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Single Domain Camelid Antibodies that Neutralize Negative Strand Viruses

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1. Introduction

1.1 Conventional antibodies

Recombinant antibodies (Abs) are widely regarded as one of the main, if not the most promising tools against cancer and auto-immune, inflammatory, neurodegenerative and infectious diseases (Stiehm *et al.*, 2008). Conventional antibodies are complex molecules consisting of pairs of heavy and light chains, whose N-terminal domain is more variable than the rest of the protein sequence. The antibody heavy chain usually consists of three constant domains (CH1, CH2 and CH3) and a variable domain (VH). The light chain has only two domains, the constant light (CL) and the variable light (VL). Important Glycosylations on the CH2 domain are necessary for antibody effector functions, such as Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement–Dependent Cytolysis (CDC), and for regulating antibody half time in serum (Fig. 1, A). Antigen-binding is determined by the three hypervariable Complementary Determining Regions (CDR1, CDR2 and CDR3) present in both the VH and VL domains. These regions are located in juxtaposed loops, creating a continuous surface of ~ 1000 Å^2 that specifically binds to the epitope in an antigen. Although all CDRs can potentially make contact with the antigen, CDR3 contacts with the epitope are generally more extensive. The structural diversity of the antigen-binding sites of a conventional antibody depends on the size of the CDR3 in the VH and the conjunction with the VL at different angles and distances. These are grouped in three different classes, according to the size and type of antigen: cavities (fitting haptens), grooves (fitting peptides) and planar sites (fitting surface patches of proteins) (Johnson *et al.*, 2010).

1.2 The single variable domain of the heavy chain antibodies

In 1993 a surprising observation was made in members of the Artiodactyl Tylopoda family (camelids). Next to conventional IgG antibodies, camelids also naturally produce Heavy Chain antibodies (HCAbs) that lack the light chain (Hamers-Casterman *et al.*, 1993). Two years later, similar single chain antibodies were discovered in cartilaginous fish (sharks) (Greenberg *et al.*, 1995). Although the CH2 and CH3 of the HCAbs and the conventional Abs

are highly homologous, there is no CH1 domain in the camelid HCAbs. The single variable domain, called VHH, is the only domain of HCAbs that makes contact with the antigen. Surprisingly, although the VHH have only three CDR regions, their affinity for antigens reaches the low nanomolar to even picomolar range, matching the best affinities of classical antibodies. When expressed as single domains (often referred to as nanobodies, Nb), the VHHs retain their strong epitope specificity and affinity, a feature that might be explained by the VHH architecture (Fig. 1, B). Just like the VHs of conventional antibodies, the amino acid (AA) sequence of VHHs is organised in three hypervariable regions (CDR1, CDR2 and CDR3) separated by four Framework regions (FR1-FR4) (Muyldermans *et al.*, 1994). As the
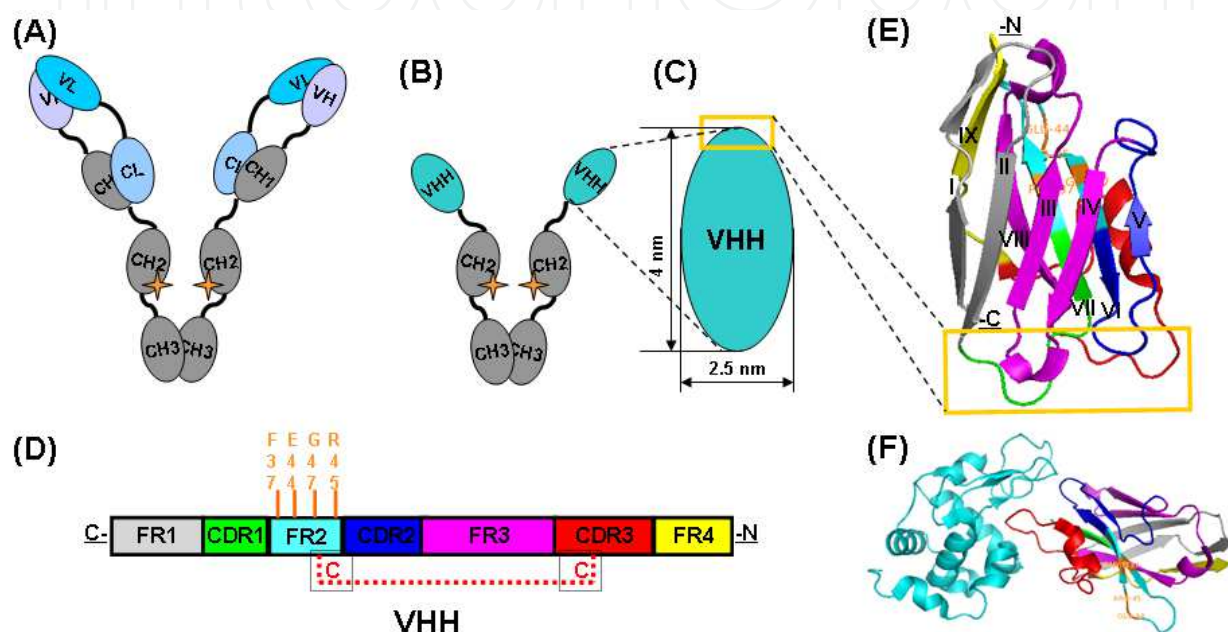

Fig. 1. Representative diagrams of a conventional antibody, an HCAb, and a VHH. (A) A conventional IgG antibody is a dimeric molecule, and each monomer comprises a heavy chain and a light chain. The heavy chain consists of the constant domains (CH1, CH2 and CH3) and the variable domain (VH). The light chain has only one conserved domain (CL) and a variable domain (VL). Important glycosylation sites (orange stars) are present in CH2, which are responsible for effector functions and the flexibility of the molecule. (B) The HCAb devoid of the light chain and CH1 contains the paratope (yellow box) present only in the single variable domain (VHH). (C) The VHH can be expressed as a prolate-shaped, soluble molecule of ~15 kDa. The yellow box shows the antigen binding site. (D) The VHH sequence is made of four Framework Regions (FR1, light gray; FR2, cyan; FR3, magenta and FR4, yellow), and three Complementary Determining Regions (CDR1, green; CDR2, blue and CDR3, red). Residues F37, E44, G47and R45 (orange) are located in the FR2 and mask a hydrophobic patch. C, C- terminal; N, N-terminal. The dotted red line represents a disulfide bond between the FR2 and the CDR3; this bond stabilizes the molecule and is present in dromedaries. (E) A three-dimensional structure of an anti lysozyme VHH, showing the Ig folding of β sheets, five strands in the front (roman numerals: I – V) and four strands in the back (VI – IX). The enlarged yellow box shows the antigen binding site, formed by juxtaposition of three CDRs. (F) The VHH shown in (F) is drawn in complex with lysozyme (light blue). A protruding paratope consisting mainly of CDR3 (red) recognizes and binds the catalytic cleft of lysozyme, inhibiting its activity.

AA sequence of the VHH FRs is highly similar to those of conventional VHs it was not surprising that the overall architecture of VHHs closely resembles that of VHs (Muyldermans *et al.*, 1994). Both VHH and VH domains fold into two β-sheets with the three CDRs that link these two sheets at one end of the barrel (or domain) (De Genst *et al.*, 2006; Desmyter *et al.*, 1996) (Fig. 1, C,E). However, there are striking structural differences between VHHs and conventional VH. Evidently, VHHs lack an interacting VL domain. Because of this, the hydrophobic amino acids present at the VH surface that is normally interacting with the VL, are substituted by hydrophilic AA (Fig.1, D). This enhances the solubility of VHH single domain proteins compared to engineered VH single domain proteins.

The absence of the additional CDRs in VHHs is likely compensated by structural features. First, the CDR3 regions of camelid VHHs are generally longer (13-17 amino acids) than the CDR3 regions of mouse and human VHs (9-12 and 9-17 AA respectively) (Wu *et al.*, 1993). In contrast to conventional Abs, in which the antigen binding surface is often a flat surface, a cavity or a groove, the long CDR3 loop may extend from the antigen binding surface (Desmyter *et al.*, 1996). This enlarges the paratope surface and hence the potential affinity and repertoire of camelid HCAbs. In addition, especially in dromedaries, the CDR1 and CDR3 regions contain a cysteine, which allows formation of a second disulfide bridge next to the single disulfide bridge in conventional VHs (Muyldermans *et al.*, 1994). This extra bridge likely stabilizes the CDR loops, thereby reducing their flexibility. This probably also contributes to the affinity (less entropy is lost upon antigen binding) and structural diversity of VHHs. Long extending CDR3 loops that are stabilized by an extra disulfide bridge can explain the tendency of VHHs to bind to clefts and concaves surfaces more readily than conventional antibodies do (Fig. 1, F) (De Genst *et al.*, 2006). Indeed comparison of multiple structures of hen egg white lysozyme interacting with either several conventional human antibodies or several camelid VHHs clearly illustrated that VHHs tends to bind to the concave substrate-binding pocket, whereas conventional antibodies favor epitopes on the "flat" surface of the antigen (Fig. 1, C). In addition, whereas each of the three CDRs of conventional VHs contributes considerably to the interaction with antigen, VHHs depended mainly on the CDR 3 loop for this interaction. Other antigens that are hard to target by conventional antibodies, but can be targeted by camelid VHHs are ion channels, GPCRs, haptens and enzymatic sites (Lauwereys *et al.*, 1998; Rasmussen *et al.*, 2011).

Next to an extended CDR3, the AA sequence of the H1 loop that precedes and comprises CDR1 appears to be particularly more variable in camelid VHHs than in conventional VHs. This might be interpreted as an extension of the VHH CDR1 (Vu *et al.*, 1997). Associated with this high variability in camelid VHHs, CH1 loops adopt conformations that deviate from the canonical H1 structures of conventional VHs (Barre *et al.*, 1994; Decanniere *et al.*, 1999; Decanniere *et al.*, 2000). Camelid VHH CH1 loops appear to fold into a more diverse repertoire of structures. The high variability in the AA sequence and conformations of the CH1 loop contribute to the VHH paratope size (850-1150 Å^2), which approaches that of conventional antibodies (VH + VL) (Desmyter *et al.*, 2002). Clearly, different biochemical and structural features of camelid VHHs compensate for the lack of a VL domain, thus allowing a broad repertoire of specific high affinity antigen interactions. In addition, due to their small size and typical extruding CDR3 regions, camelid VHH tend to bind in cavities that are not readily accessible for conventional antibodies. Next to these particular features,

VHH single domain protein is exceptionally stable and soluble, even under stringent conditions. As VHH are small and naturally monomeric, they can be easily formatted. In addition, the small size of VHHs allows them penetrate deeper into tissue (e.g. tumor tissue) and to occasionally cross the blood-brain barrier. On the down side, the small size of single domain VHH contributes to their rapid clearance from circulation.

Using display technologies, it is possible to select VHHs from large, synthetic or naive libraries (Verheesen et al., 2006). The phage display generated from an immune VHH repertoire is the most widely and powerful technique used nowadays to rapidly select VHHs with the desired specificity (Arbabi Ghahroudi et al., 1997). VHH are easily produced in bacterial or yeast systems in miligram quantities per liter of culture. Their stability, solubility, ease of production and small size make them excellent candidates for multivalent formatting. Tailor-made constructions using VHHs as building blocks enhance the avidity of the molecule even in a 3 log scale, and several constructions are being tested in clinical trials (Els Conrath et al., 2001; Hmila et al., 2010). Their high potential as therapeutics has prompted the creation in Belgium of the company Ablynx in 2001. Because of the publicity surrounding nanotechnology and the small size of the VHH, Ablynx named the VHH as "Nanobody (Nb)", and retains full intellectual property rights of the use of Nbs in therapeutics and diagnosis. The combined features of VHHs makes them ideal tools for many applications. In this chapter, we focus on the development and use of VHHs for anti-viral therapy. It is interesting to point out that only one monoclonal antibody is used today (Synagis) as a therapeutic against infectious disease (Groothuis & Simoes, 1993).

2. Influenza virus

The main prophylactic measure against influenza is vaccination. Therapeutic options for influenza are small molecule drugs targeting the viral proteins Neuraminidase (NA) or matrix protein 2 (M2). Influenza virus poses a great and continuous threat to humans and zoonotic infections also pose a dangerous challenge to human. In the last decade, two important viruses have emerged as pandemic or potentially pandemic outbreaks: the recent pandemic outbreak in the 2009 by the swine-derived H1N1 influenza virus (also called the Mexican Flu) and Highly Pathogenic Avian influenza (HPAV) viruses of the H5N1 subtype, mainly in Asiatic countries. The 2009 H1N1 pandemic presents an interesting case. It was a zoonotic infection that could be transmitted between humans, but had a low mortality rate. On the other hand, the HPAV H5N1 virus infections present a high replication efficiency, broader cell tropism and possible systemic spread in patients. Fulminant pneumonia, multi-organ failure caused by a high viral load and an intense inflammatory response (cytokine storm) are responsible of a mortality rate of 60 % (de Jong et al., 2006). Vaccines to prevent HPAV infection are not available, but NA inhibitors (osetalmivir) are used as antiviral drugs. A combination of antiviral drugs and immunomodulators was used to control infection by HPAV H5N1 in patients, but its use was considered as a risk. On the other hand, passive immunization has been a successful alternative. Immunoglobulins in immune sera derived from animals or humans exposed to a homologous virus had been used to treat HPAV-infected humans (Luke & Subbarao, 2006; Zhou et al., 2007). The genetic shift and drift of the influenza virus underline the need for new antiviral approaches. In addition, the emergence of drug resistant strains poses an extra concern. The Tamiflu Resistant strain

(resulting mainly from the H274Y mutation, Wang *et al.*, 2002) is evidence of the urgent need for new anti-influenza drugs. It is also urgent to develop new and better antiviral tools against the zoonotic influenza virus, including HPAV. The characteristics of Nbs mentioned above makes them a potentially effective antiviral approach. Several attempts have been made to target conserved epitopes of proteins in the surface proteins of influenza viruses. The main antigenic target in influenza virus is the HA protein. However, the genetic shift of this viral protein, especially in its antigenic regions, complicates this approach. Even though this strategy has been successful in current seasonal vaccines, it is costly and far from optimal: it is not suitable for emerging pandemic viruses, as has been proven not suitable as an immediately available vaccine against the Mexican flu in 2009.

2.1 Targeting influenza HA: the Nb approach

The work of Hultberg and colleagues (Hultberg *et al.*, 2011) is the first report of the use of Nb technology as an antiviral tool against influenza. That study proved the binding of Nbs to an influenza protein and the neutralization of the binding of the virion to its cellular receptor in mammalian cells. These results are the proof of principle of the use of Nbs as antivirals. We discuss the most relevant results in scope of the potential further use of Nbs. To obtain Nbs directed against H5N1 viruses, llamas were immunized with recombinant H5N1 HA (H5, A/Vietnam/1203/04). The nanobody repertoire of the hyperimmune animals was cloned into a phage display library, and two promising HA-binding VHHs were isolated. The VHH of the HCab or Nb was cloned, produced as monovalent molecules, purified and screened for specific binding to the antigen, using as competitor the HA surrogate receptor fetuin. Two of the specific binders (B12 and C8) had high affinity to HA (K_D = 9.91 and 30.1 nM) as determined by surface plasmon resonance. In addition, in a MLV (H5) pseudotyped neutralization assay both Nbs neutralized the parental virus A/Vietnam/1203/04 and also another clade 1 virus (A/Vietnam/1194/04) with a minimal inhibition concentration (IC_{50}) of 75 nM. The possibility of cross reactivity among different H5N1 clades was also tested. The Nbs efficiency in neutralizing other clades of influenza virus decreased proportionally with the antigenic distance from the virus A/Vietnam/1203/04. Three viruses from clade 2.2 were inhibited by the monovalent Nbs in a similar range as clade 1 (IC_{50} = 50–150 nM). On the other hand, one virus of clade 2.3.4 and one virus from clade 2.5 showed little or no inhibition. As mentioned above, Nbs are potentially good building blocks for multivalent molecules due their small size, high affinity, and efficacy as a production platform. Bivalent and trivalent constructs were made, based on Nb C-8, using Gly4/Ser linkers (GS) of different lengths. The neutralization potential of the bivalent and trivalent constructs was greatly enhanced against the A/Vietnam/1194/04 virus ($IC_{50} \leq 1$ pM). Inhibition of this clade 1 virus was confirmed by a micronetralization assay in NIBRG-14 infected cells. NIBRG-14 is an engineered recombinant virus whose HA and NA are derived from the A/Vietnam/1194/04 virus. Surprisingly, in the bivalent and trivalent Nbs the IC_{50} neutralization activity (9 and 3 pM, respectively) decreased by more than 3 logs, compared to the monovalent Nb. These results show that the multimeric molecules outperformed a previously developed monoclonal antibody CR 261, against NIBRG-14 (Throsby *et al.*, 2008). These results were also confirmed in a hemagglutination inhibition assay, which showed an IC_{50} of 2 nM for the bivalent and trivalent construction, compared to 156 nm of the monovalent.

The multivalency format also resulted in the potential for neutralization of influenza virus of different clades. For three clade 2.2 viruses, two bivalent constructions of the Nb C-8 (9 GS and 15 GS) did not show any decrease in the IC_{50}. On the other hand, the 10 GS linker trivalent molecule showed a 10 to 40-fold increase in the neutralization potential, but the 20 GS linker trivalent showed only two-fold decrease in the IC_{50}, or none at all. Nevertheless, using the monovalent Nb the neutralization of virus from clades 2.3.4 or 2.5 was in the high nM range or absent, respectively. This result confirms the previous result showing that both bivalent and one trivalent (10GS) constructions decrease the IC_{50} to a low nM range. It is worth mentioning that the retroviral pseudovirus A/Vietnam/1194/04 and the influenza virus NIBRG-14 share the same HA, but different results were obtained using the MLV pseudotyped neutralization assay and the infected cells microneutralization. Using microneutralization, the reported IC_{50} of the monovalent, bivalent and trivalent molecules was reduced ten-fold as compared to the IC_{50} obtained by the pseudotyped neutralization. The difference in sensitivity of the assays emphasizes the need to confirm the neutralization results of the different influenza clades in infected cells based assays. The validation of the anti HA in an *in vivo* model was performed in a mouse model by our group (Ibañez *et al.*, 2011).

To confirm the *in vivo* efficacy of the Nbs, Ibañez and colleagues used an H5N1 NIBRG-14 mouse adapted virus strain (NIBRG-14 ma). It is important to point out that the Nbs were administered intranasally in all mouse experiments, in order to enhance penetration in the respiratory tract. Initially, to evaluate the antiviral potential using the bivalent Nb (C-8, 15 GS) *in vivo*, a dose of 5 mg/kg (100 µg) was used in mice. This dose completely prevented loss of body weight at 4, 24 and 48 h before a challenge with 1 LD_{50} of NIBRG-14 ma, compared to the controls after 4 days of monitoring. Using the same set up, on day 4 after challenge, no detectable lung virus titers were observed when mice had been treated at 4 and 24 hrs before challenge, and at 48 hrs the titer was 50-fold lower than in controls. These results suggested that the bivalent Nb provide strong protection against 1 LD_{50}, but it is important to consider the half life of the molecule. In previous *in vitro* results, the bivalent Nb neutralization activity was even 3 logs higher than that of the monovalent Nb, but *in vivo* there was also a significant improvement using the bivalent. The difference in virus neutralization capacity between the monovalent and bivalent Nbs and the minimal protective dose was assessed by administration of Nbs at different doses at 24 h before challenge with 1 LD_{50} NIBRG-14. The doses of Nbs ranged from 3 to 0.025 mg/kg, and complete neutralization was confirmed for the highest doses of both constructs. In addition, administration of the highest dose (60 µg, 3 mg/kg) of bivalent Nb 24 h before challenge with 4 LD_{50} also resulted in complete protection. The monovalent neutralization activity was dependent on the amount of Nb, but it was also statistically significant for doses of 6 or 1.2 µg of Nb per mouse. Remarkably, very low or no lung virus titer was detected in mice treated with the bivalent Nb, even for the lowest doses used (2.5–0.5 µg). These results strongly confirmed the neutralization efficacy of the bivalent Nb when used as prophylactic tool against a NIBRG-14 ma, a highly pathogenic influenza virus model.

The therapeutic efficacy of the bivalent Nb was also tested in the same model. The administration of 60 µg of bivalent Nb prevented the drop in body weight and showed a reduction in the lung viral titers when administered 4, 24 and 48 h after 1 LD_{50} challenge. On the other hand, 72 h after challenge, the drop in body weight was similar to that of the controls, but statistically significant reduction in lung viral titers was observed. The decrease in viral titers was also confirmed by measuring the amount of viral RNA by RT-PCR. In

addition, 48 h after challenge of mice (treated with this dose of bivalent Nb) with 4 LD_{50} of NIBRG-14 ma, weight loss was observed and also a delay in mortality compared with the controls.

The antigenic site of the HA was mapped by selecting escape mutants in the presence of the monovalent or bivalent Nb. Three escape mutants were selected in the presence of monovalent Nb, K189E/N and N154D/S mutations were found, they are contiguous in the antigenic B site of the HA (Wiley *et al.*, 1981; Yamada *et al.*, 2006). It is noteworthy to mention that N154D/S removes an N-glycosylation site, a possible adaptation to mask an antigenic site (Fig. 2). The escape mutants selected in presence of the bivalent Nb presented not only the K189E/N mutation, but an additional D145N mutation located in the stalk of HA2, 40 residues upstream of the membrane anchor. The results of the hemaglutination assays and microneutralization experiments suggest that mutation K189N/E is necessary and sufficient to abolish binding to the Nb in a monovalent or bivalent conformation, indicating a close proximity between the antigenic B site and the receptor binding domain. Those results are the first one reported of the potential antiviral activity of a Nb against the influenza virus.
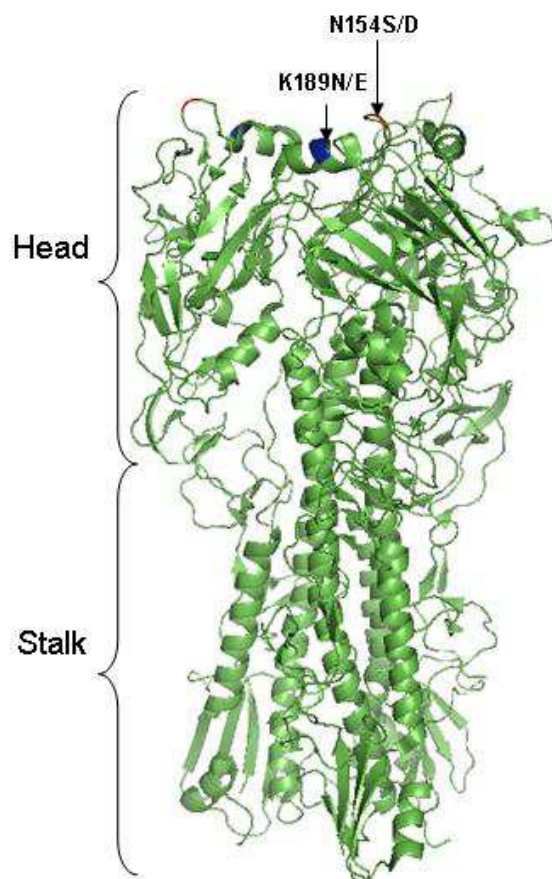

Fig. 2. Ribbon representation of the H5N1 HA trimeric protein. Two mutations in the head of the trimer confer resistance to the monovalent and bivalent VHH C-8. The mutation K189N/E was necessary and sufficient to prevent binding of both mono and bivalent VHHs. (PDB : 2IBX)

The Nb viral neutralization activity against a trimeric HA molecule (HA) was greatly enhanced when presented as bivalent and trimeric molecule, but the dynamics and details

of the binding are not clear. It has been demonstrated that during intramolecular binding, a multivalent molecule has greater avidity than its monovalent counterpart. But a very interesting question is whether intermolecular binding occurs during Nb binding to the HA. In recent reports, the existence of intermolecular binding was proved to enhance an antiviral effect (Wang & Yang, 2010). Intermolecular binding could explain the increase in the neutralization activity: sterically, the hindrance of the HA for its cellular receptor is enhanced, and the flexibility of the HA is decreased.

3. RSV virus

Respiratory Syncytial Virus (RSV) infections are the leading cause of acute lower respiratory tract infections (ALRI) in children and associated hospitalizations world wide (Falsey *et al.*, 2005; Nair *et al.*, 2010). There is no specific antiviral therapy for RSV infection available. Each year 66, 000 – 199,000 children die worldwide due to RSV ALRI. Most pediatric cases of fatal RSV infections occur in developing countries. As RSV infections do not evoke protective immunity, infections occur throughout life, causing severe morbidity in young infants, the elderly, and immune-comprised adults (Boyce *et al.*, 2000; Falsey *et al.*, 2005).

Although high levels of RSV neutralizing antibodies correlate with lower frequencies of RSV-associated ALRI, no RSV vaccine is available (Glezen *et al.*, 1981). However, monthly administration of large amounts of a humanized RSV neutralizing antibody, palivizumab (Synagis), reduces RSV-associated hospitalization of high risk infants by about 78-39% (Groothuis & Simoes, 1993). Palivizumab is currently the sole monoclonal antibody that is approved for preventing viral infection. Palivizumab blocks fusion of the RSV membrane with the membrane of the target cell by binding to the RSV fusion protein (F) (Huang *et al.*, 2010). However, due to the high cost of palivizumab, there is an urgent need for new anti-virals that can prevent or treat RSV infections. RSV neutralizing Nbs have been developed as an alternative to existing antibodies (Hultberg *et al.*, 2011).

3.1 RSV binding VHHs antiviral effect: comparison with Synagis Mab.

To investigate if Nbs could be used for antiviral therapy, Nbs that bind to the palivizumab epitope were developed. For this purpose, two llamas were immunized with recombinant RSV A F protein (RSV F_{TM}-) lacking the transmembrane region (Hultberg *et al.*, 2011). This protein folds into trimers that resemble the native RSV F protein in its post-fusion conformation (Ruiz-Arguello *et al.*, 2004). Remarkably, RSV F_{TM}- proteins can be readily recognized by RSV F neutralizing antibodies that, just like palivizumab, bind to the antigenic site II (McLellan *et al.*, 2011; Swanson *et al.*, 2011). In this way, RSV F_{TM}- immunization can potentially induce RSV F antigenic site II specific camelid HCAbs. HCAbs that specifically bind to the RSV F antigenic region II were enriched by biopanning using RSV F_{TM}- protein and competitive elution in the presence of excess of palivizumab antibody. From these HCAbs, VHHs (or Nbs) were produced and tested for binding to the RSV F_{TM}- protein. Twelve VHHs that bound to the RSV F protein were tested for neutralization of RSV Long strain (RSV A subtype) virus in a micro-neutralization assay. Two VHHs (RSV-C4 and the RSV-D3) could neutralize RSV in the high nanomolar range (IC$_{50}$: 640 nM and 300 nM, respectively), which is similar to the neutralization activity as the Synagis Fab (IC$_{50}$: 549.2 nM) and about 100-fold less effective than the Synagis Mab (IC$_{50}$: 3.02 nM). However, in contrast to palivizumab, neither RSV-C4 nor RSV-D3 VHHs could neutralize RSV B

subtype virus *in vitro*. On the contrary, another VHH (RSV-E4) could neutralize RSV B infection to some extent.

The epitopes of different VHHs were determined by antibody competition assays and diverse antibody escape RSV mutants. Whereas RSV-C4 and RSV-D3 VHHs readily competed with palivizumab for binding to recombinant RSV F_{TM}- or inactivated RSV virions, RSV-E4 competed with 101 Fab, which is known to bind to the antigenic region IV-VI (Wu *et al.*, 2007). These data are in line with the observation that AA substitutions within antigenic regions II and IV-VI, respectively, affected the binding of both RSV-D4 and RSV-C3 VHHs and RSV-E4 VHH. These data strongly suggest that both RSV-C3 and RSV-D4 bind to antigenic region II (palivizumab epitope) (Crowe *et al.*, 1998) whereas RSV-E4 VHH binds to antigenic regions IV-VI, explaining the observed differences in neutralization.

The affinity of the three VHHs, Synagis Mab and Synagis Fab was determined by Surface Plasmon Resonance using recombinant RSV F $_{TM}$- as bait. The K_D of RSV-D3, RSV-E4 and RSV-E4 were in the low nanomolar range: 9.24 nM, 1.78 nM and 0.45 nM, respectively. Although RSV-D3 was more effective than RSV-C4 at neutralizing RSV A, it had a lower affinity for F_{TM}- than RSV-C4. However, the efficient binding of RSV-E4 VHH to a neutralizing epitope (antigenic region IV-VI) was not associated with neutralization of RSV A. This suggests that the affinity of VHHs for the recombinant RSV F_{TM}-, which likely represents the F protein in its post-fusion conformation, does not correlate directly with neutralization of living RSV (Table 1.)

	F-RSV-D3m	F-RSV-D3b	Synagys
In vitro neutralization (IC50, nM)	300	0.05 – 0.14 nM*	1.03 – 5.5 nM*
Prophylactic minimal protective dose (mg/kg)	ND	12 μg	ND
Prophylactic protective extension (mg/kg)	ND	48 hrs	ND
Therapeutisch protection extension	ND	24 hrs	ND

*Obtained from two different cell based assays, microneutralization and plaque assay

Table 1. Inhibition and protection of the RSV virus A binding by Nb RSV-D3. ND = not determined.

The avidity of a binding molecule can be increased by using a multivalent format (Rudge *et al.*, 2007; Wang & Yang, 2010). To increase the antiviral potential of RSV-D3 we formatted it into a bivalent molecule, by using a flexible linker, Gly$_4$/Ser (GS). Surprisingly, bivalent RSV-D3 VHHs with GC linkers of different sizes neutralized RSV A Long virus between 2421 and 4181 times more efficient than monovalent RSV-D3 VHHs, reaching picomolar

range (IC$_{50}$: 190-110 pM). In contrast, Synagis Mab was only 200 times more efficient in neutralizing RSV A virus (IC$_{50}$: 6.5 nM) than its corresponding Fab fragment. In this way, bivalent RSV F specific VHHs outperform the Synagis antibody in RSV neutralization. Moreover, in contrast to its monovalent format, bivalent RSV-D3 could also neutralize RSV B1 strain virus. Also, neutralization was notably boosted against RSV A and B virus subtypes by linking two different VHHs (RSV-D3 and RSV-E4) which target different epitopes. The enhancement of the activity by linking two VHHs is likely due to the flexibility of the linker. Experiments aiming to characterize the binding dynamics of the RSV-D3 to the F protein are necessary for characterizing intra- or intermolecular binding.

The RSV F is responsible for fusion of the viral lipid membrane with the host membrane, but also participates in attachment of the RSV virions to target cells. In addition, it was recently demonstrated that RSV F protein can bind to nucleolin expressed at the surface of target cells, and that this interaction is crucial for RSV infection *in vitro* and *in vivo* (Tayyari *et al.*, 2011). After viral attachment, the RSV F protein mediates fusion of the viral membrane with the plasma membrane of the target cell, thereby releasing the viral genome into the cytoplasm of the host cell. This process involves a series of conformational changes in the F protein from a metastable pre-fusion to a stable post-fusion conformation. We recently demonstrated that bivalent RSV-D3 VHHs can prevent RSV infection both before and after viral attachment and can inhibit syncytia formation, but cannot hamper RSV attachment (Schepens *et al.*, 2011). Together, these observations constantly indicate that, by a similar mechanism as palivizumab, bivalent RSV-D3 VHHs prevent RSV infection by blocking fusion. Although the conformations of the RSV F antigenic regions II and IV-VI are maintained in the post-fusion form, it is more plausible that the RSV VHHs block viral fusion and syncytia formation by binding to the RSV F protein in either its pre-fusion or intermediate conformations (Fig. 3). Possibly, binding of the VHHs to the antigenic region II interferes with the conformational changes of the F protein that are required for fusion.

Immune compromised Balb/c mice (cyclophosphamide treatment) were used to test whether bivalent RSV-D3 VHHs can protect against RSV infection *in vivo* (Schepens *et al.*, 2011). As VHHs are known to remain active in the respiratory tract after nebulisation, bivalent RSV-D3 and control VHHs were administered intranasally (patent application WO 2009/147248). Prophylactic treatment of mice with 5 mg/kg of bivalent RSV-D3 VHH or palivizumab reduced RSV pulmonary titers below the detection limit of the RSV plaque assay. This strong reduction was confirmed by qPCR analysis. Remarkably, as low as 0.6 mg/kg bivalent RSV-D3 could prevent or strongly reduce (at least 100-fold) pulmonary RSV replication. In comparison, monovalent RSV-D3 VHH protected against pulmonary RSV replication about 25 times less efficiently than its bivalent counterpart. For prophylactic treatment to be valuable, even if is easy to administer, its effect should be long lasting. We demonstrate that intranasal administration of bivalent RSV-D3 VHHs can protect against RSV infection for at least 48 hours. Prophylactic treatment with palivizumab in high risk infants reduces RSV associated hospitalization, but no effective therapeutic is available. Therefore, RSV-D3 VHHs were also evaluated as therapeutic treatments. Intranasal administration of RSV-D3 VHHs 4 or 24 hours after infection strongly reduced pulmonary RSV replication (at least 100-fold). Plaque assays also indicated that administration of bivalent RSV-D3 VHHs 72 hours after RSV treatment can reduce pulmonary RSV replication. However, the lung homogenates used to quantify the pulmonary RSV titer in mice that were treated 72 hours after infection still contained neutralizing RSV VHHs. Therefore, it is not clear to which extent treatment at this time point

reduced RSV replication *in vivo*. The potential of bivalent VHHs for preventing morbidity and pulmonary inflammation upon RSV infection was assessed in a non immunocompromised mouse model. Prophylactic administration of bivalent RSV-D3 VHHs (1 mg/kg) completely prevented body weight loss and pulmonary cell infiltration that was observed in mice treated with control VHHs. Therapeutic treatment with bivalent RSV-D3 VHHs 24 h after infection partially reduced body weight loss and pulmonary cell infiltration. These observations confirm the *in vivo* antiviral potential of neutralizing VHHs.
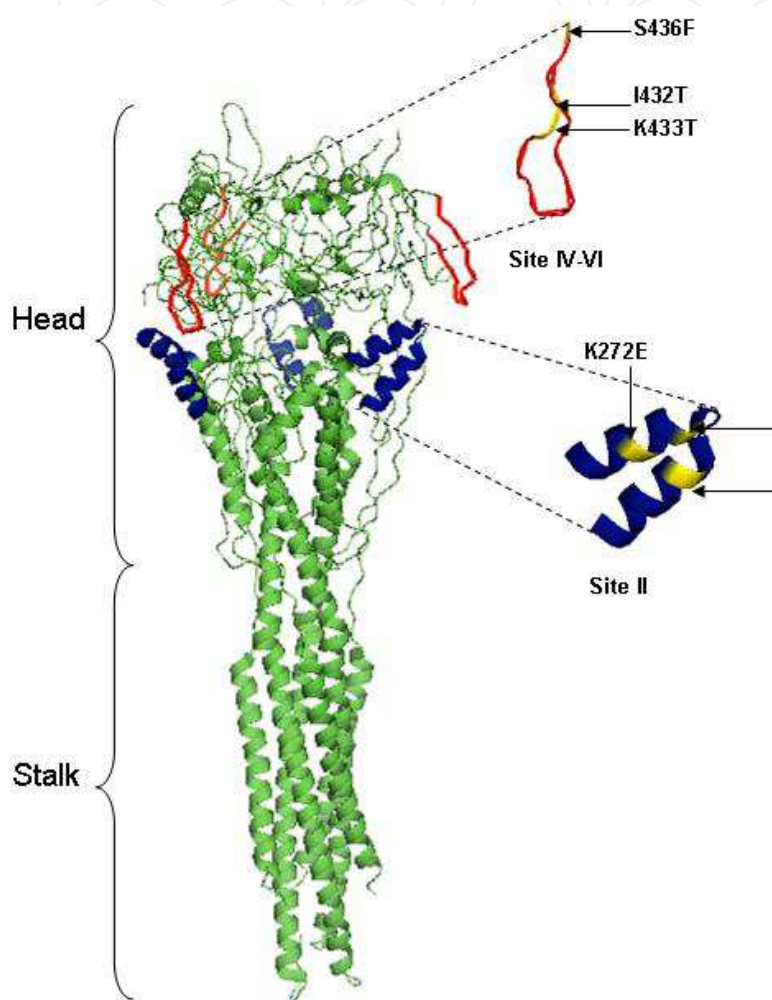
Fig. 3. Ribbon representation of the structure of the RSV F protein trimer in its post-fusion form. The head and stalk of this recombinant protein are depicted, lacking the fusion peptide, transmembrane region and cytoplasmic domain. The immunogenic epitopes recognized by Mab 101F (site II) and palimuzab (site IV-VI) are in red and blue, respectively, and the rest of the F protein is in green. The RSV-D3 and RSV-C4 resistant *in vitro* escape mutants are shown in yellow. Mutations I432T, K433T and S436F in site II disrupt the binding of the RSV-C4. The K262Y, N268I and K272E in the site IV-IV result in loss of binding of the RSV-D3 molecule. (PDB: 3RKI).

Currently Ablynx is preparing a phase I clinical trial to evaluate the safety of a trivalent RSV neutralizing VHH format consisting of three identical VHHs. Preclinical evaluation of this lead candidate can readily neutralize a broad spectrum of clinical RSV A and RSV B subtype

viruses more efficiently than Synagis (abstract, 7th international RSV symposium, Rotterdam, 2010). *In vivo* studies demonstrated that both prophylactic and therapeutic treatment with this RSV neutralizing VHH can readily reduce RSV replication in the upper and lower respiratory tract of cotton rats (abstract, 7th international RSV symposium, Rotterdam, 2010).

In summary, neutralizing RSV VHHs are promising new candidates as anti-RSV therapeutics for different reasons. First, VHHs allow versatile formatting including the creation of multivalent formats by the use of flexible linkers. This feature enabled the creation of bivalent and trivalent VHH which can neutralize RSV at picomolar range, more than 1000-fold more efficient than their monovalent counterpart. Second, by linking two different VHHs which neutralize different virus strains (such as the RSV A versus the RSV B subtype strains) cross-reactive VHH constructs can be obtained. Moreover, as a result of avidity effects, cross linking VHH with different specificity can considerably improve the neutralizing activity. Third, the VHHs small size and protruding paratopes can contribute to its neutralization activity. As structural models and electron microscopic analysis indicate that the antigenic region II is located at the side of the RSV F protein trimer, at the dense surface of RSV virions, this region is likely more accessible for small and flexible VHH formats than for large and more rigid antibodies (McLellan *et al.*, 2011; Ruiz-Arguello *et al.*, 2004) (Fig. 3). Fourth, due to their high stability at stringent conditions, VHHs can be administered via nebulisation, which allows a rapid accumulation of high amounts of neutralizing VHH at the site of respiratory viral infections. In addition, due to the high stability of VHHs and the ease of intranasal or pulmonary administration, VHH therapy could potentially be applied more generally, even in developing countries.

4. Rabies virus

Rabies virus (RV) is a single stranded RNA virus of the *Rhabdoviridae* family, genus *Lyssavirus.* Infection with RV in humans causes acute encephalitis, with a mortality rate of almost 100%. It is transmitted to humans by bites from a carnivore or a quiroptera vector and most cases occur in Asia or Africa. The long incubation period following infection by RV presents a paradox, because of the absence or very weak antiviral immune response (Johnson *et al.*, 2010). The small amount of virus inoculated after infection and the neurotropism of RV are believed to contribute to the absence of effective antibodies in the patient. After the bite, wound cleaning can reduce the chances of a productive infection in humans. Passive immunization and vaccination promptly after exposure is the only effective therapeutic tool available now. Modern vaccines are inactivated virus produced from continuous cell cultures, like the vaccine by Aventis Pasteur (human diploid cells). Nevertheless, in underdeveloped countries, the established RV therapy (attenuated virus, Mab anti RV) is too expensive for most of the population. RV has a genome of 12 kDa coding for 5 proteins: nucleoprotein, phosphoprotein, matrix, RNA-dependent RNA polymerase and the Glycoprotein (RVG). In the virus particle, the RVG is the only viral protein exposed as a trimeric spike, and it is responsible for recognition of cellular receptors, virulence and antigenicity.

4.1 Nbs present a broad protection against Rabies virus

For more than 25 years, two well-defined antigenic sites in the RVG have been characterized by Mabs: antigenic sites II and III (Lafon *et al.*, 1990). Other epitopes have also been

characterized, but their contribution to antigenicity is minor. Antigenic site III extends from 330 to 340 amino acids and is linear (Seif *et al.*, 1985). Mutations in this site affect virulence and the host range of the virus. On the contrary, antigenic site II is conformational and discontinuous and is determined by two regions, amino acids 34-42 and 198-200. Site II is responsible for about 70 % of the known Mabs against RVG (Benmansour *et al.*, 1991).

RGV is an interesting target for the VHH platform because alternative cost effective antirabies tools are needed. By using an approach similar to those previously discussed for influenza and RSV, a llama was immunized with recombinant RVG and five VHHs were obtained (Rab – E8, H7, F8, E6 and C12). The neutralizing activity of those VHHs was validated against 10 Rabies genotype 1 viruses: 3 laboratory strains (CVS-11 as prototype, ERA, CB-1) and 7 field isolates and one rabies genotype 5 virus (EBL-V1) was included to validate broad cross neutralization. A cell based assay was used, the Rapid Fluorescent Focus Inhibition Test (RFFIT) (Vene *et al.*, 1998). This assay has been internationally recognized as the *in vitro* standard for testing virus neutralizing antibodies. Mab 8-32, which recognizes antigenic site II of RVG was also included as positive control (Montano-Hirose *et al.*, 1993). VHHs F8, E6, H7 and C12 neutralized the genotype 1 strains: CB-1 and ERA with an IC_{50} in the low nanomolar range and the CVS-11 strain in the low to high nanomolar range. They could also neutralize several RV field isolates. On the other hand, E8 efficiently neutralized only CB-1 and CVS-11, in the low and high nanomolar range, respectively. C12 and E6 had better neutralization activity than Mab 8-2 against the ERA and CB-1 strains. Using a similar approach as described above for influeza and RSV, the authors also generated the bivalent against the Rabies genotype 1 CVS -11 and the genotype 5 EBLV-1. Bivalent monoparatopic VHHs were constructed using a Gly4/Ser linker, using the 12, H7, E8 and F8 VHHs. The neutralization IC_{50} of these constructions was reduced from two to 180-fold relative to the monovalent protein, indicating enhancement of the neutralization. Nevertheless, the best results were obtained when biparatopic molecules were used. The E6/H7 and the H7/F8 molecules increased the neutralization potency by a 2 log factor, while the E8/H7 increased 3 log-fold, compared with the monovalents. E8/H7 even outperformed Mab 8 -2 against the CVS-11. On the contrary, in the case of the genotype 5 strain EBLV-1, the monovalent molecules showed modest neutralization or none at all. The enhanced neutralization of biparatopic molecules was confirmed by E8/C12, which presented an increase in the neutralization potential of 147-fold (IC_{50} = 3.76 nM) relative to the monovalent moiety, but not as low as Mab 8 – 2 (IC_{50} = 0.12 nM). The results of competition assays of the 5 VHHs against the Mab 8- 2 showed that E6, E8, F8 and H7 compete for the same epitope. On the other hand, C12 did not compete which indicates that it recognizes a different epitope. The difference in epitope recognition could be one of the causes of the strong and broad effect of biparatopic molecules, especially for E8/C12. Experiments using VHHs against Rabies mutant virus, carrying substitutions in the known residues in the antigenic site II could localize the exact binding sites of these new antibodies. For example, it has been reported that substitution K198E of the glycoprotein abolish the binding of the Mab 8–2 (Montano-Hirose *et al.*, 1993). Unfortunately, the crystallographic structure of the RVG protein has not been reported. The use of vesicular stomatitis virus glycoprotein is accepted as a modeling reference and as a surrogate template for RVG structure (Cibulski *et al.*, 2009; Tomar *et al.*, 2010). We used the alignment of this protein with the RVG as reference to show the possible structure of antigenic site II (Fig. 4). The purpose of this estimate is to

show the tendency of the VHHs to recognize conformational rather than linear epitopes. In line with the results of the neutralizing VHHs against influenza and RSV, the results of the broadness and the strong potency against both RV genotypes indicate the RV neutralizing VHHs as a promising. Nevertheless, in contrast with the previous cases of the influenza and RSV VHHs, there is not *in vivo* validation of the RV neutralizing VHHs available.
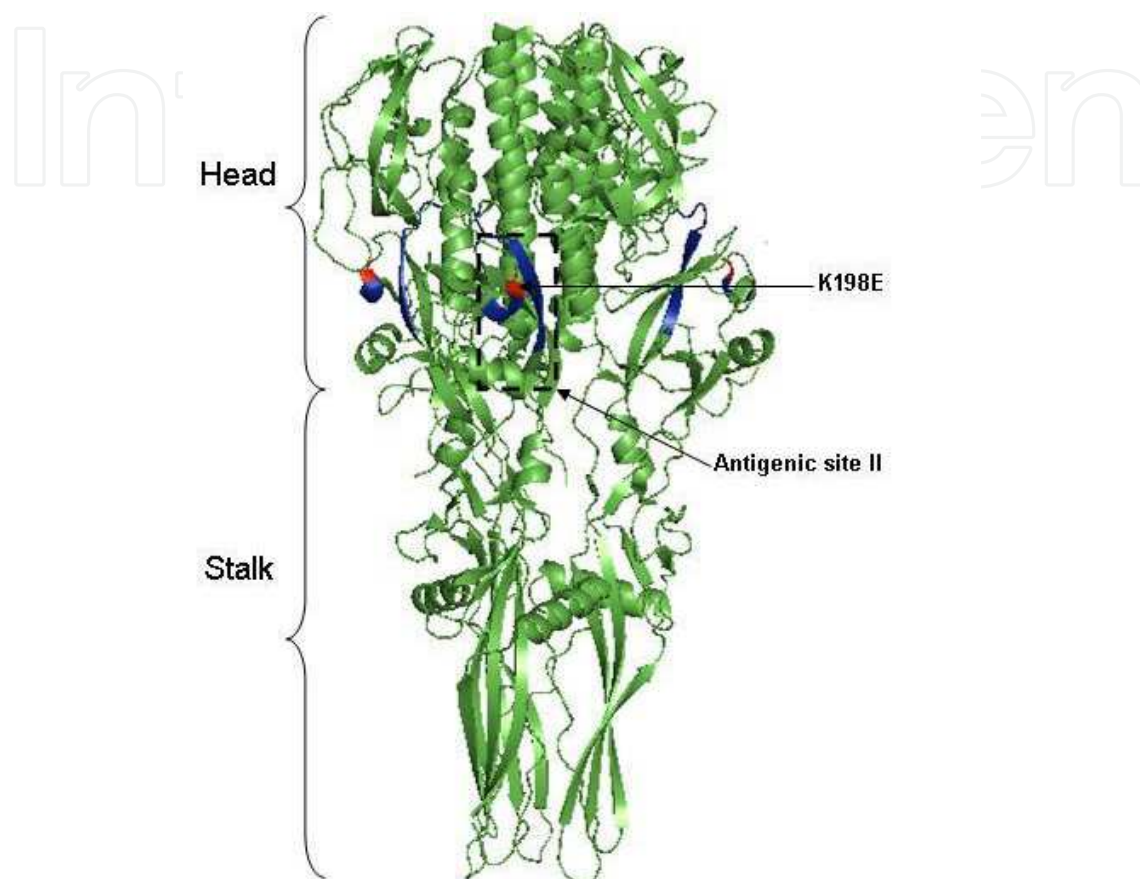

Fig. 4. Schematic representation of the vesicular stomatitis virus protein G trimer. This protein is taken as reference to depict the amino acids corresponding to the antigenic site II of the Rabies Virus glycoprotein (residues 34-42, and 198-200, in blue). The localization of the K198E mutation that prevents the binding of the Mab 8-2 is shown in red. The VHHs E8, F8, E6 and H7 compete with the Mab 8- 2 for the binding, which means that their epitopes might be within antigenic site II. (PDB: 2CMZ).

5. Conclusion

The Nb platform is a new and promising antiviral tool. The ease of producing Nbs in bacterial and lower eukaryotic cells, and the possibility of producing tailor-made constructions makes them an attractive and cost effective alternative to some established antiviral drugs. This approach may be useful for the treatment of infectious orphan diseases (including viral) and in developing countries, where the "standard" prophylaxis or therapy is prohibitively expensive or not available. In this work we have discussed findings on recently developed Nbs directed against three viruses affecting humans: the generation and *in vitro* validation of the Nbs or VHHs against influenza H5N1, RSV and RV (Hultberg *et al.*,

2011); as well as the *in vivo* validation of the influenza HA binding VHH (Ibañez *et al.*, 2011) and RSV (Schepens *et al.*, 2011). In the case of protection against influenza infection the bivalent format of the Nbs proved superior *in vitro* and *in vivo*. But, as indicated by the successful *in vivo* validation of one of the H5N1 strains, it is imperative to extend this validation to other influenza strains. Furthermore, it would be worthwhile to isolate and characterize Nbs that recognize conserved domains in HA, such as the stalk. In line with the influenza results, the activity of RSV and RVG neutralizing Nbs was significantly higher for the bivalent than the monovalent format: both the cross neutralization activity and potency were higher. Those results manifest the advantages of using a multimeric format against multimeric viral targets. The enhanced antiviral potential of the multimeric format could be due to increased avidity and/or the intra- or intermolecular binding could contribute in the enhancement. Experiments to assess the binding mechanism could lead to further improvements. The results overall confirm two important points: the high potential of the Nbs as prophylactic and therapeutic tools, and the possibility of using Nbs directed against other infectious diseases. There is limited function and sequence similarity among the three proteins used as antigens (HA, RSVF and RVG) other than their trimeric architecture and antigenicity. Nevertheless, the HA and the RVG are functionally similar and both are involved in the cellular receptor binding, whereas RSVF participates also in virion receptor binding, it s main function is in membrane fusion. In all three cases, showed capacity to neutralize the viral target by blocking binding or hampering necessary conformational changes, indicating the great versatility and efficiency of the antiviral discussed here. In competition assays, the recognition of non conventional epitopes by these antiviral was not observed, but could be the focus of research. Nbs recognized well known antigenic sites that are also targeted by Mabs. The HA, RSV F and RVG are not enzymes, and lack extensive antigenic clefts. This could be one reason why Nbs showed preference for recognition of "classical" epitopes in these viral proteins. If the presence of antigenic clefts could lead to recognition of non "classical" epitopes in the viral proteins, targeting viral enzymes could be an interesting approach. Enzymes such as the influenza Neuraminidase are potential targets. This viral sialidase presents a catalytic cleft, in which the framework and substrate contact residues are conserved in most of the influenza strains. The coming years will probably bring potent novel anti-viral Nbs directed against different viruses and it is likely that some of these Nbs will reach clinical trials.

6. Acknowledgement

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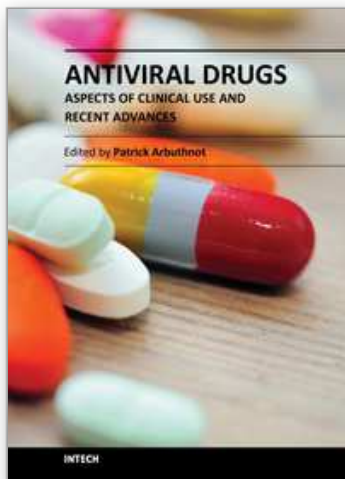
Antiviral Drugs - Aspects of Clinical Use and Recent Advances

Edited by Dr. Patrick Arbuthnot

The articles that appear in Antiviral Drugs - Aspects of Clinical Use and Recent Advances cover several topics that reflect the varied mechanisms of viral disease pathogenesis and treatment. Clinical management and new developments in the treatment of virus-related diseases are the two main sections of the book. The first part reviews the treatment of hepatitis C virus infection, the management of virus-related acute retinal necrosis, the use of leflunomide therapy in renal transplant patients, and mathematical modeling of HIV-1 treatment responses. Basic research topics are dealt with in the second half of the book. New developments in the treatment of the influenza virus, the use of animal models for HIV-1 drug development, the use of single chain camelid antibodies against negative strand RNA viruses, countering norovirus infection, and the use of plant extracts to treat herpes simplex virus infection are described. The content of the book is not intended to be comprehensive, but aims to provide the reader with insights into selected aspects of established and new viral therapies.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

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