption of late stages in the HIV replication process (24, 85). This might also be the mode of action towards FIV for which INF-α2 was found to be active (89).

3.2 The coronavirus life cycle

Adsorption: reaching the host cell [stage 1]
No preventive chemotherapeutical measures have so far been reported to prevent coronaviruses from entering the body [*stage 1*]. As the transmission encompasses the faecal-oral or oral-oral route, the use of microbicides, as described in the lentivirus section, is not feasible.

Attachment, fusion and uncoating [stages 2 to 4]
The binding to the coronavirus receptor [*stage 2*] provokes a series of conformational changes in the spike protein resulting in fusion of the envelope with the cell membrane [*stage 3*]. The process of uncoating [*stage 4*] involves cellular factors leading to a release of RNA into the cellular cytoplasm. Attachment of spike (S) glycoprotein to the cellular receptor can be blocked by (monoclonal) antibodies (60). Alternatively, specific binding inhibiting compounds like aurintricarboxylic acid (ATA), described originally to prevent HIV - CD4 binding (81), also showed antiviral activity towards coronaviral infections (44). Subsequent membrane fusion could be inhibited by plant lectins (48) and by peptides specifically targeting the coronavirus S protein (15, 76, 78).

From genomic RNA to new genomic RNA and nested mRNAs [stages 5 to 9]
The genomic coronavirus RNA introduced in the host cell can be used for translation [*stage 5*]. The replicase-transcriptase polyproteins are directly expressed from the POL 1a and 1b genes. These large proteins are cleaved by viral proteases [*stage 6*]. The resulting polymerase complex, which consists of RNA dependent RNA polymerase (RdRp), additional proteins and genomic RNA, produces negative stranded RNA [*stage 7*]. This is used, in turn, as a template to produce positive stranded subgenomic mRNAs, via discontinuous transcription, as well as new minus strand genomic RNA, via continuous transcription (52, 79). The subgenomic positive strand mRNAs consist of a nested set; each mRNA encodes one protein (monocistronic). The name *nidovirales* is used for the order of viruses to which the family *coronaviridae* belongs, *Nido*, from Latin *nidus*, meaning "nest", which refers to the nested set of subgenomic mRNAs (47). The RdRp enzyme, essential for the polymerase complex, can be regarded as an interesting target. Agents can be developed that bind to the enzyme's active site or, alternatively, nucleoside analogue inhibitors might be used.

From translation to protein assembly and budding [stages 10 to 12]
Structural proteins translated [*stage 10*] from sg mRNAs are assembled into virions in the Golgi-ER intermediate compartment, the actual site of budding [*stage 12*]. While the viral proteins S, M and E interact in the intracellular membrane, the N protein in the cytoplasm complexes with genomic RNA, forming helical structures which interact with M proteins to drive budding [*stage 11 and 12*]. No specific inhibitors of these processes have been described but interferon (IFN) (22), which was reported to inhibit coronavirus replication , might act during one of these stages, in analogy to its inhibition of lentivirus replication.

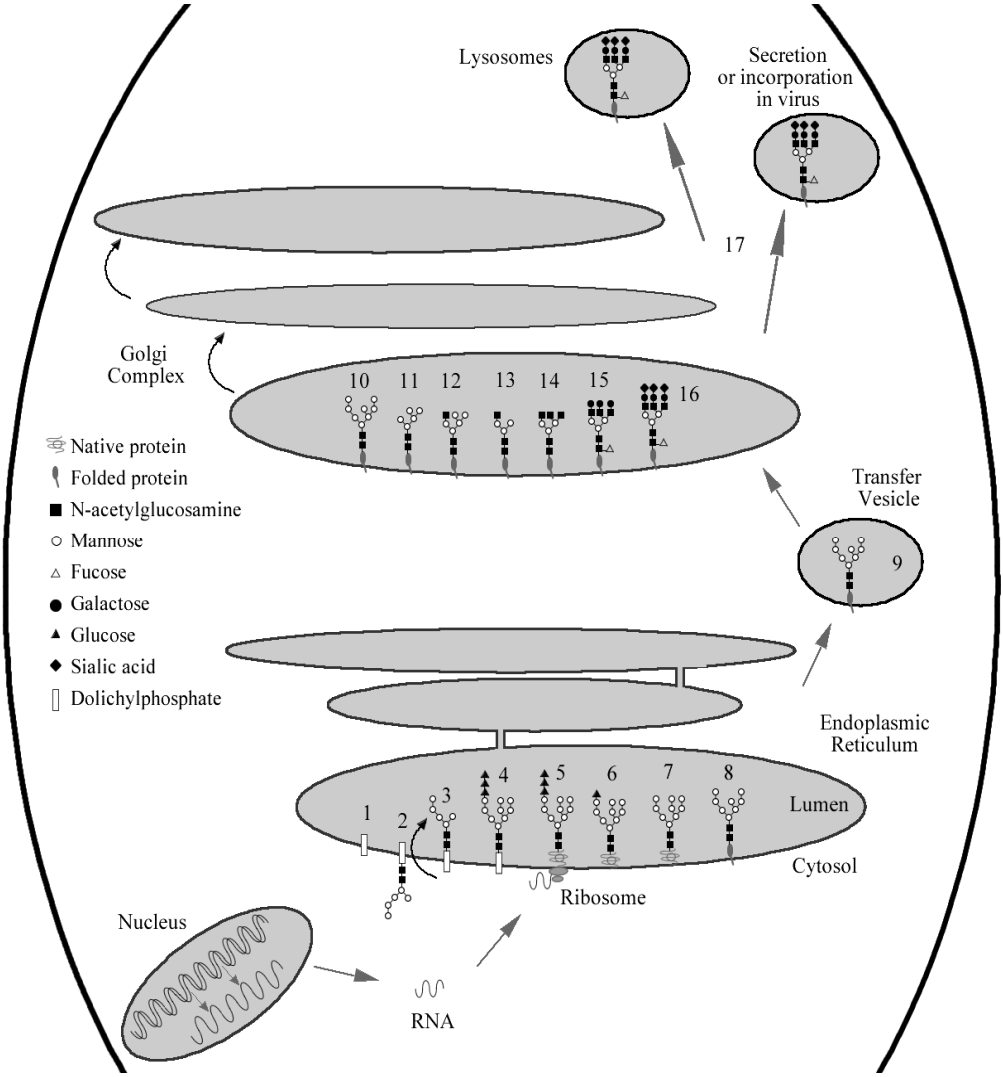
Transport to cell surface and virion release [stages 13-14]
Virus containing vesicles fuse with the cell membrane [*stage 13*] and are released by exocytosis [*stage 14*]. Also for these stages no inhibiting compounds have been described. This stage may not be sufficiently virus-specific to qualify as an antiviral target.

4. A new antiviral target: protein glycosylation

In this paragraph the process of protein glycosylation in the endoplasmic reticulum (ER) is summarized (5, 61, 77). Viral membrane proteins are usually glycosylated, often extensively, by the addition of oligosaccharide side chains to Asn residues (N-glycosylation). The lentivirus SU glycoprotein, for instance, consists for about 50% of its weight of sugars (5). These glycoproteins are eventually incorporated into the viral envelope.
The first step in N-glycosylation involves the assembly of an oligosaccharide precursor structure linked to dolichylphosphate (Dol-P) [*stage 1*]. The initial linkages involving the two core GlcNAc monosaccharides and the first five mannose residues [*stage 2*] occur on the cytosolic side of the ER membrane. The resulting Man5GlcNAc2-Dol precursor 'flips' [*stage 3*] across the membrane bilayer to become oriented to the lumen of the ER. Here four mannoses are added followed by three glucose residues [*stage 4*]. The completed oligosaccharide precursor is then ready to be transferred from the dolichylphosphate to an asparagine residue of a nascent polypeptide [*stage 5*]. Only asparagines occurring in a specific context are used as acceptor, i.e. when present in the motif Asn-X-Thr/Ser, where X can be any amino acid except for proline. Glucosidases I and II present in the lumen of the ER act to remove all three glucoses sequentially [*stage 6*]. While the proper protein folding of the polypeptide is taking place, the three glucoses and one mannose are trimmed away [*stage 7*] by different enzymes. Following glucose trimming and release from the ER, N-glycans become available for glycosidase reactions in the Golgi apparatus. In this stage the N-glycans are referred to as the high-mannose subtype, indicating that they terminate in unsubstituted mannose residues. Using vesicles [*stages 8-10*], the glycoprotein is then transferred to the Golgi complex. In the Golgi complex additional modifications take place. Distinct α-mannosidase enzymes I and II located in the ER and Golgi apparatus sequentially process (maturate) the high-mannose N-glycans. The terminal α(1,2) mannose residue is removed in the ER, followed by the α(1,3) and α(1,6) mannoses that are trimmed in the Golgi complex. The processed high-mannose Man5GlcNAc2-Asn N-glycan serves as a substrate for the further, sometimes heterogeneous maturation of N-glycans by the addition of

terminal sugar residues, e.g. GlcNAc, fucose, galactose and sialic acid, in the Golgi apparatus [*stage 11-16*].
Extracellular N-glycans in vertebrate glycoproteins occur as high-mannose, hybrid, or complex subtypes. Hybrid structures consist of both substituted (GlcNAc linkage) and unsubstituted mannose residues. In complex N-glycans both the α3- and α6-linked mannose residues are substituted with GlcNAc moieties. Most vertebrate extracellular N-glycans are found to be of the complex subtype. Multiple N-glycosylation sites on the same protein may contain different glycan structures. It appears that also factors other than protein sequence influence N-glycan diversification. Such factors may include sugar nucleotide metabolism, transport rates in the ER and Golgi complex, and the localization of transferases, and depend as well on the type of protein, the particular cell and the species. For HIV it was shown that the N-linked sugars of the envelope glycoprotein consist in particular of α(1,2), α(1,3) and α(1,6) mannose oligomers at the surface of the gp120 subunit (SU) (58). When the glycosylation process is completed the glycoprotein is generally exported out of the Golgi complex to be incorporated into the plasma membrane, to be secreted, or to be incorporated into the virus or it is transferred to a lysosome for degradation when improperly folded [*stage 17*].
There is no evidence that any virus encodes enzymes for the biosynthesis of its own N-linked glycans. As a consequence, whenever viruses carry oligosaccharides on their surface they are rely on their host cell's glycosylation apparatus. The characteristics of viral N-linked sugars are similar to the N-linked glycans found on glycoproteins of the host cell.
The various stages of glycosylation are indicated in figure 4.

Figure 4. Protein N-glycosylation in vertebrate cells.



5. Scope of the thesis

As mentioned above, the options for therapeutic treatment of viral infections are presently limited to a small number of viruses, but these few examples collectively testify to the potential power of antiviral drugs. As humans are obviously the prime target species for antiviral applications, there is a strong need for suitable systems, both *in vivo* and *in vitro*, to evaluate the efficacy of the compounds, their mechanism of action and the responses of the virus to the drug. Such systems should obviously reflect the host-pathogen interaction as close as possible. The scope of this thesis therefore is to study, using representatives of two RNA virus families, two classes of antiviral compounds, i.e. more classical nucleoside derivatives and new carbohydrate-binding agents.

With the aim of obtaining a suitable *in vitro* system for studies of antivirals with FIV, we started with the establishment of a dendritic cell-T cell co-culture infection system. As was shown for HIV, dendritic cells are probably the first immune cells a newly introduced lentivirus encounters in its host and they are instrumental in presenting the virus to the T cells. The results of this work are described in chapter 2.

The efficacy of antiviral compounds as determined *in vitro* is obviously dependent on the experimental conditions used. One important variable can be the infection system. To investigate whether and how the observed effective concentrations can vary when using different FIV infection systems we compared the activity of different antiviral compounds, as presented in chapter 3. Besides studying nucleoside derivatives we also analyzed carbohydrate-binding agents (CBA) for their inhibitory effect toward FIV. CBA are a new class of antivirals that target N-glycan structures and may hence be candidate inhibitors of enveloped viruses.

In chapters 4-6 the use of these CBA against coronavirus infections is described. Their antiviral activity towards different members of the *Nidovirales* order is described in chapter 4. Here three different approaches are evaluated to establish a 50% effective concentration.

In chapter 5 special attention was paid to the mode of action of CBA towards coronaviruses. The targets in the coronavirus envelope were identified and the influence of differences in glycan processing on the antiviral efficacy was studied.

In chapter 6 the specificity of the anti-coronaviral activity of CBA was determined by studying the generation of escape mutants. The selection of viral mutants able to resist CBA inhibition proves that the antiviral behaviour is due to a direct effect of the drug on virus infection.

Finally, the results obtained are summarized in chapter 7. In addition, suggestions for further research on antivirals and the perspectives of these compounds are discussed.

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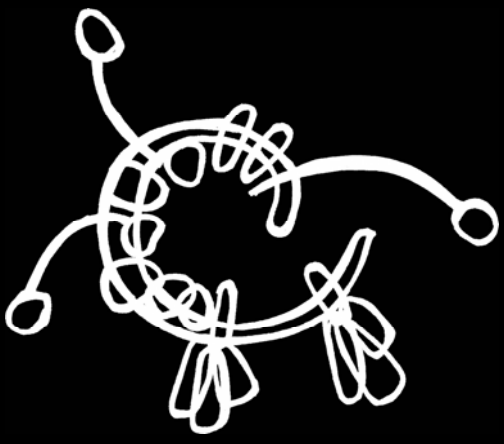

Chapter 2

Feline Immunodeficiency Virus infection is enhanced by feline bone marrow derived dendritic cells

F.J.U.M. van der Meer, N.M.P. Schuurman and H.F. Egberink

Department of Infectious Diseases and Immunology
Division of Virology, Faculty of Veterinary Medicine,
Utrecht University, Utrecht, The Netherlands

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Summary
In the pathogenesis of *feline immunodeficiency virus* (FIV) infection feline dendritic cells (feDC) are thought to play an important role. As with DC in other species feline DC are believed to transport viral particles to lymph nodes and transfer them to lymphocytes. Our investigation has focused on the ability of feDC to influence the infection of syngeneic PBMC and allogeneic thymocytes. FeDC were derived from bone marrow mononuclear cells which were cultured under the influence of feline IL4 and feline GM-CSF. Using these feDC in coculture with resting PBMC we were able to show an upregulation of FIV replication. An enhancement of FIV infection was also detected when cocultures of feDC-feline thymocytes were infected. To obtain this enhancement direct contact of the cells in the coculture was necessary; transwell cultures showed that the involvement of only soluble factors produced by feDC in this process is not likely. These feDC were also able to induce the proliferation of resting thymocytes, which might explain the enhanced FIV replication observed. Together these data suggest that feDC have similar abilities as has been shown for simian and human DC in the interaction with leukocytes. This system is suitable for further investigations on the interplay of DC and T cells during FIV infection *in vitro*.

Introduction

Feline immunodeficiency virus (FIV) is a lentivirus that causes an AIDS like syndrome in cats (28). An important stage in the pathogenesis of FIV is the earliest phase in which the virus is introduced in the cat. The infection will occur mainly via bite wounds, so the place of entry will be in most cases the skin (41). In the early stages of an HIV-1 and SIV infection, macrophages and dendritic cells (DC) play an important role in the uptake and dissemination of introduced virus particles. When DC encounters an immunological stimulus they migrate to search for antigen specific T cells. (2). Reports of an association of FIV with cells that showed characteristics of DC suggest an involvement of this cell type in the dissemination of the FIV particle to lymphoid organs (5, 27, 38). Besides this first mechanism, another contribution of DC to lentivirus infection of the host can be observed. Virions are efficiently presented to T-cells when associated with macrophages or DC. For HIV-1 and SIV infections this has been shown (9, 17, 31). For FIV this is unexplored, however, as FIV replicates both *in vivo* and *in vitro* in $CD4^+$ and $CD8^+$ T-lymphocytes (6) and DC can stimulate the proliferation of both cell types (1) we can assume that feline dendritic cells (feDC) will also play an important role in the pathogenesis of FIV infections.
Until recently feDC were not characterized, which hampered the research on this cell type. FeDC share many characteristics with DC found in other species. They are non-adherent *in vitro* and show processes, which can be regarded as dendrites. FeDC were shown to express CD1a and MHC II and having the ability of stimulating allogeneic T cells (4, 14, 34). Our goal was to evaluate the role of feDC in FIV infections *in vitro*. This study has focused on the influence of feDC on FIV infections in allogeneic thymocytes and syngeneic PBMC.

Methods

Cells and virus

Bone marrow was obtained from femurs of SPF cats. By clipping the femur with pincers the bone marrow was exposed. After rinsing of the femur cavity with PBS EDTA the cell suspension was strained through a 70µm filter (Cell Strainer BD Falcon Bedford, MA, USA), centrifuged 10 min at 600 g to remove excessive bone marrow fat and resuspended in 20 ml PBS EDTA. The bone marrow mononuclear cells (BMMC) were isolated by density gradient centrifugation (1.077 g/l, Lymfoprep® Axis-Shield PoC AS, Oslo, Norway) for 30 min at 1500 rpm. Cells were washed with Iscove's Modified Dulbecco's Medium (IMDM) and stored at -80 °C using DMSO/FCS until use. After thawing, BMMC were plated in 6 well dishes (Costar® Corning Inc., Corning, NY, USA) with IMDM containing Glutamax I (Sigma, St. Louis, MO, USA), 10% heat-inactivated FBS (Hyclone,

Logan, UT, USA), 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol, 10 ng/ml rfe GM-CSF (R&D systems, Minneapolis, MN, USA) and 10 ng/ml rfe IL4 (R&D systems, Minneapolis, MN, USA). After 24 hours non-adherent cells were removed by gentle rinsing with pre-warmed medium. The non-adherent cells were discarded and the remaining adherent cells were further cultured during 6 days in the presence of cytokines. Fresh cytokines were added on day 3. On day 6 the new formed non adherent cells derived from the culture were harvested and used for further experiments.
The macrophages were derived by the same procedure as the feDC except for the addition of rfe GM-CSF and rfe IL4. The cells obtained were adherent and multinucleated after a culture period of 6 days. Removal of the adherent macrophages took place by rinsing the cells with PBS EDTA.
PBMC were derived by isolating them from heparinized blood samples via density gradient centrifugation (1.077 g/l, Lymfoprep® Axis-Shield PoC AS, Oslo, Norway) 30 min at 1500 rpm. Cells were washed with culture medium and stored at -80 °C using DMSO/FCS until use.
Thymocytes derived from specific pathogen free cats were stimulated with concanavilin A (5 µg/ml) and kept in culture with recombinant human interleukin 2 (100 units/ml) as described previously (12). Thymocytes were maintained on IMDM containing Glutamax I (Sigma Chem. Co., St. Louis, MO), 10% heat-inactivated FBS (Hyclone, Logan, Ut, USA), 100 U/ml penicillin, 100 U/ml streptomycin and 50 µM 2-mercaptoethanol. Two days after removal of concanavilin A the thymocytes were regarded as stimulated (Ts). For the experiments with resting thymocytes (Tr) the concanavilin A was removed from the thymocytes 9 days before the experiments were performed.
Feline immunodeficiency virus was propagated on thymocytes for 5 days and designated FIV Utrecht 113.

Flow cytometry, functional properties and morphology of feDC
The functional and morphological characteristics of the cells were assessed as described earlier (4) with some minor modifications. Antibodies specific for CD1a (Fe1.5F4), CD1c (Fe5.5C1), CD11b (Ca16.3E10) and MHCII (42.3) were used for FACS analysis (all from the Leukocyte Antigen Biology Laboratory, Davis, CA, USA). Bound antibodies were detected with FITC- labeled secondary antibodies (Becton Dickinson). Dendritic cells or macrophages, which were cultured for 6 days, were collected by centrifugation for 5 min at 1200 rpm, washed twice with FACS buffer (PBS, 1% fetal bovine serum, 0.1% sodium azide) and consecutively incubated with antibodies and conjugates for 60 min at room temperature. Between each step the cells were washed twice with FACS buffer. Finally, the cells were washed twice with FACS buffer and resuspended in PBS, containing 2%

paraformaldehyde and stored at 4 °C until analyzed. For each sample, 50,000 cells were analyzed, employing a FACScalibur™ flow cytometer (Becton Dickinson) and the Windows-based WinMDI software (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA). In all these procedures isotype and secondary antibody matched controls were included.
Non specific esterase activity was detected using an esterase kit (Sigma, St. Louis, MO, USA) with α-naphtyl acetate as substrate.

Design of the infection experiments
All experiments were performed in triplicates.
PBMC, thymocytes and feDC were counted using trypan blue in a Glasstic Slide 10 (Hycor Biomedical inc. Garden Grove, CA, USA). 5.5 $x10^4$ cells were added to each well of a round bottom 96 wells plate (Costar, Cambridge, Mass). The amounts of PBMC or thymocytes were equal in cocultures and monocultures. The wells either contained only thymocytes, PBMC, only feDC or a coculture of feDC and thymocytes or feDC and syngeneic PBMC in a feDC-T or feDC-PBMC ratio of 1:10 (or 1:100 in the experiment in figure 3). To each well IMDM complete medium was added containing 0, 10 or 100 $TCID_{50}$ of FIV Utrecht 113. The final volume of each well was 200µl.

After a 2 hours incubation period at 37 °C, cells were washed 2 times with IMDM complete medium, and incubated for 6 days on medium containing rhu IL2. The infection was evaluated by determining p24 antigen production in the supernatant using an ELISA as described earlier (13).

Transwell cultures
All tests were performed in 4 fold. Concanavilin A stimulation of thymocytes was terminated 9 days prior to the start of the infection experiments and the obtained cells were regarded as resting thymocytes (Tr). Tr and feDC were combined in a transwell system. Tr ($5x10^4$) or feDC ($5x10^3$) were incubated with 100 $TCID_{50}$ FIV Utrecht 113 for two hours in the lower chamber of a round bottom 96 wells plate (Costar, Cambridge, Mass). After 2 washings with IMDM $5x10^3$ feDC were added in the transwell upper chamber (Becton Dickinson Falcon inserts 0.4 µm) of the well containing thymocytes (Tw feDC-Tr), and $5x10^4$ Tr were added in the transwell upper chamber of the well containing feDC (Tw Tr-feDC). Controls consisting of $5x10^4$ Tr or $5x10^3$ feDC and cocultures of both cell types (feDC-Tr ratio 1:10) without transwells were included. All cultures were incubated for 6 days at 37 °C and 5% CO_2 in a total volume of 200µl.
At day 6 supernatant was harvested for p24 antigen detection using an ELISA (13).

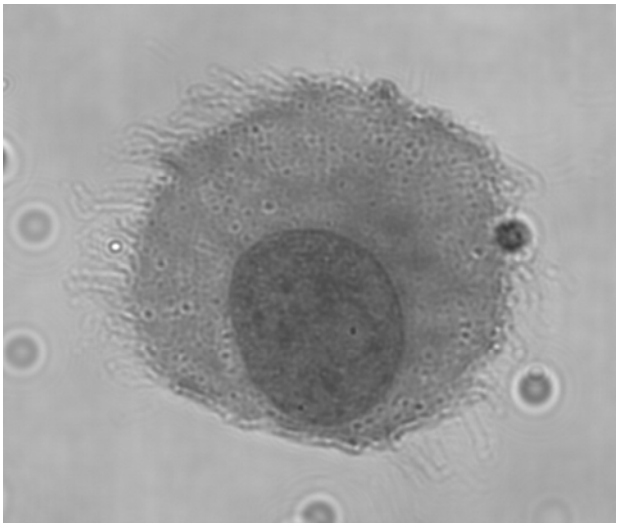
Proliferation assay
The incorporation of ^{3}H-thymidine (tritium) in replicating cells was tested in 96 well microtiter plates (Costar, Cambridge, Mass). The tests were performed in triplicate and were repeated twice. Dendritic cells were incubated with resting thymocytes (Tr) or with fresh thawed syngeneic PBMC derived from the same cat which were never concanavilin A stimulated at any time, in a ratio 1:10 ($5x10^3$ feDC : $5x10^4$ PBMC or Tr). As controls monocultures of $5x10^4$ Tr, $5x10^4$ PBMC and $5x10^3$ feDC were used. All cells were incubated for 6 days at 37 °C and 5% CO_2. Eighteen hours before ending the incubation period, 0.4 μCi of ^{3}H-thymidine in 30μl IMDM was added to each well.
The plates were stored at -20 °C until harvested. Cells were harvested onto glass fiber filters, and the incorporation of ^{3}H-thymidine was measured by liquid scintillation counting during 60 seconds.

Statistical analysis
Statistical analyses were performed with the two-sample *t*-test (two sided).

Results
Generation and evaluation of feline dendritic cells (feDC)
After 6 days of culture the bone marrow mononuclear cells subject to rfe IL4 and rfe GM-CSF stimulation (indicated as feDC hereafter) showed a distinct morphology compared to the cells cultured without cytokines (considered to be macrophages). FeDC were non-adherent, showed an irregular surface, and contained characteristic processes (figure 1). Macrophage like morphology became visible after 5-7 days. Generally, these cells were strong adherent, flat, large and multinucleated. The non-specific esterase activity was abundant in macrophage cultures; in feDC cultures it was hardly noticeable.
A selection of antibodies to discriminate macrophage cells from dendritic cells was used. Antibodies specific for CD1a, CD1c, MHC II and CD11b stained the cells of the feDC cultures indicating the presence of these surface markers (figure 2). The macrophages expressed MHC II whereas other markers were low (CD11b) or absent (CD1a and CD1c).

FIV infection of feDC-thymocyte cocultures
The feDC as defined by morphological and biological properties were then used for further studies.
The effect of feDC on FIV infection of thymocytes was evaluated by coculturing feDC in different ratios with thymocytes inoculated with a fixed amount of FIV Utrecht 113 (10 $TCID_{50}$).

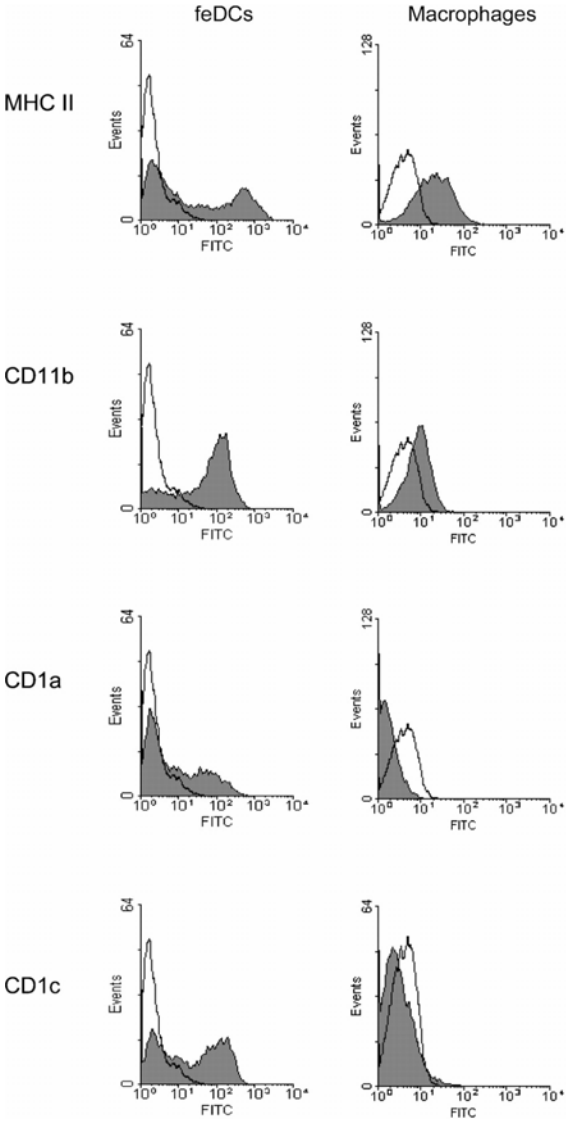
Figure 1.



Feline dendritic cell (feDC) showing distinct processes (dendrites) throughout the cell surface. The small bubbles are trapped air.

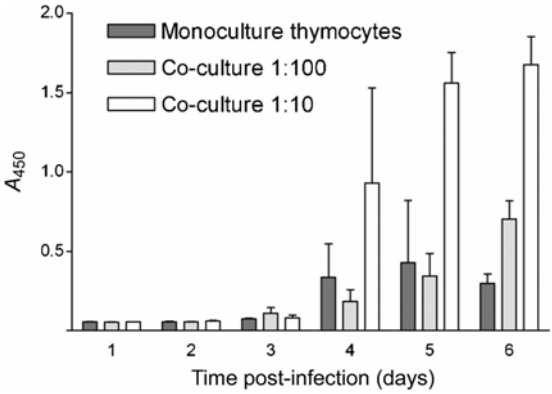
After 6 days of culture the cocultures showed dispersed clusters of cells as were seen using a phase-contrast microscope. Supernatants of feDC-thymocyte cocultures at a ratio of 1:10 and 1:100 were harvested daily during 6 days following infection. Supernatant samples were screened for FIV p24 as a determinant for virus production. Compared to the infected thymocyte monoculture, a difference in p24 production was already noted at 4 days post infection in the coculture with a feDC-T ratio of 1:10. This difference became statistically significant at day 5 ($p<0.05$). In the feDC-T coculture with a 1:100 ratio an significant upregulation of FIV infection was evident at day 6 (figure 3; $p<0.01$). The 1:10 feDC-T ratio was used for further experiments.
Next the feDC-T cocultures were infected with either 0.01, 0.1, 1, 10 or 100 $TCID_{50}$ FIV Utrecht 113 to determine the sensitivity of the system. An upregulation of p24 production could be observed when using only 1 $TCID_{50}$ FIV Utrecht 113. However, the results with this amount of virus were not consistent when the experiments were repeated. Infection experiments using 10 or 100 $TCID_{50}$ FIV Utrecht 113 showed similar levels of p24 production (figure 4), monocultures of either feDC or thymocytes differed significantly from cocultured feDC-thymocytes ($p<0.01$).

Figure 2. The immunophenotype of feline dendritic cells (feDC) and macrophages after 6 days of culture.



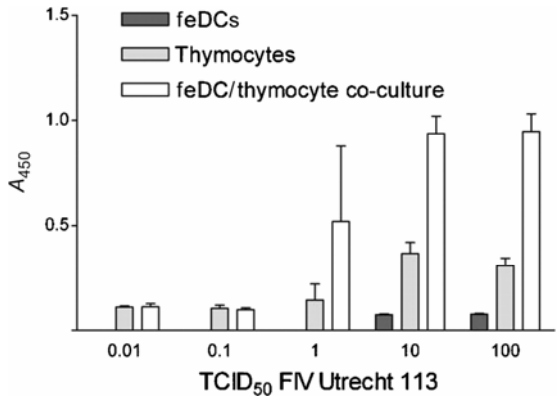
FeDC were generated by culturing monocytes with GM-CSF and IL4. Macrophages were cultured on IMDM without the addition of cytokines. Analysis by flow-cytometry in which non-colored graph represent iso-type matched control antibodies.

Figure 3.



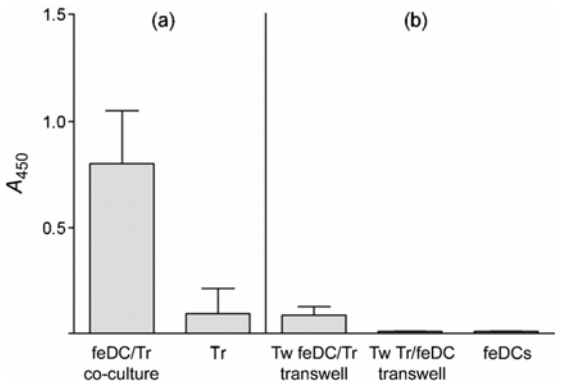
Different feDC/thymocyte co-cultures with 1 : 100 or 1 : 10 ratios subjected to a 10 $TCID_{50}$ FIV Utrecht 113 infection at day 0. The amount of FIV produced was evaluated by using a p24 ELISA on the supernatant of the culture (measured by A_{450}). Until day 4, no significant differences were detected; at day 5, the 1 : 10 co-culture differed from the 1 : 100 co-culture (P<0.01) and the thymocyte monoculture (P<0.05). At day 6, all differences in p24 values were significant (P<0.01). The data presented are the means of triplicate wells and error bars indicate SD from one representative experiment (out of two experiments).

Figure 4.



FIV Utrecht 113 titration on monocultures of thymocytes or feDCs and feDC/thymocyte co-cultures (1 : 10). After 6 days culture, supernatants were evaluated by using a p24 ELISA. Tests were performed in duplicate. Where feDC columns are not present, tests were not performed. A_{450} was measured by using a p24 ELISA. Infections with 0.01, 0.1 and 1 $TCID_{50}$ provided no statistically significant differences. The p24 values of the different cultures that were infected with either 10 or 100 $TCID_{50}$ were all statistically significant (P<0.01). The data presented are the means of triplicate wells and error bars indicate SD from one representative experiment (out of two experiments).

Figure 5.



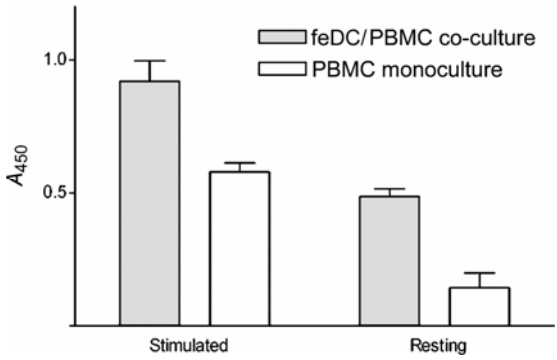
(a) The first bar represents the co-culture of feDCs and resting thymocytes (Tr) (1 : 10 ratio); the second bar represents a monoculture of resting thymocytes (Tr) (5x104 cells). These cells were infected with 100 TCID50 FIV Utrecht 113 as indicated in Methods. The obtained p24 values in feDC/Tr co-culture and Tr monoculture were statistically significantly different ($P<0.01$). The data presented are the means of triplicate wells and error bars indicate SD from one representative experiment (out of two experiments).
(b) In Tw feDC/Tr transwells (third bar), the thymocytes were infected with 100 TCID50 FIV Utrecht 113; after incubation and washes, feDCs were added to the transwell. Tw Tr/feDC (fourth bar) represents the cultures in which the feDCs were infected with 100 TCID50 FIV Utrecht 113 and Tr were added afterwards to the transwells. The fifth bar represents p24 production in the supernatant of a feDC (5x103 cells) monoculture after an infection with 100 TCID50 FIV Utrecht 113. A450 was measured via a p24 ELISA. No difference was detected between the p24 values in the Tw feDC/Tr transwells and Tr monocultures. The differences between the p24 values obtained in the feDC/Tr co-culture (Fig. 5a) and Tw feDC/Tr transwell or the Tw Tr/feDC transwell cultures were statistically significant ($P<0.01$). The data presented are the means of triplicate wells and error bars indicate SD from one representative experiment (out of two experiments).

The feDC are able to upregulate the FIV infection of resting thymocytes

The ability of feDC to enhance FIV infection in thymocytes was further evaluated for cells withheld from concanavilin A for 9 days (resting thymocytes (Tr)). The infections of feDC-thymocyte cocultures were compared to infected monocultured thymocytes. Results of this experiment are depicted in figure 5a. After the inoculation of the coculture with 100 $TCID_{50}$ of FIV Utrecht 113 the cells were incubated for 6 days. The p24 values showed a high production of FIV in the feDC-Tr coculture (OD= 0.8± 0.25) in contrast to the Tr monoculture (OD= 0.09± 0.1) (feDC-Tr coculture vs. Tr monoculture $p<0.01$). To evaluate the necessity of direct contact of thymocytes and feDC on the enhancement of FIV Utrecht 113 replication we used transwell systems. Transwells physically separate thymocytes

from feDC. However, allowing soluble factors derived from cells to pass the membrane. The results are shown in figure 5b. Transwell cultures in which the thymocytes were infected and feDC were added in the transwell upper chamber afterwards, showed similar p24 levels (feDC-Tr OD= 0.085± 0.04) as compared to monocultured resting thymocytes, hence no upregulation of FIV infection was detected. The p24 level of feDC monocultures and transwell cultures in which feDC were infected and subsequently Tr were added in the upper chamber of the transwell system had similar p24 levels (Tr-feDC OD= 0.009± 0.002 and feDC OD= 0.009± 0.001) and were regarded as background values. When stimulated thymocytes were evaluated in these experiments similar results were obtained, although differences were less pronounced (results not shown).

Figure 6. Influence of feDCs on stimulated and resting syngeneic PBMCs.



Resting PBMCs were removed from concanavilin A exposure 9 days before the experiment was performed, and stimulated PBMCs only 2 days. FIV Utrecht 113 (10 TCID50) was incubated for 2 h as described in Methods. Monocultures of feDCs (not shown) were used as controls, but showed no virus replication (A450=0.011±0.003). A450 was measured via a p24 ELISA. Differences in p24 values of monocultures of PBMCs and co-cultures of feDC/PBMCs were statistically significant ($P<0.01$). The data presented are the means of triplicate wells and error bars indicate SD from one representative experiment (out of two experiments).

Ability of feDC to stimulate FIV infection of stimulated and resting syngeneic PBMC cultures

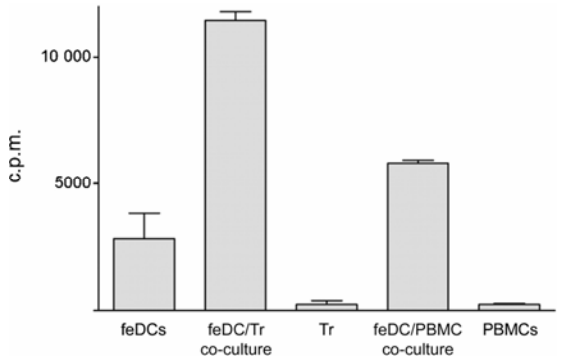
As thymocytes are allogeneic to the used feDC, the question arose whether the same phenomenon can be recorded in a syngeneic system. An evaluation of the feDC stimulatory capacity on syngeneic PBMC cultures was performed. Syngeneic PBMC were stimulated two times with concanavilin A, with a 7 days interval to

gain enough PBMC to perform the test. Analogous to the thymocyte experiments, PBMC that were deprived from concanavilin A stimulation for 2 days were regarded as stimulated PBMC, deprivation for 9 days resulted in resting PBMC. When stimulated or resting PBMC were combined with syngeneic feDC, enhancement of FIV infection was detected in both cases ($p<0.01$; figure 6). The experiment was repeated on material derived from 4 other SPF cats, all showing enhancement of FIV infection in the cocultures compared to the monocultures of stimulated syngeneic PBMC (results not shown).

FeDC-Thymocyte and feDC-PBMC proliferation assay

In order to evaluate the ability of feDC to directly stimulate either syngeneic PBMC or allogeneic thymocytes, resting PBMC or resting thymocytes (Tr) were exposed for 5 days to feDC. During the last 18 hours ^{3}H-thymidine was added to the culture and subsequently the uptake was evaluated. Cocultures of feDC-Tr as well as feDC-PBMC cocultures both showed a high amount of ^{3}H-thymidine uptake (11458 ±598 cpm and 5828 ±201 cpm respectively) compared to Tr (245 ±263 cpm) or PBMC (245 ±66 cpm) monocultures ($p<0.01$). The feDC monoculture also showed a high uptake of ^{3}H-thymidine (2822±1007 cpm). Results are shown in figure 7.

Figure 7.



Monocultures of feDCs, resting thymocytes (Tr), PBMCs (syngeneic to the feDCs) and co-cultures of feDC/Tr and of feDC/PBMC (syngeneic) were incubated with 0.4 µCi [^{3}H]thymidine during the last 18 h of the 6 day incubation period. Differences were statistically significant (feDC monoculture vs PBMC or Tr, $P<0.05$; feDC monoculture vs co-culture of feDC/Tr or feDC<PBMC, $P<0.01$; feDC/Tr co-culture vs Tr, $P<0.01$; feDC/PBMC co-culture vs PBMC monoculture, $P<0.01$). Data are expressed as the mean±SD c.p.m. The data presented are from one representative experiment (out of two experiments).

Discussion

Feline immunodeficiency virus (FIV) already serves as an appealing model for the study of lentivirus infections. The pathogenesis of FIV resembles HIV infection in many respects (3, 7). One of the earliest steps in the pathogenetic process is the interaction of dendritic cells (DC) and the lentivirus. As one of the earliest target cells for HIV-1 infection or through the capture of virions, DC are contributing to the dissemination of virus which is transmitted through the mucosa (24, 39). A similar role for feline DC can be expected. However, the early pathogenesis of FIV infection, especially the interaction of FIV with feline dendritic cells (feDC) has hardly been studied. To investigate this, a system of large-scale reliable feDC production is necessary. Feline DC generation from blood and bone marrow progenitors together with the characterization of these cells were recently described (4, 14, 34). In rat and mice bone marrow mononuclear cells (BMMC) can serve as a source for dendritic cells (20, 35). Therefore we collected also feline BMMC for the cultivation of feDC. The non adherent cells which were harvested from BMMC cultures after 6 days of feIL4 and feGM-CSF exposure were evaluated and found rich in CD11b, MHCII, and CD1c. Just a portion of these cells expressed CD1a. In earlier reports in which bone marrow cells were cultured in a similar way for 6 days, a distinct expression of these cellular markers was obtained (4). It is likely that our cultures consisted of a population of cells which are partly in the transition stage towards DC and cells which already reached the immature DC stage. By FACS analysis the cell populations evaluated seemed to be less uniform. In an attempt to gain a more monomorph cell population a MACS sort procedure was applied with the recombinant antibodies directed against huCD14 and huCD34 (Miltenyi biotec). CD14+ is a monocyte marker which can be used to sort peripheral blood samples (34). Bone marrow progenitors are CD34+ (8, 30). Unfortunately no depleted or sorted cell populations were obtained with these antibodies (results not shown). The expression of CD11b, a myeloid marker, is high on our feDC probably because bone marrow was used as monocyte source. Earlier findings of relatively high CD11b expression on murine bone marrow derived DC (20) compared to dermal and splenic derived muDC supports this. Although culture conditions were in essence similar to other studies (4, 14, 34), differences existed with regard to source (bone marrow), cytokines (recombinant feline) and culture media (Iscove's) used for the generation of feDC. These factors might have some effect on the level of cellular marker expression. Antibodies directed against or cross reacting with feline CD80, CD86 or CD40, to evaluate the maturation state of feDC are, to the best of our knowledge, not available at the moment. The cells obtained by our procedure can be regarded as dendritic cells (feDC) based on morphology, lack of esterase activity, surface marker profile and origin.

To propagate FIV efficiently *in vitro* the use of mitogens prior to infection of thymocytes or PBMC is necessary (12, 28). Dendritic cells can also fulfill this stimulating role as they are capable to stimulate T-cells and transfer retroviruses very efficiently as was shown for SIV (21, 32) and HIV-1 (17, 31, 33). This transfer leads to an enhanced viral replication in DC-$CD4^+$ lymphocyte cocultures infected with HIV-1 (9, 31, 33). For DC-T cell interaction three mechanisms are proposed (40). Transmission via a virological synapse, ligant interaction between DC and T cells and an indirect mechanism of DC mediated stimulation towards T cells, which render them more susceptible to infection. A study was performed to give a first insight in the interaction of feDC with syngeneic PBMC or allogeneic thymocytes when infected with FIV Utrecht 113. The cocultures we evaluated were infected as a whole for two hours and the p24 production was compared with infected monocultured cells. In control experiments in which feDC were incubated with FIV Utrecht 113 before the addition of thymocytes similar results were observed (not shown). The enhancement of FIV Utrecht 113 replication in both PBMC and thymocytes due to the addition of feDC was marked. The characteristics of this coculture system were evaluated by lowering the FIV Utrecht 113 amount used for infection. Even 1 $TCID_{50}$ could still lead to a detectable infection in these cocultures but not in thymocyte monocultures. However, this low virus amount led to a large variation in p24 production. Cocultures infected with 10 or 100 $TCID_{50}$ did not differ in this respect. When feDC were infected as monocultures, neither 10 nor 100 $TCID_{50}$ lead to a detectable p24 production in any of the experiments we performed so far. Hence, it seems unlikely that feDC support FIV Utrecht 113 replication. The upregulation of FIV infection through the addition of feDC to cultures of PBMC or thymocytes might also be of benefit when FIV isolation from feline blood cells is used as a diagnostic tool.

FIV Utrecht 113 is capable of replicating in resting thymocytes or resting PBMC when cocultured with feDC. In monocultures of either of these cells no or low amounts of p24 could be detected. Furthermore, the enhanced replication within resting cells cocultured with feDC suggests activation. Probably due to the excretion of soluble factors or through cell-cell interactions in which an improved environment for virus replication is created. It became clear from transwell experiments that feDC were able to enhance replication of FIV in resting thymocytes only when they were cultured in close contact. Enhancement of virus replication is likely mediated via intercellular interactions among feDC and thymocyte. This is in line with a previous study on SIV in which transwell cultures were used and only in direct contact cultures virus replication was detected (21). As rhu-IL2 was always present in the medium of the experiments it is not likely that this cytokine is involved in the enhancement of FIV Utrecht 113 in our system.

Direct contact could result, not only in optimal circumstances for the virus to be transported from one cell to the other, but also in stimulation of these cells. Both could lead to an increased amount of virus produced. The possible role of DC-SIGN as an attachment factor (11) in this process was studied by adding mannan to the co-cultures to a maximum level of 100 µg/ml. No blocking of the enhancement was observed (results not shown). This seems to be in line with previous findings (40), were no effect of mannan on SIV transmission by macaque DC and only limited effect of this compound on HIV transmission by human DC was detected. However, the lack of inhibition by mannan still does not exclude a role for a feline version of DC-SIGN. It is evident that for HIV several DC-SIGN independent mechanisms of HIV attachment and internalization exist (18). Therefore a more detailed study on the role of attachment factors for FIV on feDC is needed.
To study the possible role of stimulation of PBMC and thymocytes by feDC a proliferation assay, was performed. This assay was able to show a strong stimulation of thymocytes by allogeneic feDC (figure 7). In this respect feDC have the same capacities as DC of humans (9), monkeys (26), rabbits (10) or dogs (19) in allogeneic systems. In the syngeneic feDC-PBMC system this stimulation was still present but less pronounced. An explanation can be the more heterogeneous constitution of a PBMC culture, even when kept in culture for a longer period. Besides, syngeneic cells do not provoke a mixed leukocyte reaction, which will result in lower feDC mediated PBMC proliferation.

The FIV Utrecht 113 enhancement which occurs in an allogeneic system with DC from a SPF cat and thymocytes derived from an unrelated SPF kitten is in accordance to previous findings (9) for HIV. This interaction of DC and allogeneic T-cells is in our view mediated indirectly: DC mediated stimulation towards T cells. In the syngeneic system, in which feDC strongly enhanced FIV infection of PBMC, the interactions could be more direct and none of the above-proposed mechanisms were excluded by our experiments. When syngeneic human DC were added to T-cells Cameron *et al.* couldn't show any HIV replication (9). However, this was possibly strain dependent, since other investigators indicate that only macrophage tropic strains of HIV-1 (16, 29) or SIV (21, 25) were transmitted, and replicated efficiently in resting syngeneic PBL by immature DC. Apart from the cellular interplay as the cause of upregulation of FIV, also the virus itself can be of influence. This was illustrated by experiments with HIV (29) and SIV (25) in which the assessory gene Nef in particular was regarded important in the ability of these viruses to replicate in cocultures of immature DC and syngeneic T-cells. Even though no Nef like FIV gene is known at the moment, a gene designated as Orf A is suggested to have similarities to Nef of HIV-1 (15). Further insight in the function of this Orf A gene as Nef like 'superantigen' (36, 37) in the stimulation of

PBMC is required. The feDC in this coculturing system might induce cytotoxicity against T cells, hence inducing apoptosis. Apoptosis induction by huDC not only depends on the strength of the antigenic stimulation (22), but HIV is known to sensitize huCD4+ T cells to huDC cytotoxicity (23). Although extrapolation of these results to feline DC T-cell interaction is difficult, we cannot exclude some effects of apoptosis induced by feDC on p24 levels in our coculturing system.

In summary, we have shown that under the conditions described feDC can be generated from bone marrow derived mononuclear cells. These feDC are able to enhance the FIV Utrecht 113 infection in allogeneic thymocytes and resting syngeneic PBMC. This enhancement in feDC-thymocyte cultures was only detected when direct contact of cells was possible. FeDC were capable of inducing a proliferation in allogeneic thymocytes, which could be one of the explanations that even in an allogeneic system upregulation of FIV Utrecht 113 infection occur.

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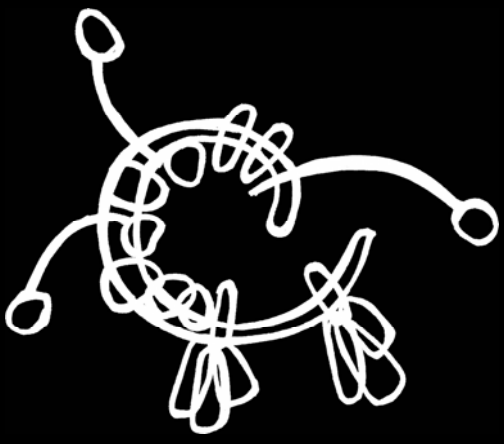

Chapter 3

Comparative evaluation of the activity of antivirals towards feline immunodeficiency virus in different cell culture systems

Short Communication

F.J.U.M. van der Meer, N.M.P. Schuurman, J. Balzarini [1] and H.F. Egberink

Department of Infectious Diseases and Immunology
Division of Virology, Faculty of Veterinary Medicine,
Utrecht University, Utrecht, The Netherlands

[1]Rega Institute for Medical Research,
K.U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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Feline immunodeficiency virus (FIV) mimics human immunodeficiency virus (HIV) infections with respect to pathogenesis, genome organisation and provoked immune responses (4, 25). On this basis FIV infections have been used to test antiviral compounds (in vitro and in vivo) for their potential activity towards human immunodeficiency virus (HIV). Thus, the antiviral activity of nucleoside reverse transcriptase inhibitors (NRTI) (6, 11, 13, 18), entry inhibitors (3, 7, 17) and protease inhibitors (12, 27) were previously studied for their inhibiting potency against FIV.

In vitro the evaluation of anti-FIV activity is generally performed on lymphocytic cells, infected with a lymphotropic FIV strain. Alternatively, a fibroblast cell line (Crandall feline kidney cells, CRFK) either freshly or persistently infected with CRFK tropic FIV strains is used. When the EC_{50} values of AZT (zidovudine) towards FIV were determined in these cell systems, differences of up to 80-fold were observed (18). These observations point to a direct effect of the particular cell system used on drug efficacy results, which could in some cases lead to the unjustified rejection of compounds as candidate antivirals. Ideally, the in vitro model system resembles the in vivo situation closely, thereby enhancing the predictability of antiviral activity in patients (15). To further assess the influences of the cell system on the observed EC_{50} values of different agents against FIV the present study was performed.

In a first set of experiments, two specific entry inhibitors, i.e. carbohydrate binding agents *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum* hybrid agglutinin (HHA) (20), previously shown to block HIV infection (1), were evaluated for their ability to inhibit FIV entry using established lymphocytic-, PBMC- and CRFK-based culture systems. FIV uses different entry mechanisms to infect these host cells. To enter thymocytes and PBMC, the virus uses CD134 (5) as its primary receptor, whereas only the chemokine coreceptor CXCR4 (26) is needed to efficiently infect CRFK. In a second set of experiments we evaluated NRTIs such as PMEA (adefovir) (6), the R enantiomer of (R)-PMPDAP (18) and AZT (zidovudine) (18), in a new dendritical cell (DC) - thymocyte coculture system. The presence of DCs in the DC-thymocyte cocultures has recently been shown to enhance FIV infections (21). Since DCs are involved in the early pathogenesis of retrovirus infections (14, 23), testing the activity of antivirals in this cell model is obviously of special importance, particularly for those NRTI known already to inhibit FIV in thymocyte monocultures.

Plant lectins like GNA and HHA target the mannose residues of N-linked oligosaccharides attached to the viral envelope glycoproteins (1). It is, however, well-known that host cells are important in determining the structure of the glycan structures present on the viral glycoproteins (16). FIV Utrecht 113 strains FIV-113_{Th}, FIV-113_{PBMC} and FIV-113_{CRFK} were propagated in their respective host

cells, i.e. thymocytes, peripheral blood mononuclear cells (PBMC) and CRFK. 100 $TCID_{50}$ (50% tissue culture infective dose) of each FIV was used to infect their homologous host cells in the presence of various concentrations of GNA or HHA. To determine and quantify infection of the cells, viral antigen released into the culture supernatant was evaluated at 6 days post infection by p24 ELISA (8). Our results (table 1) indicated that FIV-113_{CRFK} was very sensitive to the inhibitory effect of plant lectins in CRFK cultures (EC_{50}: 0.8×10^{-3} - 1.4×10^{-3} μM). This high inhibitory potency was in sharp contrast to the low sensitivity of FIV-113_{Th} in thymocyte cultures (EC_{50}: 0.3 - 1.8 μM). When the antiviral activities of these lectins were subsequently determined in PBMC derived FIV-113_{PBMC} the lectin efficacy was comparable to the values obtained in the FIV-113_{Th} – thymocytes infection system.

Table 1. The antiviral activity of GNA and HHA towards FIV Utrecht 113 derived from different cell types.

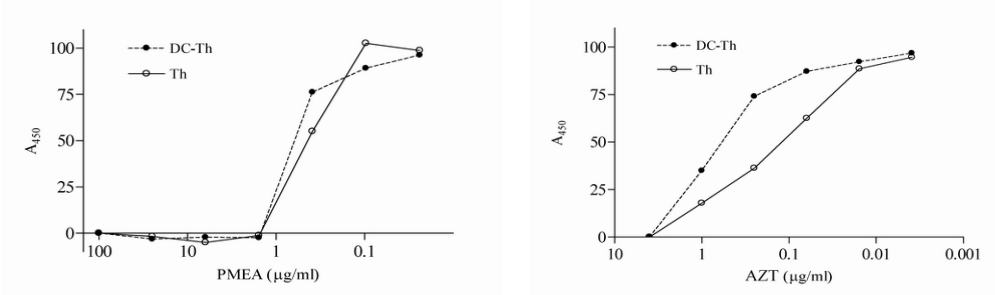
Lectin	FIV-113_{CRFK}	FIV-113_{Th}	FIV-113_{PBMC}
GNA	$1.4 \times 10^{-3} \pm 1.6 \times 10^{-3}$	1.8 ± 0.06	1.4 ± 0.08
HHA	$0.8 \times 10^{-3} \pm 0.8 \times 10^{-3}$	0.3 ± 0.06	0.4 ± 0.02

FIV-113_{CRFK} was derived from CRFK ATCC, FIV-113_{Th} was derived from thymocytes and FIV-113_{PBMC} from peripheral blood mononuclear cells harvested from an SPF cat. The antiviral activity is expressed in μM ± SD.

These results suggested that the oligosaccharides carried by FIV-113_{Th} and FIV-113_{PBMC} differ from those present on FIV-113_{CRFK}. FIV-113_{CRFK} and FIV-113_{Th} envelope proteins differ only in a single amino acid, not related to glycosylation (19). We therefore hypothesised that the host cell glycosylation machinery influenced the maturation of the glycan structures, thereby determining the differences in FIV lectin sensitivity. To confirm this we attempted several times to propagate FIV-113_{CRFK} on thymocytes in order to obtain FIV-113_{CRFK} with a thymocyte-like glycosylation pattern on its envelope glycoproteins. These attempts all failed. Even though the p24 capsid protein production as assessed by ELISA was high, infectious progeny virus was undetectable by virus titration on thymocytes. Interestingly, this observation indicates that FIV-113_{CRFK} is able to infect thymocytes and even initiate replication in these cells, without the production of infectious thymotropic virus. The high sensitivity of CRFK-tropic viruses to plant lectins might be related to the presence of specific glycan structures

(i.e. high-mannose type glycans) on their envelope when propagated in CRFK cultures. The reason for the much lower activity of the CBA against FIV in PBMC and thymocytes is still unclear. It would be interesting to determine the nature of the N-glycans of the envelope glycoproteins of the different virus strains to establish whether our hypothesis is correct. Alternatively, the observed differences might be related to specific properties of their earlier-mentioned entry mechanism. When the fusion time of FIV-113_{CRFK} on CRFK would be significantly longer compared to that of FIV-113_{Th} or FIV-113_{PBMC}, the lectin binding sites on the viral envelope glycoproteins might be exposed much longer to the carbohydrate recognition domains of HHA and GNA. Hence, inhibition of FIV infection by HHA or GNA in CRFK would be more efficacious. Based on the FIV-113_{CRFK} results plant lectins are promising antivirals against FIV. However, based on the FIV-113_{PBMC} and FIV-113_{Th} data, the high EC_{50} values are rather disappointing in terms of antiviral efficacy of the plant lectins.

Figure 1. The influence of PMEA or AZT on feline thymocyte monocultures or feline dendritical cell-thymocyte cocultures.



The X-axis represents the concentration antiviral compound, the Y-axis indicates the normalized p24 antigen production as determined by ELISA (8).

The antiviral activity of NRTI (PMEA, (R)-PMPDAP and AZT) in a thymocyte monoculture was compared to that in the DC-thymocyte coculture using the thymotropic FIV strain. In these experiments a 1:10 DC-thymocyte ratio was used. FIV-113_{Th} was used to infect the thymocyte cell cultures in the presence of various concentrations of the NRTI. The infection was evaluated at 6 days post infection by determining p24 antigen present in the supernatant using the FIV p24-specific ELISA. Statistical analyses were performed using a student's t-test. In both in vitro systems PMEA, (R)-PMPDAP and AZT were significantly active against FIV (figure 1; for (R)-PMPDAP, results not shown). PMEA and (R)-PMPDAP showed

similar antiviral activity curves in thymocytes and DC-thymocyte cultures. The EC_{50} values of PMEA and (R)-PMPDAP determined in these cell cultures were not significantly different (EC_{50} PMEA: DC-thymocyte 0.65 ± 0.20 μg/ml; thymocytes 0.42 ± 0.17 μg/ml; EC_{50} (R)-PMPDAP: DC-thymocyte 0.07 ± 0.02 μg/ml; thymocytes 0.11 ± 0.04 μg/ml). However, AZT showed a 6-fold ($p<0.01$) less inhibitory potency in DC-thymocyte cocultures than in thymocyte monocultures (EC_{50} AZT: DC-thymocyte 0.98 ± 0.62 μg/ml; thymocytes 0.15 ± 0.07 μg/ml). For PMEA and AZT the EC_{50} values are the average ± SD of six independent tests, for (R)-PMPDAP they are the result of three independent tests. Previously, we showed that addition of DC to thymocytes induced proliferation of the thymocytes (21). This will probably give rise to differences of drug metabolism (9, 10) due to an enhanced phosphorylating nucleoside kinase activity in the stimulated thymocytes. As AZT activity is dependent on cellular phophorylation its antiviral activity would be expected to increase as well. However, other phenomena may partly counteract the AZT efficacy. Indeed, FIV also replicates to markedly higher levels in activated thymocytes, and the expansion of the endogenous dideoxynucleoside 5'-triphosphates pools, in particular dTTP, will compete with the AZT-5'-triphosphate levels (22).

The changed balance between these phenomena in DC-stimulated thymocytes may explain the eventual decrease in antiviral efficacy of AZT, but not PMEA and (R)-PMPDAP. The metabolism of the latter drugs is indeed known to be much more independent on the metabolic condition of the cells than AZT. Acyclic nucleoside phosphonates like PMEA and (R)-PMPDAP have a long-lasting antiretroviral activity due to the relatively slow metabolism of the phosphorylated PMEA derivatives and, especially, to the relatively long intracellular half-life of the active metabolites (the diphosphorylated analogues). The intracellular breakdown of AZT-TP is much faster (2, 24), which might partly explain the efficacy differences observed.

In conclusion, we examined three different FIV-based antiviral evaluation systems and obtained marked differences in EC_{50} values, especially for CBA entry inhibitors. Cell cultures used for antiviral testing are in most cases based on similar culture systems as routinely used to propagate the viruses. Our study confirms and extends earlier observed differences between cell systems used for the evaluation of the activity of antivirals towards FIV (18). For the correct interpretation and extrapolation of the obtained EC_{50} values to the in vivo situation the degree of similarity between the used in vitro models and the actual in vivo situation must be taken into consideration.

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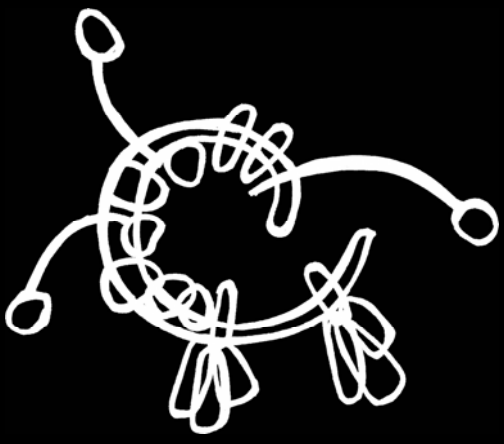

Chapter 4

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

F.J.U.M. van der Meer [a], C.A.M. de Haan [a], N.M.P. Schuurman [a], B.J. Haijema [a], W.J. Peumans [b], E.J.M. Van Damme [b], P.L. Delputte [c], J. Balzarini [d] and H.F. Egberink [a]

[a] Department of Immunology and Infectious Diseases Division of Virology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[b] Department of Molecular Biology, UGent, Gent, Belgium
[c] Laboratory of Virology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, UGent, Merelbeke, Belgium
[d] Rega Institute for Medical Research, K.U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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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 serotype 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.

Introduction

Coronaviruses are long known as important veterinary pathogens. In humans the relevance has recently increased considerably with the emergence of new human coronaviruses such as SARS-CoV (25), HKU1 and NL63 (43). 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 (18). This *Nidovirales* order consists 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 (28).

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 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 (18, 19) and it became clear that the application of carbohydrate binding agents (CBA) directed against the glycosylated envelope of these viruses may show promising results (2).

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 (3-5, 30, 45, 60), cytomegalovirus (4, 5, 29), Ebola virus (12), hepatitis C virus (32), influenza A and B virus strains (38) and to a limited extent for coronaviruses (11, 56, 61). 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 (3, 53). Moreover, systemic application of these compounds in mice did not result in acute toxic effects (3, 7) 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 kD tetramers with an α(1,3) and/or α(1,6) mannose tropism. CA (*Cymbidium* sp. agglutinin) is a 25 kD 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 kD in size, with a N-acetylglucosamine specificity (54). 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 (39) and human immunodeficiency virus (HIV) binding capacities (50). 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 (15, 24).
To evaluate the antiviral efficacy of compounds against viruses several techniques may be used. Classically the compound is added to the infectious virus and their host cells. In this way inhibition of virus production or cytopathogenicity can be monitored, the latter either by light microscopy or colorimetric methods that evaluate cell viability (13, 41). These methods evaluate antiviral efficacy on multiple rounds of infection, including the budding and transmission processes. Immune fluorescence techniques or immunoperoxidase staining can be used to detect viral antigen expression in infected cells. Using these methods a reduction of infected cells due to antivirals can be determined and so the efficacy of the antiviral compound.
Reporter gene expression can also be used for the evaluation of virus infections. In these assays, a reporter gene is either incorporated in the viral genome or is cloned behind a virus specific promotor. Expression of the reporter gene correlates with the amount of infected cells and virus replication. The effect of antiviral compounds on attachment and entry processes and protein expression can be studied in a single round of infection (40, 58).
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.

Materials and methods

Test compounds

The mannose-specific plant lectins from *Galanthus nivalis* (GNA), *Hippeastrum* hybrid (HHA), *Cymbidium* hybrid (CA), and the N-acetylglucosamine (GlcNAc) specific lectin from *Urtica dioica* (UDA) were derived and purified from these plants, as described previously (54). 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 (36).

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 gastro-enteritis 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 (22). The feline coronaviruses have evolved into several sublineages in which two serotypes can be distinguished. We evaluated both feline coronaviruses serotype I and II.

Feline FCWF cells (obtained from N. C. Pedersen) were used for the antiviral experiments with, and propagation of, FCoV serotype II FIPV (strain 79-1146), FCoV (strain 79-1683) and FIPV-Δ3abcFL (20) and the FCoV serotype I FIPV Black TN406HP (42). Mouse LR7 cells, a L-2 murine fibroblast cell line stably expressing the MHV receptor (46) were used for the experiments with, and propagation of, MHV (strain A59) and MHV-EFLM (21).

MHV-EFLM and FIPV-Δ3abcFL are viruses containing a firefly luciferase gene, respectively in a MHV A59 and FIPV 79-1146 background (20). Growth properties and infectivity are similar to the parental viruses (21).

Porcine ST cells were used for the experiments with, and propagation of, TGEV (strain Purdue). Simian Vero cells were used for the experiments with, and propagation of, the Vero-cell adapted IBV strain Beaudette. Berne virus and equine arteritis virus (EAV) strain Bucyrus were grown on equine dermis (Ederm) cells (American Type Culture Collection). 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. PAMs were obtained as described earlier (23). 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 100x, Gibco BRL), 1 mM sodium pyruvate, and a mixture of antibiotics in a humidified 5% CO_2 atmosphere at 37°C.

Antiviral assays

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

Cell viability was evaluated as described previously (41) 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 hours incubation at 37 ºC and 5% CO_2 the medium containing the MTT was removed and the cells were lysed by addition of 200µl DMSO to each well. Following 10 minutes 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 ºC and 5% CO_2 for 1 hour and added to the cells after a single wash with PBS DEAE. The mixture was removed after 1 hour. Cells were washed with PBS Ca^{++}/Mg^{++} and the test compounds were added again at the same concentrations. The MTT assay was performed two days (FIPV 79-1146, FCoV 79-1683) or three 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 ºC and 5% CO_2 in the presence of various concentrations of the test compounds during three days. The compound concentration that decreased the viability of 50% of the cells was defined as the 50% cytotoxic concentration (CC_{50}).

EMA staining of porcine alveolar macrophages (PAMs) for the determination of cytotoxicity
PAMs were incubated for 24 hours 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 (17). 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}).

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 ºC and 5% CO_2 for 1 hour and added to the cells after a single wash with PBS DEAE. The mixture was removed after 1 hour. Cells were washed with PBS Ca^{++}/Mg^{++} and the test compounds were added again at the same concentrations. At 6 hours post infection (or 16 hours for FIPV Black TN406HP infected FCWF) the cells were fixed during 15 minutes with 4% formaldehyde, and

subsequently permeabilized with 70% ethanol for 5 minutes. When FIPV Black TN406HP was assessed, the cells were fixed at 16 hours 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) (47) 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 was described earlier (23). Virus (MOI 1) was preincubated for 1 hour at 37°C with different concentrations of the test compound and added to the PAMs for 1 hour. Cells were washed with medium without fetal bovine serum (FBS) to remove unbound virus. PAMs were fixed at 10 hours post infection by a 20 minutes 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 (59) 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.

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 ºC and 5% CO_2 for 1 hour and added to the cells after a single wash with PBS DEAE. The mixture was removed after 1 hour. Cells were washed with PBS Ca^{++}/Mg^{++} 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.

Results

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- to 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 full cytopathogenic effect 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.

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 (table 2 and 3).

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} ± SD) and are expressed in μM. ND= could not be determined due to syncytium formation; NT=not tested.

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 *Galanthus nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA), *Urtica 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} ± SD) 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 *Galanthus nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA), *Cymbidium* sp. agglutinin (CA), *Urtica 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} ± SD) and are expressed in μM.

The plant lectin EC_{50} as determined by IPOX ranged from 0.04 to 0.53 μM against MHV-EFLM and 0.03 to 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-

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 to 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).

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 *Galanthus nivalis* agglutinin (GNA), *Hippeastrum* hybrid agglutinin (HHA), *Cymbidium* sp. agglutinin (CA), *Urtica 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} ± SD) and are expressed in μM. NT = not tested.

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-, 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.

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.

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 2-10 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.

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 (table 5) using the NetNGlyc server (see web reference). No clear relation could be found between the number of predicted glycosylation sites and the EC_{50} concentrations for the various CBA.

Table 5. N-Glycosylation of nidoviruses

Virus	GenBank Accession Number	Envelope Protein	Potential N-glycosylated sites	Predicted N-glycosylated sites (≥ +)
FIPV 79-1146	**DQ010921**	S	35	33
serotype II		M	3	3
FCoV 79-1683	**X80799**	S	33	30
serotype II	**AB086904**	M	4	4
FIPV Black	**AB088223**	S	36	26
serotype I	**AB086903**	M	4	3
MHV A59	**NC_001846**	S	23	19 **
		M	1	1
IBV Beaudette	**NC_001451**	S	29	27 *
		M	2	2
TGEV Purdue	**DQ811789**	S	32	28
		M	3	3
Torovirus Berne	**X52506**	GP precursor	19	11
EAV Bucyrus	**NC_002532**	GP 2b	1	1
		GP 3	6	5
		GP 4	4	4 *
		GP 5	1	1
PRRSV	**NC_001961**	GP 2	2	2
		GP 3	7	7
		GP 4	4	4
		GP 5	4	4

The prediction of the N-glycosylated sites using the NetNGlyc server (R. Gupta, E. Jung, and S. Brunak, unpublished data) based on sequences derived from the GenBank (accession numbers are indicated in column 2). The potential glycosylated sites occur on Asparagines in the Asn-Xaa-Ser/Thr motif. Predictions indicated in the NetNGlyc server output as +, ++ or +++ are included in column 5. An * indicates the occurrence of a Proline just after the Asparagine in one of the predicted sites. This renders the Asparagine inaccessible in most cases, precluding N-linked glycosylation.

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) (3, 7). 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 a 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.

In the MTT assay the efficacy of GNA and HHA against nidoviruses was very high (EC_{50} < 0.01 to 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 (3) and the SARS

coronavirus (11). 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) (4). Cytomegalovirus, SIV and HIV-1 infections could all be very efficiently inhibited by UDA (4). 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 (23).

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 (6, 51). Despite the fact that PRM-A has a specificity for D-mannose structures (26), 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 (35) 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 (10, 15) 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 serotype I and II might be due to the different receptor usage influencing the entire infection process (49). Serotype II FCoV utilize fAPN (52) as primary receptor whereas for serotype I coronaviruses the attachment to the host cell is relying on a different molecule, currently unknown (34). 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- 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 (54). In the natural situation they may also play an important role in the defense mechanism of the plant (55). Similar molecules are participating in the innate immune system of vertebrates, a-specifically binding the glycosylated proteins of pathogens (31). 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.

For HIV the envelope sugars are suggested to be involved in the retroviral mechanism to escape host immunity (14, 44, 48, 57). The glycosylation on coronavirus envelope proteins is suggested to serve a similar purpose (1, 16). 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) (6, 8-10). As a consequence the immunogenic epitopes will be exposed to the immune system (2) 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 (27, 33, 37).

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.

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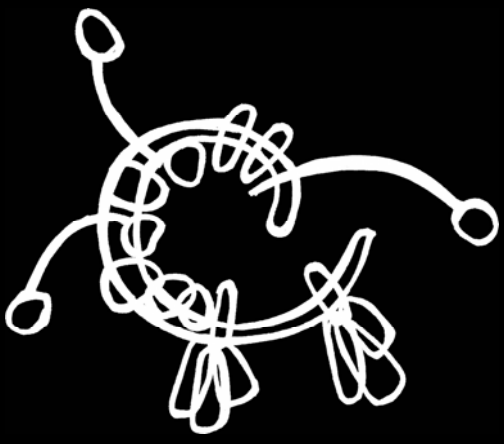

Chapter 5

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

F.J.U.M. van der Meer, C.A.M. de Haan, N.M.P. Schuurman, B.J. Haijema, M.H. Verheije, B.J. Bosch, J. Balzarini [1] and H.F. Egberink

Department of Infectious Diseases and Immunology
Division of Virology, Faculty of Veterinary Medicine
Utrecht University, Utrecht, The Netherlands

[1]Rega Institute for Medical Research,
K.U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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Synopsis
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 (GNA), *Hippeastrum* hybrid agglutinin (HHA) and *Urtica dioica* agglutinin (UDA) and the non-peptidic mannose-binding antibiotic pradimicin A (PRM-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 carbohydrate-binding agents exhibit promising capabilities to inhibit coronavirus infections.

Introduction

Development of intervention strategies for coronavirus infections has been boosted after the SARS coronavirus epidemic. Successes were recorded but the demand for antiviral chemotherapeutics, which are safe and active in low concentrations, perpetuates the search for new compounds. The use of interferons (22) and human monoclonal antibodies (40, 42) 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 (13, 23). 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 (34, 37, 39).

Compounds that specifically bind to or alter carbohydrate structures on these exterior glycoproteins were recently evaluated for their properties as antiviral agents (2, 21). It has been demonstrated that a variety of carbohydrate-binding agents (CBA) attach to N-glycosylated molecules and possess antiviral activity (1, 10). 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 (6-8, 47).

An interesting group of CBA are the plant lectins (44). *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum* hybrid agglutinin (HHA) are tetrameric α(1,3) and/or α(1,6) mannose-binding proteins that were previously found active towards human, simian and feline retroviruses, cytomegalovirus (2-4) and members of the *Nidovirales* order (45). *Urtica dioica* agglutinin (UDA)(3, 6), is a 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 (38). 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 (1, 9-11, 50). PRM-A is an actinomycete (*Actinomadura hibisca*)-derived D-mannose binding agent (33) described as a “lectin-mimic antibiotic” (20). It was shown to be active on fungi, yeast (33), HIV-1 (5, 41) and several viruses from the *Nidovirales* order (45). Strikingly, PRM-A demonstrated antiviral activity against serotype I but not serotype II feline coronaviruses (FCoV)(45). The exact PRM-A tropism is currently not known, but it is suggested that α(1,2)-mannose configurations on the N-glycans are important for recognition by PRM-A (25).

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 *Galanthus nivalis* (GNA), *Hippeastrum* hybrid (HHA), and the N-acetylglucosamine (GlcNAc) specific *Urtica dioica* (UDA) were derived and purified as described previously(44) and kindly provided by E. Van Damme (Ghent, Belgium). Pradimicin A (PRM-A) was obtained from T. Oki and Y. Igarashi, Japan.

Cells and viruses

Felis cattus 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), FIPV-Δ3abcFL and FIPV 79-1146 is a serotype II feline coronavirus. FIPV-Δ3abcFL contains a firefly luciferase gene in a FIPV 79-1146 background.(16, 17) Mouse LR7 cells, a L-2 murine fibroblast cell line stably expressing the murine hepatitis virus receptor (35) were used for the experiments with, and propagation of, MHV (strain A59), the M gene MHV mutants Alb138, Alb 244, Alb248 and MHV-EFLM. M gene MHV mutants designated Alb138, Alb 244, Alb248 contained respectively an O-glycosylated, a N-glycosylated or an unglycosylated M protein at the amino terminal ectodomain (15). MHV-EFLM contains a firefly luciferase gene in a MHV A59 background (16, 17). All 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 tests were performed on the same medium containing only 5% FBS. MHV-EFLM$_{HeLa}$ is a 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-Δ3abcFL$_{HeLa}$ HeLa cells stably expressing the feline coronavirus receptor, feline aminopeptidase N (fAPN) were used (48). Both cell lines were maintained on DMEM containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.5 mg/ml G418 (Life Technologies, Ltd., Paisley, United Kingdom).

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 of 5. After a 1-hour infection period, the cells were washed 3 times with PBS Ca^{++}/Mg^{++}. Subsequently the cells were incubated in the presence of 50 µg/ml GNA, HHA or PRM-A or 6.25 µg/ml UDA. Uninfected and infected cells without CBA addition were used as controls. After an additional 6 hours incubation period at 37 ºC and 5% CO_2 the cells were fixated at -20 ºC for 10 minutes 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-Δ3abcFL or MHV-EFLM respectively, in the presence of various concentrations of the test compounds. FCWF or LR7 cell monolayers were infected at a multiplicity of infection (MOI) of 0.5. The virus-drug mixture was preincubated at 37 ºC and 5% CO_2 for 1 hour and added to the cells after a single wash with DEAE PBS. The mixture was removed after 1 hour. Cells were washed with PBS Ca^{++}/Mg^{++} and new test compounds in DMEM supplemented with 5% FBS were added in the same concentration. 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 compared to the mock treated cells (EC_{50}) was calculated. The EC_{90} was the concentration antiviral compound capable of reducing 90% of the luciferase expression compared to mock treated cells.

Immunoperoxidase (IPOX) assay
Antiviral activity measurements were based on the reduction of focus forming units (FFU) when infected in the presence of various concentrations of the test compound. The cell monolayer was infected at a multiplicity of infection of 0.5. The virus-drug mixture was preincubated at 37 ºC and 5% CO_2 for 1 hour and added to the cells after a single wash with DEAE PBS. The mixture was removed after 1 hour. Cells were washed with PBS Ca^{++}/Mg^{++} and new test compounds in DMEM supplemented with 5% FBS were added. At 6 hours post infection the cells were fixated during 15 minutes with formaldehyde 4%, and subsequently permeabilized with 70% ethanol for 5 minutes. Immunoperoxidase (IPOX) detection of MHV A59 or the M gene MHV mutants (Alb138, Alb 244, Alb248) positive cells was carried out by using a rabbit polyclonal antibody against MHV (K135)(36) 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 FIPV 79-1146 combined with a HRP goat-anti-cat (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 to the mock-treated cells (EC_{50}) was calculated.

Virus cell entry assay
The efficacy of 50 µg/ml 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 wells plates with DMEM containing 5% fetal bovine serum (FBS), 100 IU per ml penicillin and 100 µg per ml streptomycin. MHV-EFLM was preincubated with or without CBA on melting ice for 1 hour. LR7 cells were also preincubated on melting ice for 15 minutes, washed with ice cold DEAE PBS and inoculated with MHV-EFLM at an MOI of 0.5 in the presence or absence of the antiviral compound, at 4 ºC. One hour post infection, the cells were washed three times with ice cold PBS Ca^{++}/Mg^{++}. To each cell 200 µl prewarmed (37 ºC) medium was added in the presence or absence of antiviral compound. At 6 hours post infection 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 a multiplicity of infection of 0.5 following a prior wash with DEAE PBS. One hour after the onset of infection the inoculum was removed, cells were washed three times with PBS Ca^{++}/Mg^{++} and further incubated

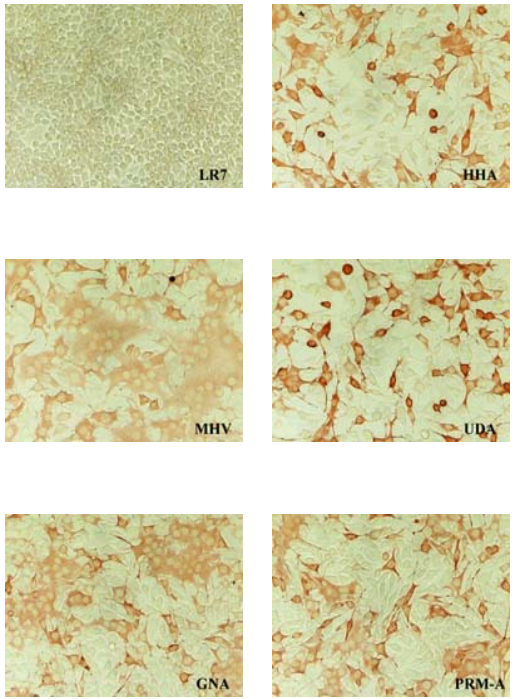
in DMEM containing 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin and 1mM 1-deoxymannojirimycin (dMM) (Sigma Chemical Co., St. Louis, MO, USA). At 9 hr post infection the medium was harvested and stored at -80 ºC. Virus derived from dMM treated cells was designated MHV-EFLM$_{dMM}$ or FIPV-Δ3abcFL$_{dMM}$. As 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 incubated with various amounts of GNA, HHA, UDA and PRM-A, ranging from 20 ng/ml to 100 µg/ml. At 6 hours post infection cells were lysed and the virus infection was scored using the luciferase assay.

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

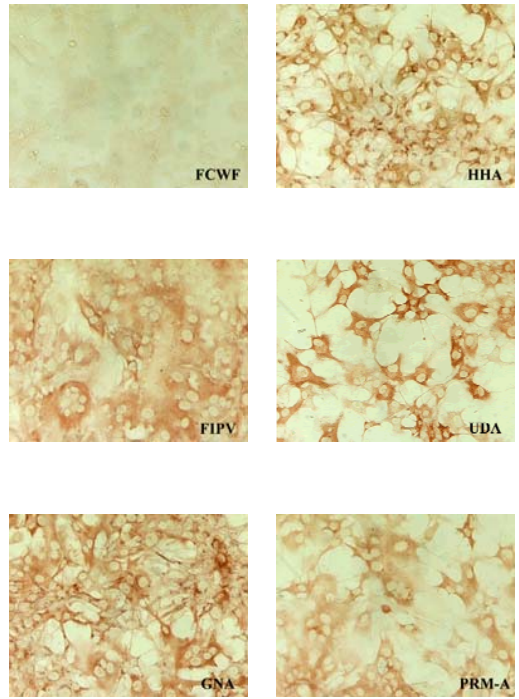
Results
CBA prevention of syncytium formation
Coronaviruses contain two glycosylated envelope proteins (M and S), which both may be targeted by CBA during virus entry. In order to discriminate between CBA binding to either the M or S protein, the influence of CBA on syncytium formation was studied. The expression of coronavirus S proteins on the cell surface is solely responsible for the fusion of 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 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 (Alb 138), N-linked sugars (Alb 244) or no sugars attached (Alb 248) (15). Wildtype 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).

Figure 1. Immunoperoxidase staining of LR7 cells (figure 1a) and FCWF cells (figure 1b) infected with respectively MHV A59 and FIPV 79-1146.



1a. LR7 cells infected with MHV A59



1b. FCWF cells infected with FIPV 79-1146

Upper left panels of figure 1a and 1b (designated LR7 and FCWF) are non-infected controls. Panels indicated with either MHV or FIPV are infected but not treated with CBA. After 1 hour infection CBA were added (50 µg/ml GNA, HHA and PRM-A; 6.25 µg/ml UDA) for 6 hours as indicated in the panels.

Table 1. Influence of M glycosylation on the sensitivity of the virus to CBA.

Glycosylation M protein	Mutant	GNA	HHA	UDA	PRM-A
O – N +	Alb 248	0.4 ± 0.3	1.2 ± 0.4	0.7 ± 0.2	2.0 ± 0.5
O – N –	Alb 244	3.7 ± 4.1	2.9 ± 0.6	2.8 ± 2.2	4.1 ± 1.8
O + N –	Alb 138	1.8 ± 0.5	1.8 ± 0.5	2.7 ± 1.3	5.7 ± 0.3

Introduction of a N-glycosylation site (Alb 248), the presence of no glycosylation site (Alb 244) or an O-glycosylation site (Alb 138) at the aminoterminal ectodomain of the M protein of MHV A59 was performed. The EC_{50} was determined using the immunoperoxidase assay. EC_{50} ± SD in µg/ml. The Alb 248 (O-N+) EC_{50} values were significantly different from the Alb 244 (O-N-) EC_{50} values ($p<0.01$).

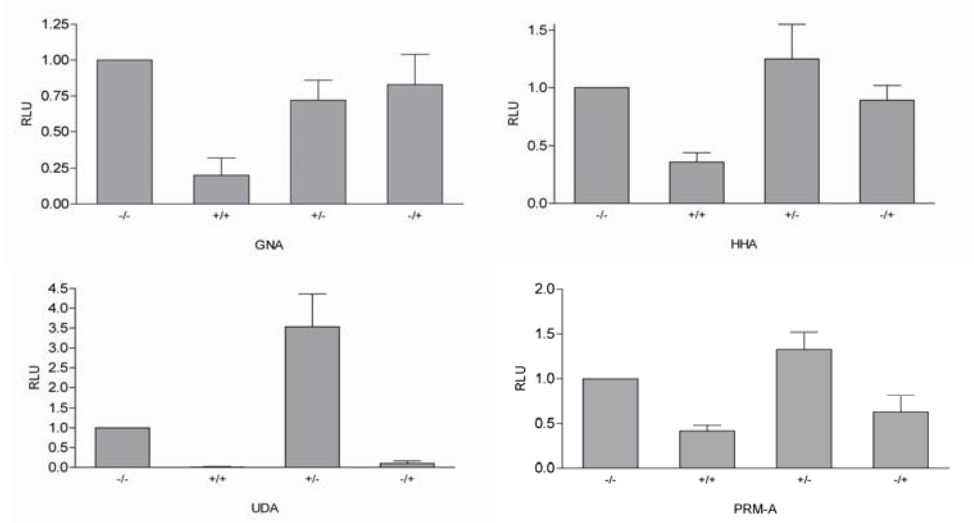
Recombinant virus Alb 248 (O-N+) showed the highest sensitivity for the CBA (EC_{50} 0.4-1.2 µg/ml for the plant lectins and 2.0 µg/ml for PRM-A) among all mutant virus strains. The obtained Alb 248 (O-N+) EC_{50} values were significantly different ($p<0.01$) from the Alb 244 (O-N-) EC_{50} values (EC_{50} 2.8 - 4.1 µg/ml). The CBA EC_{50} values of Alb 138 (O+N-) (EC_{50} 1.8 to 2.7 µg/ml) did not differ from the EC_{50} values obtained for Alb 244 ($p>0.05$). 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 carbohydrate-binding agents

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 performed at a temperature of 4 ºC, 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 ºC. When cells and virus were inoculated at 4 ºC and subsequently incubated at 37 ºC (+/+), 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. 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.

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 4 graphs the first bar represents the relative light unit (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 ºC but absent during the 37 ºC incubation period (+/-). The last, fourth bar, shows the RLU production when CBA was present only during the 37ºC period and not during the 4ºC incubation period (-/+). The bars represent the average value of three separate tests; the error bars represent the standard deviation normalized to the (-/-) experiment.

Figure 3. CBA efficacy against viruses derived from dMM treated host cells (legend next page).
Figure 3a. FIPV- Δ3abcFL

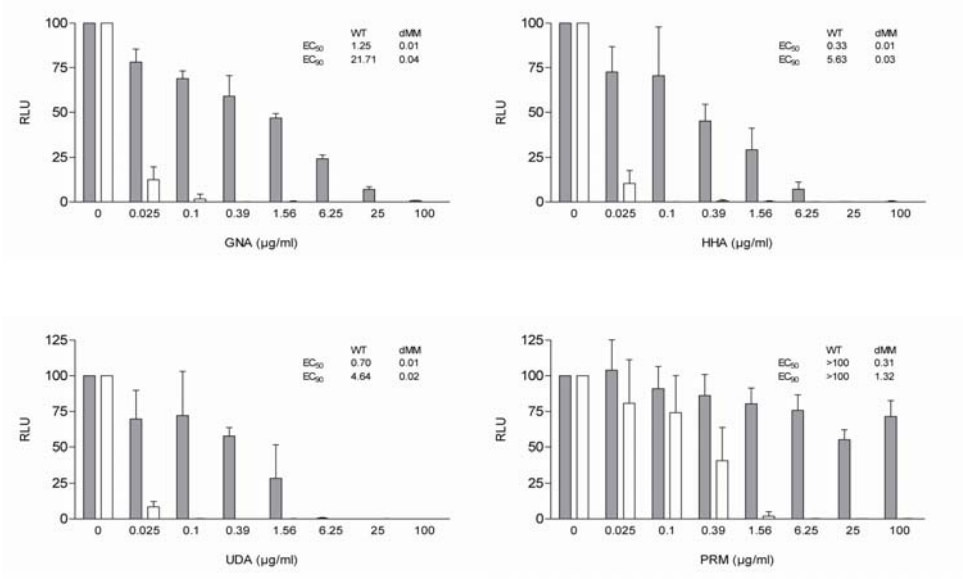
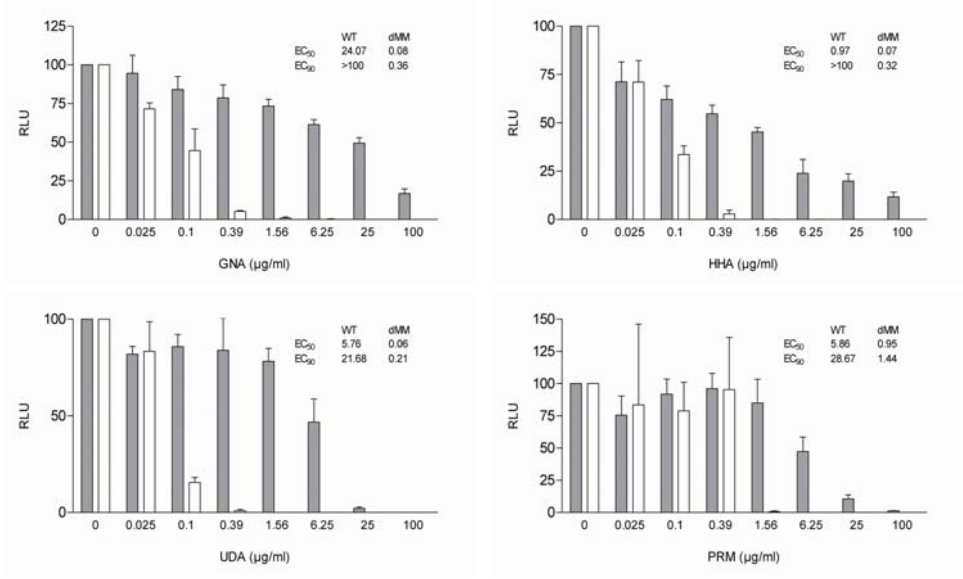


Figure 3b. MHV-EFLM



Legend figure 3: Bars represent the average of triplicate experiments. Whiskers represent standard deviation. White bars represent viruses derived from cells subjected to 1mM 1-deoxymannojirimycin (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 relative light units production (RLU) 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 μg/ml) viruses. FIPV-Δ3abcFL (figure 3a) and MHV-EFLM figure 3b) are both evaluated for their sensitivity to the lectins GNA, HHA, UDA and to PRM-A. Indicated in the figure are the EC_{50} and EC_{90} values for both WT- and dMM-virus in μg/ml.

Effectivity 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 of influence on the inhibitory capacity of CBA. To study this in more detail, viruses were grown on cells treated with 1-deoxymannojirimycin (dMM). Since dMM inhibits mannosidase activity in the Golgi complex, 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-$EFLM_{dMM}$ and FIPV-$\Delta3abcFL_{dMM}$). MHV-$EFLM_{dMM}$ showed in all cases a much higher sensitivity for CBA inhibition compared to 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 (45). Interestingly, FIPV-$\Delta3abcFL_{dMM}$ was clearly sensitive to PRM-A. Moreover, the GlcNAc-binding lectin UDA showed a higher inhibitory potency towards MHV-$EFLM_{dMM}$ and FIPV-$\Delta3abcFL_{dMM}$ infection of cell cultures compared to non-dMM treated viruses. This gain in antiviral activity of CBA to virus derived from dMM treated compared to non-dMM-treated (WT) cells was represented in the EC_{50} and EC_{90} 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.

Effectivity 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-$\Delta3abcFL_{HeLa}$ and MHV-$EFLM_{HeLa}$ 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 to FCWF- or LR7 cell-derived virus strains.

FCWF cell-derived FIPV-Δ3abcFL$_{FCWF}$ was, as expected, still refractory to PRM-A exposure but FIPV-Δ3abcFL$_{HeLa}$ increased in susceptibility towards PRM-A (EC_{50} FIPV-Δ3abcFL$_{HeLa}$: 4.8 µg/ml; EC_{50} FIPV-Δ3abcFL$_{FCWF}$: >100 µg/ml). Pradimicin A was also more effective towards MHV-EFLM$_{HeLa}$ compared to MHV-EFLM$_{LR7}$ (EC_{50} MHV-EFLM$_{HeLa}$: 0.25 µg/ml; EC_{50} MHV-EFLM$_{LR7}$: 5.4 µg/ml) (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$_{HeLa}$ to a lesser extent than MHV-EFLM$_{HeLa}$. 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 it self.

Figure 4. Host cell influences on CBA efficacy.
Figure 4a

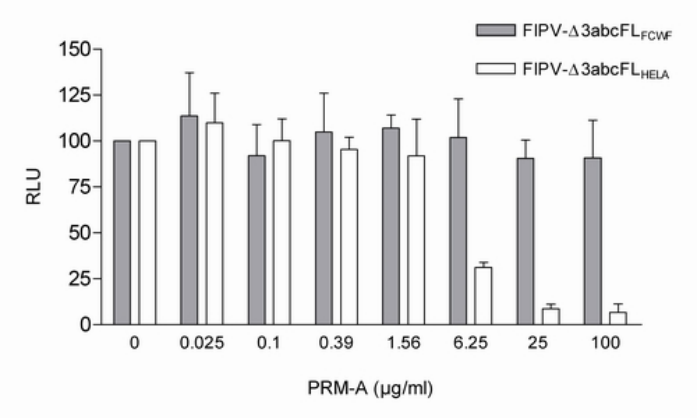
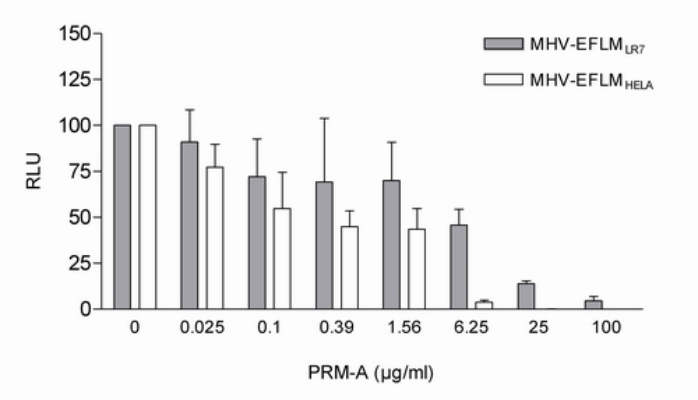


Figure 4b



Legend figure 4a. FIPV-Δ3abcFL derived from fAPN expressing human HeLa cells (FIPV-Δ3abcFL$_{HeLa}$) were compared to FIPV-Δ3abcFL$_{FCWF}$ derived from feline FCWF cells. The relative light unit (RLU) production of the viruses subject to different amounts of PRM-A was normalized to the mock-treated virus (0 μg/ml).
Figure 4b. MHV-EFLM derived from mCAECAM expressing human HeLa cells (MHV-EFLM$_{HeLa}$) were compared to the same virus derived from murine LR7 cells (MHV-EFLM$_{LR7}$). The relative light unit (RLU) production of the viruses subject to different amounts of PRM-A was normalized to the mock-treated virus (0 μg/ml).
RLU were determined by the luciferase assay and displayed on the Y-axis. PRM-A concentrations are shown on the X-axis.

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 (30, 49). It may be assumed that this entry process will be modified, slowed down or even completely blocked when disturbed by an attached carbohydrate-binding agent (CBA) at N-glycans. However, the exact nature of the influence of CBA on the viral infection process is not clear. Steric hindrance or conformational changes induced by CBA on the HIV-1 gp120 glycoprotein are thought to be the basis of the antiviral activity towards HIV (4). For the SARS coronavirus replication process even a dual mechanism of CBA action was proposed: virus fusion as well as interference at the level of exocytosis or viral egress from the cell (26). Our studies now show that CBA target the N-glycosylated M and S envelope proteins during coronavirus entry. Furthermore, the antiviral efficacy of the CBA was significantly affected by the maturation state of the N-glycans.
Following coronaviral infection of FCWF cells (with FIPV 79-1146) or LR7 cells (with MHV A59) syncytia appear. We showed that GNA, HHA, UDA and PRM-A markedly diminished the generation of multinucleated giant cells. This effect is reminiscent to inhibition of syncytium formation by CBA observed in co-cultures of persistently HIV infected HUT-78/HIV-1 and Molt-4 cells (3, 4). For HIV-1 it has been demonstrated that GNA can be used to selectively bind the gp120 protein of HIV-1 (24, 29). As syncytium formation induced by coronaviruses is solely induced by the S protein (14), a direct binding of CBA to this glycoprotein seems likely, since both HIV gp120 and CoV S glycoproteins are expressed at the outer surface of the virus.
The most abundant protein of the coronavirus envelope is the virus membrane protein (M). In case of infectious bronchitis virus of poultry and porcine transmissible gastroenteritis virus this protein is N-glycosylated (12, 27). The FIPV M protein contains one N-glycosylated residue (46), while the MHV A59 M protein becomes O-glycosylated at a single site (18, 31). We studied the influence

of M protein glycosylation using MHV recombinants, containing either N-, O- or unglycosylated sites at the MHV M ectodomain (15). A higher antiviral activity of the compounds was detected when MHV M was N-glycosylated. Thus, besides S, the other envelope protein M may also be a target for CBA when it contains glycan structures on its ectodomain. CBA bound to M glycans might pose a negative effect on virus entry, possibly by interfering with virus disassembly via cross-linking of M proteins (28).

The CBA inhibitory activity against HIV is mainly accomplished by intervention with the viral fusion process (3, 4), rather than the result of attachment inhibition of gp120 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. However, the inhibitory activity of HHA and GNA 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 approximately 50 kDa in size, PRM-A and UDA are much smaller (0.83 kDa and 8.5 kDa, respectively). We speculate that, because of steric hindrance, HHA and GNA might only be able to interfere with virus-cell fusion when bound to the S protein prior to spike-receptor binding, even though they did appear to affect virus-cell attachment itself. Strikingly, an enhancement of virus infection was noticed when some CBA were present during the attachment phase only. Hypothetically this could be due to a tethering effect of CBA facilitating virus-cell attachment and eventually leading to enhancement of infection (32). This may be possible with CBA evaluated in this study as they are able to bind multiple carbohydrate moieties (1, 10).

The processing of glycans attached to glycoproteins is of important influence on the inhibitory activity CBA against coronaviruses. The role of host cells in determining the sensitivity to CBA was underscored by the observed altered sensitivity of genetically identical viruses that had been propagated on different cell lines. Both FIPV-Δ3abcFL and MHV-EFLM sensitivity to PRM-A increased when propagated on HeLa cells instead of their native cell lines, FCWF and LR7 respectively. Since viruses derive their envelope glycans by the host cell glycosylation machinery (34), these results indicate that cell-specific glycosylation influences the ability of PRM-A to inhibit virus infection. The role of the host cell was also implicated by interference with glycan maturation by dMM. Due to inhibition of the mannosidase I and II enzymes in the Golgi, high-mannose residues persist on the (viral) glycoproteins. It is therefore expected that the mannose content at the N-glycosylated sites determine the CBA efficacy. Indeed, MHV and FIPV strains derived from dMM-treated cells became more susceptible to the investigated CBA. 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. It is however, likely that UDA binds more efficient to GlcNAc when combined with a higher amount of mannose residues (38). Thus, we conclude that the amount of mannose present on the viral glycoproteins determines the inhibiting capacities of CBA.

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 *in vivo* has already been proposed (43). 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. It is expected that the use of CBA as an anti-coronaviral agent will be applied as a systemic therapeutic. Considering such a systemic application of CBA, the combined treatment with glycosylation-influencing compounds like dMM might be beneficial. The dosage of CBA may in those cases be lowered, hence limiting the chance of inducing side effects. The separate application of mannosidase inhibitors (19) and CBA (2, 6) showed low toxicity *in vivo*. The effect of a combined *in vivo* application however, can still be totally different. Our next goal will therefore be the establishment of a safe and systemic applicable form together with an optimised dosage regime to impede coronavirus infections.

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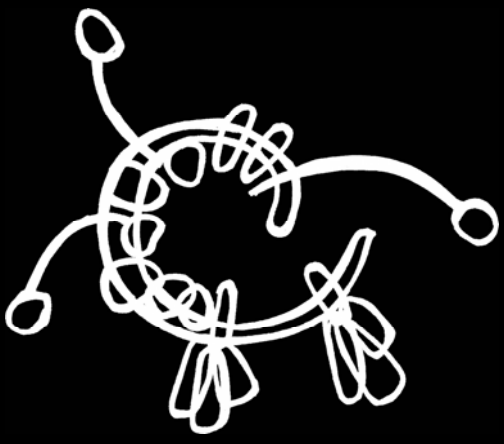


Chapter 6

Resistance of the coronaviruses murine hepatitis virus and feline infectious peritonitis virus to *Galanthus nivalis* agglutinin and *Urtica dioica* agglutinin

F.J.U.M. van der Meer, C.A.M. de Haan, J. Balzarini[1] and H.F. Egberink

Department of Infectious Diseases and Immunology
Division of Virology, Faculty of Veterinary Medicine
Utrecht University, Utrecht, The Netherlands

[1]Rega Institute for Medical Research,
K.U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Summary
Recently N-glycosylated structures on glycoproteins of enveloped viruses were explored as targets for new antiviral strategies. Earlier, carbohydrate-binding agents (CBA) were shown to inhibit coronavirus infections *in vitro*. In this study escalating concentrations of plant lectins (*Urtica dioica* agglutinin and *Galanthus nivalis* agglutinin) were used to select CBA resistant feline infectious peritonitis virus (FIPV) and mouse hepatitis virus (MHV). The resistance of MHV towards UDA and FIPV towards GNA was evident based on the determination of the resistance indices. The genetic evaluation of MHV and FIPV revealed that 6 out of 7 induced amino acid changes were in the close proximity of a potential N-glycosylated Asparagine residue. Only one out of these 6 mutations resulted in the removal of an Asn-Xaa-Ser sequon.
Therefore, in contrast to CBA resistant retroviruses, elimination of multiple glycosylated sites from the virus envelope glycoproteins was not observed. It is likely that the amino acid substitutions induced changes in the three dimensional structure of the spike glycoprotein rendering the lectin target sites inaccessible. However it cannot be excluded that additional mutations in the coronavirus genome contributed to the CBA resistance.

Introduction

The development of antivirals opens the prospects of controlling virus infections and limiting or even eliminating its pathological consequences. This led to a broad collection of pathogenic viruses for which antiviral products are currently under research. Only recently coronaviruses were included. Encouraged by the expected re-emergence of the SARS coronavirus (16) research to new anti-coronaviral compounds (13) developed. Also a new category of antivirals, carbohydrate binding agents (CBA), were evaluated and found active against SARS coronavirus (9, 43, 45). Already the inhibition of retroviruses, hepatitis C virus, influenza virus, Ebola virus and cytomegalovirus *in vitro* by CBA was shown earlier (1-3, 10, 21-23, 31, 34). As a consequence the application of these compounds in the prevention of HIV infection is proposed and promising *in vivo* results were obtained (39).

In this current study the generation of coronavirus resistance towards two plant lectins with a different binding preference was evaluated. Recently we demonstrated the antiviral activity of CBA, plant and prokaryotic lectins, towards coronaviruses and other members of the nidovirales order (41). These lectins interacted with the N-glycosylation of coronaviruses envelope proteins during the entry phase (42). The *Galanthus nivalis* agglutinin (GNA) of snowdrop binds to α(1,3) and α(1,6) mannose residues of the glycan shield, whereas the lectin derived from the stinging nettle: *Urtica dioica* agglutinin (UDA) possesses a N-acetylglucosamine (GlcNAc) specificity (40).

We used the feline infectious peritonitis virus (FIPV) strain 79-1146, which causes a progressive systemic infection in cats, and mouse hepatitis virus (MHV) strain A59 inducing a neuropathy and inflammation of the liver in mice. Both FIPV and MHV infections are eventually lethal. MHV and FIPV are enveloped spherical viruses containing four essential structural proteins: the membrane (M), the envelope (E), the spike (S), and the nucleocapsid (N) protein. The N protein wraps the genomic RNA into a nucleocapsid and is not exposed on the outside of the virus particle. A lipid membrane with the S, M, and E proteins forms the envelope. Trimers of the heavy glycosylated S protein (19) protrude from the virion membrane. The attachment of the S protein to mCEACAM1a (MHV) (11) or fAPN (FIPV) (38) is the first step in the viral entry process. Moreover, the S protein is responsible for cell-cell fusion (15).

To confirm that an observed antiviral effect is virus specific and not mediated by changes in host cell physiology, a study evaluating the CBA antiviral profile is warranted (24). Additionally the induction of coronavirus CBA escape mutants might be helpful in order to gain further insight in the envelope glycosylation and early virus-host interactions. For HIV the subcultivation under escalating CBA concentrations resulted in deletions of N-glycosylated amino acid residues on the gp120 envelope glycoprotein (4, 5, 7, 44). This resistance pattern is unique as other

HIV entry inhibiting compounds were still antiviral active towards the generated mutants (5). However, the flexibility of HIV to escape CBA antiviral pressure *in vitro* seems to be limited. Apart from the elimination of glycosylated sites no other resistant mutants were derived.

Materials and methods

Test compounds

The plant lectins *Galanthus nivalis* agglutinin (GNA) and *Urtica dioica* agglutinin (UDA) were derived and purified from these plants, as described previously (40).

Viruses and cells

Feline infectious peritonitis virus 79-1146 (FIPV) was propagated in Crandell feline kidney cells (CRFK; American Type Culture Collection). Plaque assays, plaque purification and antiviral selection were performed with CRFK cells. Murine hepatitis virus A59 (MHV) was propagated in LR7 cells a L-2 murine fibroblast cell line stably expressing the MHV receptor (35). Plaque assays, plaque purification and antiviral selection were performed with LR7 cells. 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. Titration's and resistance selections were performed in DMEM containing 2% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Selection and isolation of GNA resistant FIPV strains and UDA resistant MHV strains

FIPV and MHV were subjected to subcultivations in 24 wells plates in the presence of plant lectins. Viruses were plaque purified to minimize genetic variation of parental viruses. From each virus three different strains were subcultivated. The amount of virus used during the subcultivation was 100 $TCID_{50}$ (50% tissue culture infective dose). The start concentration of UDA in the MHV subcultures was 8 µg/ml (0.9 µM) but was reduced following passage 3 to 4.5 µg/ml due to a lack of progeny virus in all three subcultivated strains. For GNA in the FIPV cultures the start concentration was 10 µg/ml. Following 1 hour preincubation of FIPV with GNA or MHV with UDA the mixture was added to the cells in a total volume of 0.5 ml.

When a cytopathogenic effect (CPE) was present, supernatants were harvested, aliquotted, titrated and stored at -80 ºC. When no CPE became visible after 4 days of incubation the supernatant was harvested and titrated to detect the presence of virus. At each subcultivation, the same concentration of test compound was administered; an increased drug concentration was used when no significant

decline of virus titre was observed and/or the interval between infection and CPE in the cultures with plant lectins became shorter than 3 days. When a subcultivation failed to produce progeny virus, one additional subcultivation was attempted with the same virus amount. Eventually a third attempt was performed with 1000 $TCID_{50}$ of MHV. Additionally the lectin concentration could be levelled or lowered.

Screening for coronavirus resistance towards plant lectins using the resistance index

A modification of the antibody neutralization index used to identify viruses was applied to examine viruses for their sensitivity to antivirals. Stocks of subcultivated MHV and FIPV strains and their parental strains were diluted to $1x10^6$ $TCID_{50}$/ml and titrated in 10 fold dilutions in a 96 wells plate. To each well medium only or medium containing lectin was added. After 3 days virus replication was evaluated by scoring the CPE by light microscopy. The titre was calculated using the Spearman-Kärber method. The resistance index was calculated by dividing the virus titre obtained in medium only with the virus titre obtained in the presence of CBA.

Nucleic acid isolation, amplification and amino acid evaluation of envelope proteins

When referred to the sequence of FIPV 79-1146 or MHV A59 GenBank accession no. **DQ 010921** for FIPV 79-1146 and **NC 001846** for MHV A59 were used. The S gene was sequenced in three separate overlapping pcr products (designated S1, S2 and S3), The E and M gene were sequenced in one stretch (designated EM). Two independent pcr reactions for each product were evaluated to be able to correct for errors introduced during amplification. Primer details are presented in table 1. First, from 140 µl virus-containing culture supernatant, viral RNA was isolated using a QIAGEN viral RNA isolation kit (according to the manufacturer). This was followed by reverse transcription with the isolated RNA under standard conditions using Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, USA) and the reverse primers: 1533 for product FIPV S1, 1645 for product FIPV S2, 1514 for product FIPV S3 and 2110 for product FIPV EM. PCR was performed using the primer combinations: 1644 - 1533 to obtain FIPV S1, 1712 - 1645 to obtain FIPV S2, 1911 - 1514 to obtain FIPV S3 and 2027 - 2110 to obtain FIPV EM applying the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Indianapolis, USA).

Table 1. Primers used for the genetic evaluation of FIPV and MHV envelope proteins E, M and S

Primer number	Virus	Sequence (5' - 3')	Forward/ Reverse	Position (on viral genome)
1514	FIPV	TAATGACTAATAAGTTTAG	Reverse	24602-24620
1533	FIPV	GGTTGAATCTAATAACATCCACTGTGTTAT	Reverse	21215-21244
1574	MHV	CGATCCTAGGGTATATTGGTGATTTTAGAT	Forward	23966-23989
1644	FIPV	TGGACTGTGTTTTGTACAAGTG	Forward	19871-19892
1645	FIPV	CCATTGCAAGTGCTTGTTCAATAG	Reverse	22868-22891
1712	FIPV	TTGTTAGTGGCAGGTTTGTA	Forward	21080-21099
1911	FIPV	GCAAGTTGAATACATGCAGG	Forward	22749-22768
2027	FIPV	ATGACGTTCCCTAGGGCA	Forward	25722-25739
2110	FIPV	CAGTTGACGCGTTGTCCCTGTG	Reverse	26787-26810
2259	MHV	CAGCTTGGTAACTCTGGATT	Forward	25138-25157
2461	MHV	GTGTTACTATAAGCTCGAGAC	Forward	26402-26422
2575	MHV	CATTATTCTTAGGCAAGGTG	Reverse	25195-25214
2578	MHV	CCTATGCATCCAAGTAGAGG	Reverse	26467-26486
2701	MHV	TTAGATTCTCAACAATGCG	Reverse	29636-29654
2702	MHV	ATGTTTAATTTATTCCTTACAGA	Forward	28706-28728
3199	MHV	GTCAATCCTCATGAGAG	Reverse	27888-27904
3448	FIPV	GTAACAGTCACATTAATAACAT	Forward	21665-21686
3449	FIPV	CAGGCTAGACTTAATTATGTTG	Forward	23344-23365
MHV S 26893	MHV	GTTTGATGCAACCAATTCTG	Forward	26889-26908

For the sequencing of the products FIPV S2 and FIPV S3 two additional sequencing primers were designed: 3448 and 3449 respectively.
For MHV strains a similar procedure was followed as described for FIPV using the reverse primers 2575 for product MHV S1, 2578 for product MHV S2, 3199 for product MHV S3 and 2702 for product MHV EM. PCR was performed using the

primer combinations: 1574 - 2575 to obtain MHV S1, 2259 - 2578 to obtain MHV S2, 2461 - 3199 to obtain MHV S3 and 2701 - 2702 to obtain MHV EM. For the sequencing of the product MHV S3 an additional sequencing primer was designed: MHV S 26893.

Results

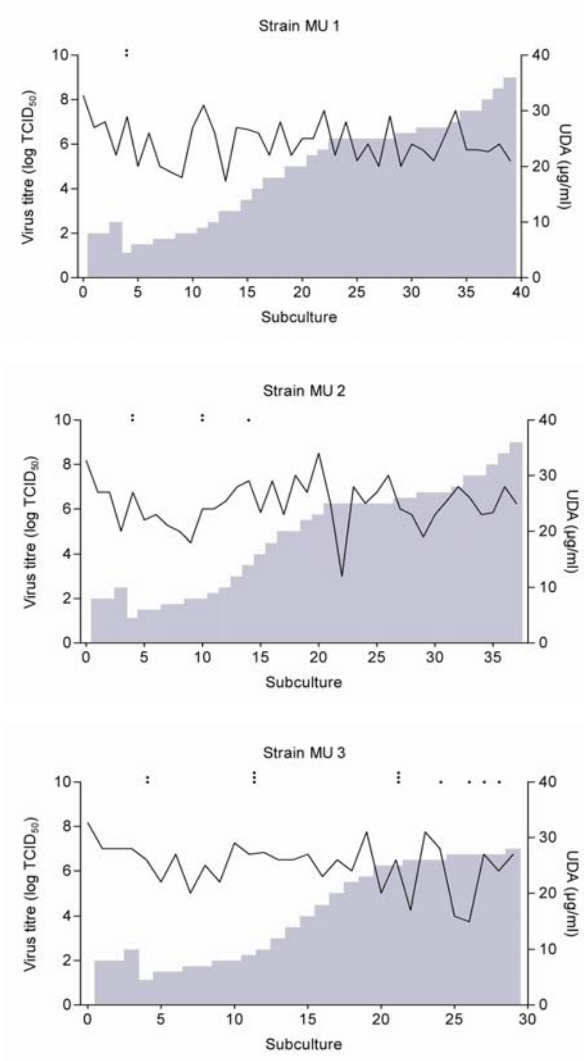
Selection of resistant coronavirus strains

a. UDA resistant MHV (MU)

MHV A59 strains resistant to UDA were selected by serial passage of MHV A59 in the presence of increasing concentrations of UDA (figure 1).

The obtained passages were designated MU, for MHV subcultivated with UDA followed by the subcultivation and strain numbers: for example MU 25.1 (MHV cultivated with UDA subcultivation 25 strain 1). Three passages were lost during the process MU 2 to 4; so the parental strain of MU 5 is MU 1. Therefore, for example MU 25 is displayed in figure 1 as subcultivation 22. Eventually strain MU 42.1 and MU 40.2 replicated in the presence of 36 µg/ml (4.1 µM) UDA, about 8 times the EC_{50}; strain MU 32.3 in 28 µg/ml (3.2 µM) UDA, about 6 times the EC_{50}. Not all subcultivations led to the production of progeny virus in these cases no CPE was observed and the titration of the harvested supernatant did not indicate the presence of residual MHV.

Figure 1. Subculturing of UDA resistant MHV strains

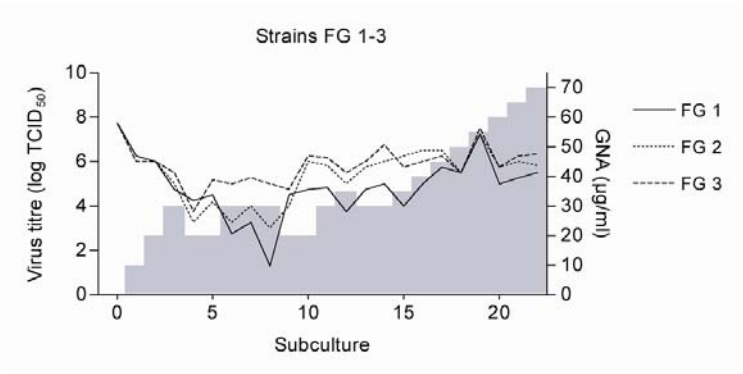


Three independent selection pathways to generate UDA resistant MHV A59 strains are displayed. At each subcultivation, the same concentration of test compound was administered; an increased drug concentration was used when no significant decline of virus titre was observed and/or the interval between infection and CPE in the cultures with plant lectins became shorter than 3 days. The unsuccessful subcultivation attempts in which no progeny virus was derived are indicated with a dot. The line represents the virus titre determined on successful passages, calibrated on the left Y-axis. The grey bars represent the amount of GNA used in the subculture (X-axis). The GNA amount is depicted on the right Y-axis.

b. GNA resistant FIPV (FG)

FIPV 79-1146 resistant strains to GNA were selected by serial passage of FIPV 79-1146 in the presence of increasing concentrations of GNA (figure 2). The obtained passages were designated FG, for FIPV subcultivated with GNA followed by the subcultivation and strain numbers: for example FG 21.2 (FIPV cultivated with GNA subcultivation 21 strain 2). All subcultivations of FG produced progeny virus. At subcultivation 21 all FG strains replicated in the presence of 70 µg/ml (1.4 µM) GNA, which is about 10 times the EC_{50}.

Figure 2. Subculturing of GNA resistant FIPV strains



Three independent selection pathways to generate GNA resistant FIPV 79-1146 strains are displayed. An increased drug concentration was used when no significant decline of virus titre was observed and/or the interval between infection and CPE in the cultures with plant lectins became shorter than 3 days. As all subcultivated strains followed a similar pathway, they are displayed in one figure. The subcultivation procedure of the three independent FIPV strains was performed synchronous; therefore they are depicted in the same figure. The lines represent the virus titre determined on successful passages, calibrated on the left Y-axis. The grey bars represent the amount of GNA used in the subculture (X-axis). The GNA amount is depicted on the right Y-axis.

Screening for coronavirus resistance towards plant lectins using the resistance index.

a. UDA resistant MHV (MU)

To obtain an objective indication of resistance of MHV strains to UDA, MU 25.1, MU 23.2, MU 22.3 and the parental plaque derived wildtype MHV A59 were titrated in the presence or absence of 20 µg/ml UDA. The difference in titre results in the antiviral index and is an indication of the magnitude of resistance. When this

index is low, the virus can replicate efficiently in the presence of the antiviral compound, hence is resistant. Wildtype MHV A59 showed an index of > 4000, whereas the MU strain indices were all below 100 (table 2a). This indicated a difference in sensitivity to 20 μg/ml UDA. The resistance index for subsequent passages MU 42.1, MU 40.2 and MU 32.3 comparing titres at 0 μg/ml and 30 μg/ml also indicated this resistance (depicted in table 2b). For the parental MHV A59 an index of > 2000 was derived whereas the MU strains indices were ≤ 100.

b. GNA resistant FIPV (FG)

The resistance indices of FG 21.1, FG 21.2 and FG 21.3 (table 2c) were determined in the presence or absence of 50 μg/ml (1 μM) of GNA and compared to the parental strain. The resistance index for the wildtype FIPV 79-1146 parental strain was > 350, compared to ≤ 15 for the FG strains.

Table 2. Resistance index

Table 2a

MHV strain	Titre (^{10}log) at 0 μg/ml UDA	Titre (^{10}log) at 20 μg/ml UDA	Resistance index
Wildtype	6.41	2.80	4073
MU 25.1	7.30	5.30	100
MU 23.2	6.56	5.04	33
MU 22.3	5.56	3.80	58

Table 2b

MHV strain	Titre (^{10}log) at 0 μg/ml UDA	Titre (^{10}log) at 30 μg/ml UDA	Resistance index
Wildtype	6.15	2.80	2239
MU 42.1	6.46	5.15	20
MU 40.2	5.80	4.80	10
MU 32.3	6.80	4.80	100

Table 2. Resistance index (continued)

Table 2c

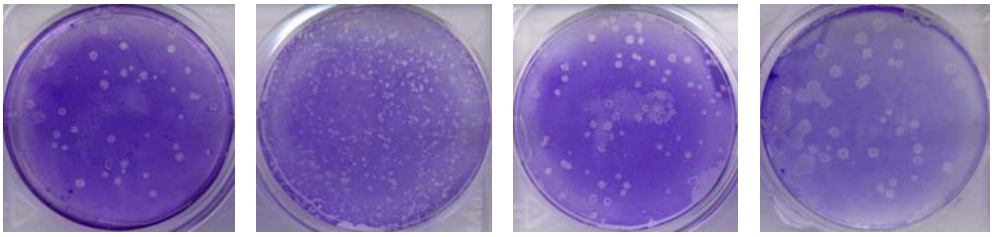
FIPV strain	Titre (^{10}log) at 0 µg/ml GNA	Titre (^{10}log) at 50 µg/ml GNA	Resistance index
Wildtype	5.58	3.02	363
FG 21.1	3.80	4.43	0.23
FG 21.2	4.43	3.26	15
FG 21.3	3.80	3.52	2

Titre comparison of resistant and wildtype viruses titrated in the presence or absence of CBA (as indicated). The difference in titre results in the antiviral index and is an indication of the magnitude of resistance. When this index is low, the virus can replicate efficiently in the presence of the antiviral compound. Titres indicated in the 2nd and 3rd column are displayed as ^{10}log.

Phenotypic characterization of UDA resistant MHV coronavirus using plaque assay

The phenotype of the MU viruses was investigated by comparing plaque sizes in a plaque assay. At 20 hours post infection the plaques of MU 40.2 and MU 32.3 were not different from wildtype MHV. Surprisingly the phenotype of MU 42.1 indicated a significant reduction in plaque size (figure 3). In retrospect MU 23.1 already contained this phenotype (not shown).

Figure 3. Plaque assays of UDA resistant MHV strains.



MHV A59 MU 42.1 MU 40.2 MU 32.3

The nomenclature is explained in the results section. The assay was terminated at 2 days post infection. The cell layer is stained using crystal violet. Subcultivation MU 42.1 displayed a small phenotype whereas MU 40.2 and MU 32.3 displayed a phenotype comparable to wildtype (MHV A59).

Amino acid evaluation of lectin resistant envelope proteins
a. UDA resistant MHV (MU)
To further investigate the observed differences in index and phenotype the genetical characterization of the envelope proteins E, M and S was performed. The MU strains (MU 30.1, MU 30.2 and 27.3) contained one amino acid substitution in the S glycoprotein when compared to the parental strain. These were all situated in the vicinity of N-glycosylated Asparagines (figure 4a). The UDA selection process induced in MU 30.1 (the small phenotype strain) a mutation in the S2 gene at position 767 resulting in an Arginine to Serine change.
The S gene of further selected MU 42.1 revealed an additional mutation in the HR1 region: the Asparagine at position 1003 changed to a Serine. Strain MU 42.1 was the only virus that apart from the first few subcultivation attempts, did not fail once in the production of progeny virus (figure 1). In MU 30.2 the glycosylation site at position 357 was found deleted due to a substitution of a Serine to a Glycine residue at position 359, removing the N-glycosylation motiv Asn-Leu-Ser. The additional S gene evaluation of MU 40.2 did not show accompanying sequence changes. The mutation in MU 27.3 was situated at position 522, resulting in a Proline to Leucine substitution. This strain 3 was only evaluated once (as MU 27.3), as we were not able to passage this strain very efficiently. All mutated amino acids are indicated in figure 4a with an arrow. The E and M proteins of all MU strains did not differ from the parental virus proteins.

b. GNA resistant FIPV (FG)
The genome evaluation revealed in all resistant FG strains one amino acid substitution at different locations in the S gene, shown in figure 4b. Strikingly all mutations were in the close proximity of a potential glycosylated site, without causing its deletion. The GNA selection of strain FG 21.1 resulted in an Isoleucine to Threonine mutation on position 170 (potential glycosylated site at position 174). The deletion of an entire codon in FG 21.2 led to the removal of a Glycine residue at position 1347. This is not only close to the 1344 glycosylated Asparagine, the Prosite prediction (25) of the Leucine zipper at position 1345-1366 in wildtype FIPV 79-1146 S no longer existed. Strain FG 21.3 contained a Leucine to Phenylalanine substitution at position 1369. The Asn-Xaa-Ser/Thr stretches are found at position 1361 and 1374. The M and E genome were similar to the parental virus.

Figure 4. Positioning on the S protein of the UDA induced MHV and the GNA induced FIPV mutations.

Figure 4a: MHV resistance to UDA

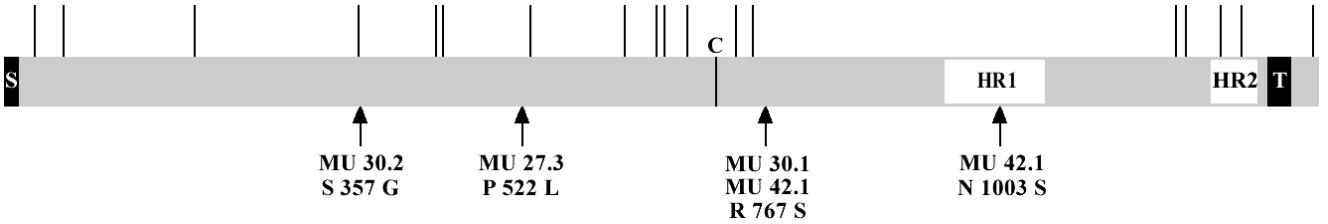
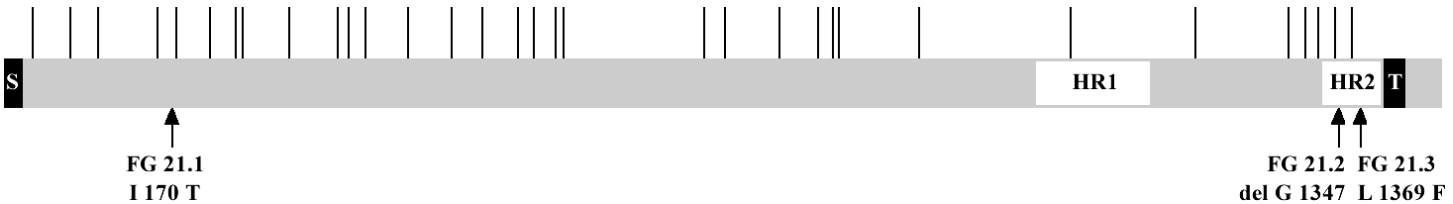


Figure 4b: FIPV resistance to GNA



The large grey bars are representing the spike proteins of MHV (figure 4a; 1324 amino acids) and FIPV (figure 4b; 1452 amino acids). The positions of potential N-glycosylated sites are indicated with a line on top of the grey bars. The mutated amino acids are pointed out with an arrow, the nomenclature of the mutated viruses as presented is explained in the results section. The HR1 and HR2 stretches are indicated in white, in black the transmembrane stretch in the carboxy terminal part of the protein contains a T, whereas the signal peptide in the amino-terminal part contains a S. Additionally the furine cleavage site in MHV A59 is indicated with a C.

Discussion

The generation of resistant viruses following antiviral exposure both *in vivo* and *in vitro* has been described (26, 33). No report of coronaviruses becoming resistant to any antiviral compound has been published so far. In this study we were able to evaluate the possibility to generate coronaviruses resistant to carbohydrate binding agents (CBA). We used a method as that was described to generate CBA resistant HIV strains (4-8, 44): propagating virus in the presence of escalating lectin concentrations. Applying this method we were able to generate viruses that can be regarded as resistant to plant lectins based on the replicating capacity at high UDA or GNA concentrations and their resistance indices.

As retroviruses like HIV, are always incorporated as provirus in persistently infected cell lines, subcultivation of retroviruses in the presence of antiviral compounds can continue until virus replication commences. The principal difference in the method of resistant coronavirus generation compared to retrovirus assays is the possibility to eliminate the virus. The detection of virus indicates the development of resistant strains. The subcultivation of coronaviruses will be unsuccessful when the antiviral compound concentration exceeds the amount partly resistant viruses can withstand. The use of coronavirus infected cell lines (20, 36) in these procedures can be considered, but the mechanism by which these viruses can persist in these cell lines remains to be elucidated.

Whether the observed small plaque phenotype of MU 42.1 can be directly related to the mutation at position 767 is not clear. This mutation is relatively close to the furine cleavage site at position 717. Changes in this region might influence plaque phenotype by reducing cell-cell spread or replicative capacity. This question can only be answered by using reverse genetics techniques, thereby introducing the mutation in a wildtype MHV A59 background. However, not necessarily a mutation in the S protein has to be the basis of this phenotype. Also mutations in other virus genes might induce changes in plaque size (30).

Both, the generation of UDA resistant MHV strains and GNA resistant FIPV strains seemed successful. As all lectin resistant HIV strains contained deletions of N-glycosylated sites (4-8, 44) in the surface protein (gp120) our focus directed towards changes in the coronavirus envelope glycoproteins, E, M and S. In most MHV strains, the S protein is cleaved posttranslational into an amino-terminal (S1) and a carboxy-terminal (S2) subunit (18, 37). FIPV does not contain a cleavage site. A globular head formed by the amino terminal part exhibits a receptor-binding activity (14, 27). Membrane fusion is mediated by the carboxy terminal subunit which contains apart from two heptad repeat regions a transmembrane domain (14), indicated in figure 4. Both MU and FG subcultivation procedures resulted in viruses containing mutated S proteins. Six out of 7 observed mutations were found in the close proximity of N-glycosylated sites. The effective removal of only one

N-glycosylation site was remarkable, as similar experiments using HIV always resulted in mutations at multiple glycosylated sites in the gp120 envelope protein. The MU strain with the lowest number of unsuccessful subcultivation attempts, strain 1, contains two mutations in this S2 part of the spike protein. The mutation in MU strain 1 at position 767 is in the close proximity of a N-glycosylated Asparagine at position 754. An additional mutation found in strain MU 42.1 is situated in the centre of the HR1 peptide, away from any glycosylated site. This might indicate that a successful UDA resistance can be achieved by changing this part of the spike protein. As the HR1 peptide is involved in the fusion process of MHV, changes in this peptide might alter the structural changes preceding host cell entry. Two separate FG strains showed mutations in the HR2 region. Virus fusion inhibition was earlier described as the mode of action for plant lectins towards HIV (2, 3).

Several mechanisms can be proposed that led to a diminished coronavirus CBA sensitivity. Selection of N-glycan deleted virus strains was expected, however these mutations were uncommon in resistant coronaviruses. As the coronaviruses exist in an infected individual as a heterogeneous virus cluster the CBA selection merely aims to identify the best-fit virus. These resistant viruses must be already present in the so called quasispecies (17). As our selection procedure was hardly able to derive N-glycan deleted coronaviruses the prevalence of these mutants in the quasispecies is expected to be very low. These structures might be essential for coronavirus protein function. An alternative to evade lectin exposure is the induction of changes in the sugar content present at N-glycosylated structures. This could eliminate the possibility to bind lectin resulting in resistance without the removal of the N-glycan site. Earlier it was shown that the HIV envelope glycan shield consist of a combination of complex, hybrid and high mannose structures (28). The way these different glycan types are assigned to an Asparagine is not exactly known, but it is unlikely that the glycan shield of MHV and FIPV will only contain high mannose structures. As N-glycan maturation and diversification is not only determined by the protein sequence, other factors like the time spend in the endoplasmic reticulum and Golgi might also have influenced the glycosylation status of the MU and FG S proteins (29). The observed mutations also could have led to adaptations in the folding of the S protein. As a consequence the lectins binding sites might become inaccessible. This strategy is also observed for HIV resistance to NNRTI. NNRTIs are compounds binding to an allosteric pocket of the reverse transcriptase enzyme of HIV. By changing the aligning amino acids, this pocket becomes inaccessible (12). The last mechanism we suggest is an acceleration of the membrane fusion kinetics. This mechanism has been proposed for the HIV entry inhibitor enfuvirtide (32). Since CBA targets structures involved in the membrane fusion process, changing the binding site exposure time will

logically modulate virus sensitivity for these compounds. We found only a limited amount of mutations in the S protein but amino acid substitutions inducing resistance are not necessarily restricted to the envelope glycoproteins. Compensatory mutations might be present in genes playing a modulating role during protein production. The coronaviral polymerase 1a and 1b are candidates for further evaluation.

We conclude that generation of lectin resistant coronaviruses is possible. The induced virus mutations only led in one occasion to the deletion of a N-glycosylated site, suggesting the importance of N-glycosylation for the normal coronavirus glycoprotein function. To investigate the impact of the observed mutations, these mutations should be introduced in the S gene present in a wildtype coronavirus backbone by a reverse genetics system. When these modifications do not result in resistance, further exploration of the MU and FG genomes is warranted. Moreover a more detailed investigation of the glycosylation structures of coronaviruses seems essential to fully appreciate their importance during viral attachment and entry processes.

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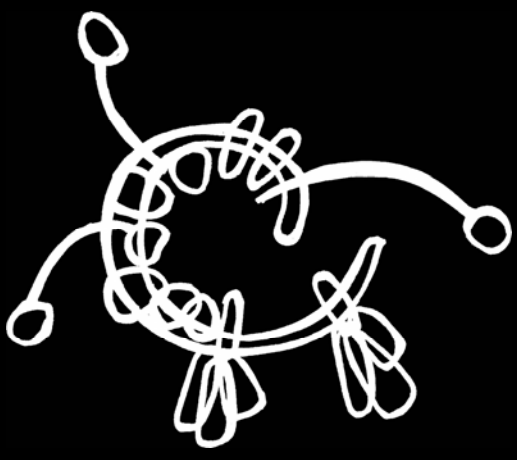

Chapter 7

General discussion

In this thesis a study is described that aimed to evaluate the effects of various antiviral agents on retrovirus and coronavirus infections. Both retro- and coronaviruses contain RNA as their genetic material and carry a lipid envelope with glycosylated proteins. Yet, the life cycle, pathogenesis and symptomatology of these viruses are fundamentally different.

In the first part of the thesis, the possibilities for improvement of antiviral *in vitro* assessments were explored. We established an *in vitro* system (chapter 2) that can be used to study the role of the dendritic cell (DC) in feline immunodeficiency virus (FIV) infection of T cells. The results demonstrated that DC-FIV-T cell interactions are very similar to those described for human DC and T cells with HIV, strengthening the significance of the FIV system as a model for HIV. Besides for antiviral studies, this cell system may assist future research on the interactions of lentiviruses with the immune system.

Next an evaluation of the activity of antivirals towards FIV infection in various cell systems was performed. We compared 'old' cell systems with 'new' antivirals, and 'new' cell systems with 'old' antivirals. It became evident that the efficacy of antiviral compounds can be dramatically influenced by differences in the host cells used (chapter 3). The conclusion could be drawn that antiviral agents performed dissimilar in different cell systems. This underlines the importance of using different cell systems for such screenings.

A common feature of retro- and nidoviruses, glycosylation of envelope glycoproteins, was used to comparatively study the potency of a new type of antiviral: the carbohydrate-binding agents (CBA). In view of the first promising observations with such agents towards HIV (5, 17)), we evaluated their effects on FIV infection (chapter 3), as well as on members of different families of the *Nidovirales* order (chapter 4-6). Until 2003 coronaviruses had not been explored as target for antivirals despite their importance as human and veterinary pathogens. From then on they became the subject of antiviral studies mainly because of the possible re-emergence of the SARS coronavirus.

The presence of carbohydrate binding compounds in our *in vitro* systems strongly inhibited the infections by coronaviruses, arteriviruses and torovirus (chapter 4). To facilitate future research of anti-nidoviral chemotherapy, we compared different assays using as read-out parameters cell viability (MTT-based colorimetric assay), infected cell number (immunoperoxidase assay) and viral protein expression level (luciferase based assay). For the latter purpose, recombinant coronaviruses expressing luciferase were found particularly easy to use and accurate tools in antiviral studies.

The exact mechanism by which the CBA act upon viral infection of vertebrate cells has not been elucidated. However, there are strong indications that the interaction of CBA during the infection process occurs mainly at the stage of virus entry (8,

11, 12)(chapter 5). Limited knowledge is available about their efficacy towards nidoviruses. Our results indicate that CBA target the two glycosylated envelope proteins, the spike (S) and the membrane (M) protein, of mouse hepatitis virus (MHV) and feline infectious peritonitis virus (FIPV). Furthermore, CBA did not inhibit virus-cell attachment, but 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 glycans. Inhibition of mannosidases in host cells rendered the progeny viruses more sensitive to mannose binding compounds and even to the N-acetylglucosamine binding compound UDA. In addition, inhibition of coronaviruses was shown to be dependent on the cell type used to grow the virus stocks (all described in chapter 5).

To prove that CBA are inhibitory to viruses and the antiviral activity is not mediated by influences on cell physiology, we generated escape mutants (chapter 6) by exposing FIPV and MHV to escalating concentrations of plant lectins (GNA and UDA, respectively). In contrast to observations with retroviruses in response to CBA, elimination of N-glycosylation sites from the coronavirus envelope glycoproteins was detected in resistant viruses only once. It is likely that the observed amino acid substitutions led to changes in the three dimensional structure of the spike glycoprotein, thereby rendering the lectin target sites inaccessible. We could not exclude, however, that additional mutations in the coronavirus genome contributed to the CBA resistance.

Our combined results clearly qualified CBA as candidate anti-nidovirus agents. In addition, these agents might as well be valuable tools for the study of the early host-nidovirus interplay and for the role of glycosylation during a nidovirus infection.

Refinement of *in vitro* cell systems for the evaluation of antivirals

> "Since every model has its limitations, a model cannot be held to any absolute standard of performance. Instead, a model is validated by comparing it to an alternative. Such validation requires expertise in modeling and experience in judging models." John D. Hawke, Jr. *Before a Conference on Measuring Financial Risk in the 21st Century; Washington, D.C.* 1999

The 'Red Queen effect' (57) [a]

The assessment of antiviral activity *in vitro* can be regarded as a first step towards the development of a new antiviral chemotherapy in patients. However, the biological and physiological context in which drugs exert their therapeutic effects in patients is vastly more complex compared to *in vitro* models (44). Viruses rely on, and will therefore adapt to, their host cells. These adaptations result in alterations in viral structural and/or non-structural proteins. The virus will change during the process of the viral pathogenesis as the site of virus introduction, replication and excretion can involve separate body compartments, all containing cells with their own characteristics. These host cell differences within an infected individual will have its repercussions on the virus protein constitution leading to differences in, for example, composition of the glycan structures on the envelope and/or cell tropism (46). This continuous evolution[b] assists the pathogen to evade host immunity (1) and the host's strategy to cope with the infection. These virus evasion mechanisms might render an otherwise potent antiviral unsuitable, due to its inability to eliminate the virus from its *in situ* hiding places. It is clear that an *in vitro* system for antiviral assessment should not only evaluate the ability to eliminate the pathogen of a certain chemical; equally important can be the ability to eliminate these pathogens in all its possible *in situ* variants. Therefore an *in vitro* assay must qualify as a model reflecting the normal physiology as close as possible.

[a] Based on a comment of the Red Queen to Alice. "Now, *here,* you see, it takes all the running you can do, to keep in the same place." Through the Looking-Glass and What Alice Found There. Chapter 2 page 46. Lewis Carroll 1862.

[b] Change in the genetic composition of a population during successive generations, as a result of natural selection acting on the genetic variation among individuals, and resulting in the development of new species.

How to approach the in vivo virus-cell interaction?
The necessity to improve *in vitro* systems used for the evaluation of antivirals presented itself in three separate experiments. The studies described in chapter 3, 4 and 5 all indicated antiviral activity varied considerably when using different host cells. Without exception the inhibitory capacities of the evaluated agents were lower when measured on immune cells (dendritic cells (DC), thymocytes, peripheral blood mononuclear cells (PBMC) and peripheral alveolar macrophages (PAM) than when using fibroblast-like cells. The use of primary cells at least involves the natural target cells in the system; however, they will never completely mimic the cells in the patient's morphology and biochemical features. Cultivation may have disrupted its natural physiology and pose an impact on the constitution of the derived viruses. Therefore an increased interest exists for the use of alternative cell cultivation methods in a 3 dimensional structure (2) which more closely resemble the normal cell-cell interactions and natural virus spread. As an additional *ex vivo* step preceding *in vivo* assessments this might be a helpful tool predicting the chances on success. In most cases the predictive value of evaluation systems is unknown therefore the results need critical appraisal.

Further development of the FIV *in vitro* model for HIV
The DC - T cell interaction has already been described in an extensive body of literature on HIV (52). In chapter 2 of this thesis, a method was established suitable for further elucidation of the early FIV pathogenesis in analogy to the HIV interaction with DC. The infection system allows us to study the interaction of feline DC and T cells assuming that the coculture conditions more optimally mimic the natural *trans* infection and replication of FIV.
The DC-pathogen interactions occur at an early stage of virus infection. As with DC in other species, feline DC are believed to transport viral particles to lymph nodes and transfer them to lymphocytes (13). It can be assumed that a feline version of DC-SIGN, a C-type lectin of dendritic cells used to bind pathogens, or similarly acting pathogen recognition molecules exist. Human DC-SIGN is known to interact with HIV (29) as well as with many other pathogens (56) in humans. Human DC-SIGN has also been shown to interact with FIV (24). Therefore we assumed analogies of HIV-DC-T cell and FIV-DC-T cell interactions. Consistently, we observed in our DC-T-cell coculture system an upregulation of FIV replication comparable to that reported for HIV (19) Our data suggest that feline DC behave similar to simian and human DC in their interaction with leukocytes. The addition of feline T cells to FIV-exposed feline DC caused the same enhanced level of infection as a direct infection of feline DC-T cocultures (unpublished results). However, we were not able to detect FIV replication in feline

DC, in contrast to the infection of human DC observed with HIV. Whether this is due to an intrinsic inability of FIV to infect and/or replicate in these cells or a consequence of the used DC cultivation method is not clear and needs further assessment. Overall, it would be interesting to further dissect the feline DC-T cell interaction in the presence or absence of a FIV infection. A further assessment of intervention strategies targeting the different stages of the interaction will be possible. Moreover, the expertise and insights obtained could as well be beneficial for the study of other feline infections in which an involvement of dendritic cells can be assumed in the pathogenesis, like FIPV and Feline leukaemia virus.
In the studies of chapter 2 we were able to generate sufficient amounts of DC for our experiments. However, to improve the study of feline DC an easy and highly specific selection method for feline monocytic cells from bone marrow or peripheral blood would be beneficial. The magnetic bead sorting systems used to select progenitors of dendritic cells from human bone marrow derived monocytes (CD34) and blood monocytes (CD14) were, when applied to feline bone marrow or blood, in our hands not able to select feline progenitors of dendritic cells Moreover, improvement might be achieved by optimization of DC propagation procedures from monocytes, as this might enhance the quality of the resulting cells (42) and thereby also the reproducibility of the results.

Targeting the N-glycosylation of viruses

> "It would appear to be a matter of semantics as to whether a substance not produced in response to an antigen should be called an antibody even though it is a protein and combines specifically with certain antigen only. It might be better to have a different word for these substances and the present writer would like to propose the word *lectin* from Latin *lectus*, the past principle of *legere* meaning to pick, choose or select" W.C. Boyd: *The proteins of immune reactions* 1954 (18)

The advantages of lectins
By their carbohydrate recognition domains, lectins can recognize glycan structures on viral proteins. Lectins are defined as proteins that bind carbohydrates without initiating further modifications through associated enzymatic activity (61). Proteins that are classified as lectins can have totally dissimilar structures, thus the definition is functional rather than structural (65). Lectins are found in most organisms, ranging from viruses and bacteria to plants and animals (23, 28, 38). In

plants, lectins are mainly involved in recognition processes either within or outside the plant by binding to carbohydrates (38, 55). In the natural situation plant lectins may play an important role in the defense mechanism of the plant (50). Yet, only for UDA[a] has an involvement in the resistance of stinging nettle to fungal colonization been clearly demonstrated (58). Also in the innate immunity of vertebrates, lectins binding with a low specificity to high-mannose glycans of pathogens (i.e. viruses) are part of a defense mechanism (26, 27, 39). The glycans that cover retrovirus particles are used as a mechanism to escape host antibody-mediated immunity (15, 45, 60). As nidoviruses also contain glycosylated envelope proteins (34), it has been suggested that these glycans are used for the same purpose (3, 20).
Lectins or CBA were recently evaluated for their antiviral activity against a variety of enveloped viruses and were shown in most cases to inhibit virus infection at low concentrations (5-8, 12, 14, 17, 30-32, 38, 43, 48, 59, 63, 64)(chapter 3). CBA can thus be considered a new class of promising virus inhibitors. Mannose (GNA[b], HHA[c], CA[d], CV-N[e] or PRM-A[f]) or N-acetylglucosamine (GlcNAc) binding agents (like UDA) are not expected to act very specific (10, 33) as mannose and GlcNAc units are part of the oligosaccharide side chains of most N-glycosylated proteins. This can be an advantage as compared to the more specifically acting neutralizing antibodies that target a particular glycan-containing epitope on a viral glycoprotein like, for example, the HIV-1 specific antibody 2G12 (10, 33). Viruses can easily escape from such antibodies by mutations either in the sequence encoding the epitope or in sequences elsewhere in the molecule that render the epitope inaccessible due to surrounding glycans. In contrast, CBA may concomitantly bind to several glycans exposed on the virus envelope. Hence, multiple protein changes will be required to reduce their inhibitory potency.

Application of CBA as anti- nidovirals
Systemically acting nidoviruses like the coronaviruses FIPV and MHV, but also the arteriviruses equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV) were included in our research (chapter 4-6). Whereas the coronaviruses exhibited a high sensitivity for CBA, the arteriviruses appeared to be significantly less sensitive to these agents. Whether these results

[a] *Urtica dioica* agglutinin
[b] *Galanthus nivalis* agglutinin
[c] *Hippeastrum* hybrid agglutinin
[d] *Cymbidium* agglutinin
[e] Cyanovirin-N
[f] Pradimicin-A

reflect the *in vivo* sensitivities needs to be established. The nidoviruses that replicate in macrophages (FIPV and PRRSV) are expected to contain glycoproteins of which only a limited fraction is of the high mannose type. Macrophages are known to process glycan structures present on virus envelopes more extensively than occurs in many other types of infected cells such as, for instance, PBMC (36, 62). This feature might reduce the efficacy of CBA as agents against enveloped viruses using macrophages as their target cell.
From the limited studies that have been done by others and us, it can be concluded that CBA constitute a very promising new class of antivirals. ,. However, apart from their *in vitro* efficacy against viruses and the development of resistance, there is only limited knowledge about important aspects such as application routes and formulations, biodistribution and toxicity. Moreover, these compounds might trigger an immune response, which might, upon repeated application, reduce the antiviral effect.
The MHV infection model seems like a suitable model to evaluate all these aspects *in vivo*. MHV induces reproducibly distinct pathological effects in mice and can be efficiently inhibited by CBA *in vitro* (chapter 4). As readout parameters survival time or virus replication levels in appropriate organs such as liver and brain can be used. The luciferase containing viruses may facilitate these evaluations.

Application of lectins to elucidate host-pathogen interactions
Viruses use a variety of carbohydrate-protein interactions when entering their target cells. For receptor recognition and early virus host interactions glycans were shown to be crucial (4, 21). For viruses such as influenza virus (49), herpes viruses (53), PRRSV (25), and many corona- and toroviruses (22), terminal sialic acids exposed by N-glycosylated proteins are the primary determinants of attachment. The ability of UDA and PRM-A to inhibit fusion but not virus attachment (chapter 5) suggests that involvement of high-mannose glycans in coronavirus binding to the receptor is rather limited. These glycan structures probably serve a different purpose, like proper folding of the protein or concealing antigens in order to avoid recognition by the immune system. The latter function has been shown for HIV (15, 45, 60) and has been suggested for coronaviruses (3, 20). A role for the high mannose glycoproteins can be expected in the virus fusion processes. Attachment of relatively large lectins to N-linked sugars can impose steric hindrance or their binding might cross link membrane glycoproteins (35). The appearance, due to exposure to lectins, of virus mutants with altered glycosylation in regions of the viral envelope protein involved in virus fusion can be beneficial for virus entry and can therefore be regarded as a viral resistance mechanism. An indication of this resistance mechanism was described in chapter 6 where we attempted to derive

lectin resistant coronaviruses. This procedure might be refined and prolonged to further assess the flexibility of coronaviruses to escape suppression by lectins.

Future perspectives

> Perception is fundamentally selective…unseen objects can momentarily re-enter awareness when they physically disappear: in some situations, you can see the disappearance of something you can't see. Mitroff SR et al. *Seeing the disappearance of unseen objects*. 2004 (40)

As already pointed out, the results described in this thesis offer new perspectives but will also need a lot of further work. Ideally, this will eventually lead to new drugs applicable for therapeutic intervention. Certainly it will provide further insight in the biology of viral infections particularly with regard to the role of glycans. This might in turn give rise to unexpected new possibilities for applications not only in the field of intervention but also for prevention, as will be illustrated.

Study of hypoglycosylated coronaviruses as vaccine strains
To induce a strong neutralizing antibody response, it would be advantageous to remove the 'invisibility cloak' that enveloped viruses carry by the glycan cover of their surface glycoproteins. Hidden behind this protective shield it is difficult and sometimes even impossible for the immune system to induce a sustainable response towards certain epitopes of these viruses. Removal of oligosaccharide side chains from envelope glycoproteins led to virus attenuation. Subsequent exposure of such hypoglycosylated viruses to the immune system resulted in a robust antibody mediated response (3, 41, 45, 47). By CBA guided selection of mutant strains lacking several N-linked sugars (9-11, 63), novel vaccine strains might be obtained. However, selection of mutated strains can be time consuming and the result is uncertain. By applying site directed mutagenesis, carefully selected N-glycosylation sites can be removed to generate attenuated hypoglycosylated coronaviruses that thereby expose otherwise hidden antigenic sites. A thorough assessment of the potentially disposable glycosylation sites of envelope glycoproteins should precede such experiments. This procedure should in principle be applicable to every virus that uses its glycan shield in immune evasion strategies.
Viral envelope proteins have often been produced for vaccination purposes by various expression systems such as vaccinia virus, bacteria or yeast. However, due

to inappropriate formation of secondary, tertiary and glycan structures the antiviral immune responses observed in vaccination studies with expressed hypoglycosylated proteins have been disappointing (16, 41). Besides the targeted removal of glycosylation sites in a viral protein, new glycan structures can be introduced at other sites of the protein, which might enhance presentation to dendritic cells (21, 37) or improve T cell recognition (51), a prerequisite for an efficient response by B and T cells. However, the ability of DC-SIGN to bind virus is dependent on the type of carbohydrate structures present on viral envelope glycoproteins and not merely on the presence of N-glycans *per se*. Therefore the additional factors, which eventually determine the glycan structure attached to an Asn-Xaa-Ser/Thr sequon in a virus glycoprotein, need further assessment to be able to use this knowledge for vaccinological purposes.

Possibilities for CBA application in veterinary medicine

The use of lectins as antivirals received recently much appreciation. Their apparent non-toxic nature and potent antiviral activity helped to instigate research to further develop these agents. As microbicides (6, 54) they may act in the prevention of HIV spread. This form of application will not be an option to avoid coronavirus infections, unless CBA can be used to prevent virus entry without interfering with nutrient absorbing capacities of enterocytes or lung epithelial cells. It does not seem likely that this can be easily achieved. A further development as anti-coronavirus agent will probably involve systemically applied agents for therapeutic or prophylactic purpose.

Systemically replicating nidoviruses (PRRSV, FIPV and EAV), not concealed in host cells, will eventually become available for elimination by CBA. These nidoviruses have to distribute themselves continuously by budding or by lytic destruction of their host cells in order to survive. Moreover, no latency stage occurs during their replication cycle. These factors offer antiviral opportunities for CBA. To be effective it seems necessary that CBA are capable of eliminating the nidoviruses from the monocytic/macrophage cell compartment (PRRSV, FIPV) or from the secondary sex glands of horses (EAV). The most promising compound to accomplish this is UDA. In our hands this compound showed good antiviral properties (chapter 4 and 5), contains a low molecular weight (8.5 kD) and can be applied systemically without acute toxicity (9). This does not rule out the risks of side effects during long-term exposure. When formulations of CBA suitable for application in pigs, horses or cats will become available, these products may be able to convert potential lethal (PRRSV and FIPV) and economical important (PRRSV and EAV) nidovirus infections into manageable diseases.

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Samenvatting in het Nederlands

Virussen zijn zeer kleine ziekteverwekkers met erfelijk materiaal. Ze kunnen zich niet buiten een gastheer vermenigvuldigen, in tegenstelling tot veel bacteriën en parasieten. Voor onderzoek naar virussen zijn dus celsystemen (*in vitro*) nodig waarin cellen als gastheer dienen voor het virus dat onderzocht wordt. Alle levende organismen hebben DNA als genetisch materiaal; RNA is een kopie van het DNA en wordt gebruikt voor het maken van eiwitten. Virussen kunnen zowel DNA als RNA als genetisch materiaal hebben, dit is uniek voor virussen. Daarnaast kan er een onderverdeling worden gemaakt van virussen met en zonder een mantel, ook wel envelop genoemd. Men spreekt van envelop bevattende virussen en naakte virussen.

Mijn onderzoek is uitgevoerd met twee verschillende groepen virussen:

1. de lentivirussen: de naam is afkomstig van 'lenti' dat 'traag' of 'langzaam' betekent. Bijvoorbeeld het immuundeficientie virus van katten: FIV, een virus dat AIDS bij katten kan veroorzaken. Dit FIV en de ziekte die dit virus veroorzaakt lijkt zoveel op het mensenvirus HIV en de ziekte AIDS dat het vaak wordt gebruikt als diermodel ten behoeve van onderzoek naar HIV en AIDS.

en

2. de coronavirussen. Het woord 'corona' dat 'kroon' betekend, is voor deze virussen gebruikt omdat een krans van uitsteeksels zichtbaar is wanneer dit virus met een electronen microscoop wordt bekeken. Hiertoe behoort het virus dat bij katten een infectieuze buikvliesontsteking veroorzaakt (FIPV) en het virus dat bij muizen een leverontsteking kan veroorzaken (MHV). Deze virussen hebben grote overeenkomsten met het SARS-coronavirus dat bij mensen SARS veroorzaakt.

De manier waarop deze virusgroepen ziekten veroorzaken en zich in het lichaam vermenigvuldigen is niet met elkaar te vergelijken. Een schematisch plaatje van deze virussen en de manier waarop ze zich vermenigvuldigen in de cel is te vinden in hoofdstuk 1, figuur 1, 2 en 3. Beide virusgroepen hebben als overeenkomst dat ze RNA als genetisch materiaal gebruiken en een envelop hebben. Zo'n virus envelop bestaat uit een vet en eiwit structuur en wordt verkregen van de gastheercel. De envelop eiwitten, proteïnen, kunnen daarnaast ook nog suikergroepen bevatten. De aanwezigheid van deze suikers heet 'glycosylatie',

vandaar dat deze suiker bevattende eiwitten ook wel ‘glycoproteïnen’ genoemd worden. De suikergroepen kunnen tot wel 50% van het totale gewicht van de glycoproteïnen uitmaken.
Deze suikergroepen op de glycoproteïnen zijn om een groot aantal redenen belangrijk voor het virus. Allereerst helpen ze het eiwit om zich goed te vormen, en dat is weer belangrijk voor de functie van het eiwit. Daarnaast helpen suikerstructuren bij het herkennen van de gastheercel. Ook kan het virus zich met behulp van deze suikerstructuren verschuilen voor het afweersysteem van de gastheer. Het virus kan op die manier langere tijd aanwezig blijven in de gastheer. Hoe deze glycosylering van eiwitten plaatsvindt staat schematisch aangegeven in figuur 4 van hoofdstuk 1. Het grote verschil in de glycosylering van de eiwitten van de gastheer zelf en de eiwitten van het virus is de aanwezigheid van relatief veel mannose groepen op virale glycoproteïnen. Mannose is net als bijvoorbeeld glucose een suiker molecuul. Eiwitten van de gastheer zelf hebben die suikergroep niet of nauwelijks.

In hoofdstuk 2 en 3 hebben we een studie beschreven naar de mogelijkheid om *in vitro*-systemen waarin virus onderzoek gedaan kan worden te verbeteren en daarnaast verschillende systemen met elkaar te vergelijken. Een nieuw celsysteem dat de eerste interactie van het virus met het afweer apparaat van de kat moet weerspiegelen werd gemaakt. Afweercellen die dendritische cellen worden genoemd zijn opgekweekt uit beenmerg van de kat. Deze dendritische cellen vormen de eerste lijn van afweer in het lichaam. Indien ze een ziektekiem tegenkomen nemen ze deze op, breken hem in kleine stukjes en verplaatsen zich naar de lymfeknopen waar ze samen met zogenaamde T-helper cellen de afweer op gang brengen. Deze T-helper cellen zullen de dendritische cellen met afgebroken ziektekiemen herkennen en andere cellen activeren die betrokken zijn bij de afweer. Dit kan resulteren in afweerstoffen als antilichamen door zogenaamde B cellen of het doden van andere cellen die met het virus geïnfecteerd zijn, dat doen de cytotoxische T cellen.
We veronderstelde dat de afweer tijdens een FIV infectie ook zo zou optreden. Echter, bij HIV infecties was al gebleken dat deze dendritische cellen het HIV virus niet goed kunnen verwerken. Doordat de dendritische cellen het intacte HIV virus naar T-helper cellen in de lymfeknopen toebrengen wordt die infectie juist versterkt. HIV infecteert namelijk deze T-helper cellen. Dendritische cellen stimuleren deze T-helper cellen zodat er een verhoogde HIV vermeerdering kan optreden in deze T-helper cellen. Met andere woorden, de dendritische cellen worden door HIV dus gebruikt als een soort Trojaans paard: in plaats van de afweer in tegen HIV in werking te stellen, brengt de dendritische cel het virus bij de cel gebracht die het virus nodig heeft om zich in te vermeerderen. Er is al veel

onderzoek gedaan naar de relatie tussen het HIV virus van mensen, dendritische cellen en T-helper cellen. Over de interactie van FIV met dit type cellen was veel minder bekend. Ons onderzoek geeft een eerste aanzet tot het vaststellen van de interactie tussen het FIV en de dendritische cellen. Dit is een belangrijk gegeven in het ontstaan van de ziekte AIDS bij katten. Daarom wilden we ook graag weten of we met antivirale middelen op dit proces kunnen ingrijpen. Dit hebben we onderzocht door verschillende antivirale middelen, genaamd AZT, (R)-PMPDAP en PMEA te testen met dit cel systeem. Deze en soortgelijke anti-HIV middelen worden op dit moment al ingezet bij veel HIV patiënten. We hebben hierbij onze celsystemen als model gebruikt en de vergelijking gemaakt tussen FIV infecties van T-cellen met en zonder dendritische cellen.. Hieruit bleek dat voor de werkzaamheid van AZT het wel degelijk uitmaakt of dendritische cellen in het celsysteem aanwezig zijn, voor PMEA en (R)-PMPDAP maakte het niet uit. Het AZT bleek in het systeem waar zowel dendritische als T cellen aanwezig waren veel minder goed te werken.

We hebben verder onderzocht of het mogelijk was een antiviraal middel te ontwikkelen dat zich zou richten op suikergroepen op glycoproteïnen die veel mannose bevatten. Dit was al eerder succesvol gebleken als antiviraal middel tegen HIV. Deze antivirale middelen worden wel lectines of koolhydraat bindende agentia (in het Engels: carbohydrate binding agents of CBA) genoemd. Om een goede studie te doen naar antivirale middelen moet er worden vastgesteld of zo'n middel ook echt alleen het virus uitschakelt en niet toevallig ook nog erg giftig is voor de gastheer cellen zelf. Ook wilden we graag weten of het werkzaam is tegen de verschillende virussen, en zo ja hoe goed dan wel. Dit drukken we uit in de effectieve concentratie die 50% van de infectie voorkomt = EC_{50}. Daarnaast hebben we onderzocht hoe het middel precies werkt en of er ook resistentie tegen kan ontstaan.

In het onderzoek als beschreven in hoofdstuk 3 is een studie gedaan naar de werkzaamheid van CBA ten opzichte van FIV. Hieruit konden we concluderen dat de EC_{50} van CBA tegen FIV afhankelijk bleek te zijn van het celsysteem waarop het virus was vermeerderd. FIV vermeerderd op katten nier cellen bleken veel gevoeliger te zijn voor CBA dan FIV verkregen van T cellen uit het bloed of uit de thymus/zwezerik. Dit komt waarschijnlijk doordat het virus zijn envelop verkrijgt van de gastheer. Hierdoor heeft de gastheercel veel invloed op de samenstelling van die envelop. Het virus zelf kan maar een beperkte invloed hierop uitoefenen. Omdat CBA aanhechten op de envelop van de gastheercel is deze cel van grote invloed op de werkzaamheid van deze middelen. Het is dus van belang om meerdere celsystemen met elkaar te vergelijken voordat de conclusie kan worden

getrokken dat een bepaald antiviraal middel wel of niet geschikt is ter bestrijding van een virusinfectie.
In het onderzoek als beschreven in hoofdstuk 4 en 5 hebben we eveneens kunnen aantonen dat CBA goed werken tegen coronavirussen, maar tevens werkzaam zijn tegen arterivirussen en torovirusen die sterk verwant zijn aan coronavirussen. De hoeveelheid mannose die aanwezig is in de suikerstructuren op de glycoproteïnen van de envelop bleek van doorslag gevend belang te zijn voor de werkzaamheid van deze CBA. Hoe meer mannose groepen aanwezig op de glycoproteïnen, hoe beter het bleek te werken.

Omdat deze middelen zich richten op de suikergroepen van de glycoproteïnen ligt het voor de hand dat een virus resistent zal worden door deze suikergroepen te verwijderen. Het virus zal veranderen, het zogenaamde muteren, en wel zo dat er nog zo min mogelijk mannose aanwezig is op de envelop. In eerdere studies bleek HIV ook in staat hele suikergroepen van de envelop te verwijderen en zo resistent te worden.
Bij coronavirussen ligt dat minder eenvoudig, bleek uit de studie welke is beschreven in hoofdstuk 6. Hoewel dit deel van het onderzoek nog voortgezet zal worden, blijken coronavirussen die minder gevoelig zijn voor CBA zich er veel aan gelegen laten liggen om de suikergroepen op hun envelop-eiwitten te behouden. Dit duidt erop dat deze structuren zeer belangrijk zijn voor het normaal functioneren van het virus. Er zijn wel veranderingen waargenomen op de envelop, met name rondom de suikergroepen, maar slechts een keer was het verdwijnen van een suikergroep een feit.

Aangezien we hier pas aan het begin van een nieuwe ontwikkeling staan moeten deze CBA nog verder worden aangepast en onderzocht. Dit om te zorgen dat ze zo weinig mogelijk bijwerkingen hebben en toch een goede antivirale activiteit laten zien. Ook zou een nader onderzoek naar de samenstelling van de suiker structuren op de proteïnen en hun functie voor het coronavirus moeten uitwijzen of we in de toekomst nieuwe levende virus varianten kunnen maken waarvan enkele suikergroepen zijn verwijderd. Als deze suikerstructuren zijn weggehaald kan het virus zich minder goed verschuilen voor het afweer systeem. Omdat daardoor hun ‘onzichtbaarheidsmantel’ is verwijderd zouden deze virus varianten misschien ingezet kunnen worden in vaccins.

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Curriculum vitae

Frank van der Meer werd geboren op 20 februari 1967 te Aalsmeer. Na een lang traject op de middelbare school (Mavo-Havo-VWO) behaalde hij in 1987 zijn VWO diploma aan het Keizer Karel College te Amstelveen. Datzelfde jaar werd een aanvang gemaakt met de studie Diergeneeskunde aan de Universiteit Utrecht. Tijdens deze studie was hij een jaar Ab-actis van de Diergeneeskundige Studenten Kring (DSK), Peerdepiet, lid van enkele commissies en had zitting in de studentengeleding van de faculteitsraad. Zijn afstudeerscriptie had als onderwerp de Ovum Pick Up techniek bij paarden, waaraan een half jaar onderzoek werd besteed. In 1996 studeerde hij af als dierenarts met als afstudeerrichting landbouwhuisdieren. Vervolgens is hij ongeveer 1.5 jaar werkzaam geweest in Veendam en Denekamp als algemeen practicus. In 1998 keerde hij terug naar de Diergeneeskundige Faculteit om te gaan werken bij de toenmalige hoofdafdeling Landbouwhuisdieren. In samenwerking onder andere het proefbedrijf voor de paardenhouderij ‘de Waiboerhoeve’ te Lelystad werd een onderzoek gedaan naar de mogelijkheden voor het immunologisch castreren van hengsten. In de tussentijd was hij ook gaan werken als docent veterinaire virologie bij afdeling Virologie van de hoofdafdeling, later departement Infectieziekten en Immunologie. Van september 2001 tot mei 2003 was hij werkzaam bij de afdeling Veterinaire Farmacologie, Farmacie en Toxicologie (VFFT) als docent/onderzoeker farmacologie, waarna hij in juni 2003 wederom in dienst trad van de afdeling Virologie, nu om een aanvang te maken met zijn promotie onderzoek met dit proefschrift als resultaat.

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