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In vitro antiviral activity of single domain antibody fragments against poliovirus

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ABSTRACT

VHHs or Nanobodies® are single-domain antigen-binding fragments derived from heavy chain antibodies found in camelids. It has already been shown that complex protein mixtures and even whole organisms elicit good immune responses in camelids; therefore we hypothesized that VHHs selected from a dromedary immunized with poliovirus type 1 might inhibit the in vitro replication of poliovirus through binding to essential biological sites on the viral capsid. In this study, we aimed to determine whether VHHs inhibit wild-type and vaccine strains of poliovirus type 1. Interestingly, VHHs showed a potent antipolio activity with EC_{50} values in the low nanomolar range. Moreover, these antibody fragments completely blocked viral multiplication at higher concentrations. Remarkably, no (immune) escape variants against some of these VHHs could be generated. In conclusion, VHHs fulfil several in vitro requirements to be assigned as potential antiviral compounds for further development of an anti-poliovirus drugs.

1. Introduction

Despite the development of effective vaccines against poliomyelitis and large-scale vaccination campaigns, the current plans to eradicate the infectious agent, the poliovirus, have not been successful. New outbreaks of poliomyelitis that are mainly due to wild polio cases and the circulation of vaccine-derived poliovirus strains still occur in the Indian subcontinent and sub-Sahara Africa (World Health Organization, 2010). Moreover, outbreaks remain possible in the future, even after the completion of polio eradication, by the accidental or intentional reintroduction of poliovirus into the wild. The awareness to have additional means such as antiviral drugs to support the current available tools and to provide rapid protection to individuals in the postpolio era, is growing among healthcare workers and policy makers. At present, no approved antiviral drugs are on the market for the treatment and prophylaxis of poliovirus infections. Therefore, the development of efficacious antiviral compounds for halting the infection and the spread of poliovirus is needed immediately and in the future (Committee on Development of a Polio Antiviral and its Potential Role in Global Poliomyelitis Eradication, 2006).

One of the best studied and promising targets for the development of an anti-poliovirus therapy is the capsid of the virus. The capsid is a proteinaceous shell surrounding the viral genome and is assembled from 60 identical monomers to form an icosahedral structure. Each monomer is further composed of four structural proteins, viral protein 1 (VP1), VP2, VP3 and VP4. The main function of the viral capsid is to protect the polio genome against degradation but also to deliver the viral RNA into the host cell. The capsid, however, is also the first viral structure encountered by the host organism and thus the primordial target for the host defense mechanisms. Protection against the infection is generally considered to be mainly based on neutralizing serum antibodies against the outer capsid proteins VP1–VP3 (Rossmann, 1989).

Neutralizing antibodies are induced by the host organism as a reaction to a natural infection or by vaccination but they can also be passively administered to individuals for the pre- and post-exposure prophylaxis of a variety of viral infections. Since the 1890s, passive immunotherapy has been widely practiced, first with pathogen-specific (or hyperimmune) serum from recovered patients or immunized volunteers and later on with purified polyclonal immunoglobulins from pooled normal donor serum (ter Meulen and Goudsmit, 2006). Nowadays, although many of these products are still on the market, recent advances in human monoclonal antibody (mAb) engineering have led to the isolation and

Abbreviations: VHH, variable part of the heavy chain of a heavy chain antibody; mAb, monoclonal antibody; CDR, complementarity determining region.

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characterization of potent human antiviral antibodies. The major strength for using monoclonal antibody therapy is that mAbs can bind to their viral targets with high affinity and exquisite specificity. Unfortunately, their therapeutic value is hampered by the high development and manufacturing costs together with the restricted mode of administration and immunogenicity (Magliani et al., 2009). In general, mAbs can only be administered by means of injection and although they have a low toxicity and are well tolerated, they are still recognized as foreign proteins in the human body and can elicit some levels of immune response even after "humanization".

Another antiviral approach targeting the capsid is the use of the so-called capsid-binding compounds. These compounds are small molecules which bind into the hydrophobic pockets within the viral capsid and exert their antiviral activities on the early picornaviral replication steps (i.e. attachment, entry and/or uncoating). A relatively large number of these capsid-binders have been synthesized from various chemical structures such as rhodamine, flavonoids, chalcones, oxazolinyl isoxazoles, aralkylamino pyridines, pyridazinamines (1992), phenoxyl imidazoles and pyridazinylpiperidines and their alkoxy benzoazole analogs (for a review see (De Palma et al., 2008)). Many of these compounds possess anti-poliovirus activity in both cell culture and animal models. Several of these compounds were considered as very promising antiviral compounds: R75761, for instance, showed a broad-spectrum activity against poliovirus (Thys et al., 2008) and disoxaril even entered clinical trials. However, the use of these compounds has encountered various problems ranging from a poor pharmacokinetic profile to severe side effects in patients. At present, one compound, called V-073, is in preclinical development for polio indications (Collett et al., 2008)

In this study, we describe the generation of a novel class of poliovirus capsid binding molecules based on the variable domains of the heavy chain of the camelid heavy-chain antibodies (VHHs or Nanobodies). VHHs are a novel class of single-domain antigen-binding fragments derived from heavy chain antibodies, which naturally occur in members of the Camelidae family (Hamers-Casterman et al., 1993). VHHs are the smallest naturally occurring intact antigen-binding domains known today (Arbabi et al., 1997) and possess several interesting features: (i) VHHs can recognize structures which are inaccessible for conventional antibodies, such as pockets and clefts (Conrath et al., 2001b), abundantly present at the outer surface of polioviruses (e.g., the canyon). Because these clefts are less exposed to the immune pressure of antibodies, they are less subjected to genetic variability rendering these structures ideal targets in terms of drug resistance; (ii) VHHs bind their targets with the same high affinity and selectivity as those of the typical protein–protein interactions involving conventional antibodies (van der Linden et al., 1999), and this in contrast to the low affinity and poor selectivity of small molecular drugs which often result in unwanted side-effects and lack of potency. (iii) VHHs are resistant to acid and alkaline pH, temperature and to attacks by proteases to a greater degree than conventional antibodies (Dumoulin et al., 2002; Harmsen et al., 2006). VHHs are able to pass the stomach and remain biologically active in the gut. This creates opportunities for oral delivery of VHHs (Van Bockstaele et al., 2009); (iv) although VHHs are derived from camelid antibodies, they are not immunogenic in primates. Moreover, there exists a general strategy to humanize camelid VHHs (Vincke et al., 2009); (v) VHHs can be easily tailored for increased serum half-life (varying from 30 min to 3 weeks). This versatility increases the range of therapeutic options available to VHHs ranging from acute to chronic (Conrath et al., 2001a; Harmsen et al., 2008); (vi) VHHs are encoded by single genes and are efficiently produced in prokaryotic and eukaryotic hosts including bacteria and yeast. The production process is scalable and multi-gram quantities of VHHs have been produced (Frenken et al., 2000; van der Linden et al., 2000); (vii) because VHHs typically exhibit a superior stability, as compared with conventional antibodies, they can be formulated as long shelf-life, ready-to-use solutions; and (viii) for therapy, these small formats can be beneficial in various other aspects not mentioned above such as bio-distribution, renal clearance, tissue penetration and target retention (Van Bockstaele et al., 2009).

The traditional (high throughput) antiviral drug screenings require not only the screening of giant libraries to identify possible lead compounds with antiviral effects free from (cyto)toxicity, but also the subsequent synthesis and analysis of hundreds of derivatives based on the lead compound in order to finally obtain an improved antiviral activity and/or impaired toxicity. In contrast, panels of target specific VHHs with high affinity can be generated in about 3 months from the start of immunization and the VHHs can enter the preclinical phase in less than 18 months from their isolation.

In this study, we present the isolation and characterization of poliovirus type 1-specific VHHs from an immune dromedary and demonstrate their in vitro antiviral activity.

2. Materials and methods

2.1. Cells, viruses, monoclonal antibody and R75761

HeLa monolayer cells were cultured as previously described (Rombaut et al., 1985). Experiments were performed with wild-type poliovirus type 1 Mahoney strain or the attenuated poliovirus Sabin strains type 1, type 2 and type 3. Different viruses were cultivated and purified as previously described (Everaert et al., 1989). The monoclonal antibody 35-1f4, a neutralizing antibody recognizing poliovirus type 1 (Brioen et al., 1982), and R75761, a pyridazinamine analogue synthesized by the Janssen Research Foundation with anti-poliovirus activity (Thys et al., 2008), were used as antiviral compounds with known activity.

2.2. Immunization of dromedary

A dromedary (Camelus dromedarius) kept at the Central Veterinary Research Laboratory (Dubai, United Arab Emirates) was injected six times subcutaneously at weekly intervals, each time with one ml poliovirus type 1 Sabin strain (approximately 1.5×10^8 infectious particles per ml, kindly provided by GSK, Rixensart, Belgium) mixed with an equal volume of Gerbu LQ 3000 adjuvant (GERBU Biochemicals). Four days after the last immunization, 50 ml of anti-coagulated blood was collected and transported to the Brussels laboratory. Plasma and peripheral blood lymphocytes were isolated with Lymphoprep™ (Nycomed) according to the instruction manual. The blood is carefully layered over Lymphoprep and centrifuged at $800 \times g$ for 20 min. The lymphocytes form a distinct band at the sample/medium interface and are collected from the interface by using a Pasteur pipette.

2.3. Fractionation of IgG subclasses

Separation of different plasma IgG subclasses was performed by differential adsorption on Hitrap-protein A and Hitrap-protein G columns (Amersham Biosciences/GE Healthcare) as previously described.

2.4. Solid-phase binding enzyme-linked immunosorbent assays (ELISAs)

MaxiSorb 96-well plates (Nunc) were coated with poliovirus type 1 Sabin strain overnight at 4 °C in 10 mM PBS buffer (pH 7.2) at a concentration of 1 μg/ml. Residual sites were blocked with protein-free blocking buffer (Thermo scientific) for 1 h at

room temperature. For the analysis of specific antigen-IgG subclass binding, serial dilutions of purified IgG subclasses were added to the wells. After incubation, bound dromedary IgG was detected with a rabbit anti-dromedary IgG antiserum. As secondary reagent, a goat anti-rabbit-alkaline phosphatase conjugate (Sigma) was used. After addition of the substrate p-nitrophenyl phosphate (Sigma), the reaction was measured after 25 min at 405 nm. Poliovirus binding of specific VHH-clones was revealed using a horseradish peroxidase-anti-M13 conjugate (Amersham Biosciences/GE Healthcare) (Conrath et al., 2001a).

2.5. Vector construction

The phage display vector pHEN4 was described previously (Arbabi et al., 1997). The pHEN6(c) vector is equivalent to the pHEN4 vector, except that the hemaglutinin tag and geneIII were replaced by a His_6 detection and purification tag. All these constructs were made by using standard cloning techniques.

2.6. Library construction and selection of specific antibody fragments

The VHH library was constructed as described previously (Conrath et al., 2001a). Basically, total RNA was extracted from peripheral blood lymphocytes and cDNA was prepared by reverse transcription (RT) with an oligo(dT) primer. VH and VHH genes were amplified with the leader-specific primer CALL001 (5′-GTCCTGGCTGCTCTTCTACAAGG-3′) and CH2-specific primer CALL002 (5′-GGTACGTGCTGTTGAACTGTTCC-3′). Two types of PCR products were obtained: one with about 700 bp length and another with about 900 bp length. The PCR products containing VHH genes (about 700 bp) were purified from agarose gel and re-amplified using nested primer A6E (5′-GATGTGCAGCTGCAGG-AGTCTGGRGGAGG-3′ where R stands for A or G) and primer 38 (5′-GGACTAGTGCGGCCGCTGGAGACGGTGACCTGGGT-3′) containing the restriction sites PstI and NotI, respectively. The final PCR products were cloned into the phagemid vector pHEN4 (Arbabi et al., 1997) and transformed into electro-competent *E. coli* TG1 cells. The VHH repertoire was displayed on phage after infection with M13K07 helper phages. Specific phage virions against poliovirus type 1 Sabin strain were enriched by three consecutive rounds of in vitro selection on microtiter plates coated with poliovirus type 1 (10 μg/well). After extensive washing with PBS/0.05% tween-20, bound phage particles were eluted with 100 mM triethylamine (pH ~10.0). The eluted particles were immediately neutralized with 1 M Tris–HCl (pH 7.4) and used to infect exponentially growing *E. coli* TG1 cells. The enrichment of phage particles carrying the antigen-specific VHHs was assessed by comparing the number of phages eluted from antigen-coated blocked wells versus empty only-blocked wells. After the second and third rounds of panning, individual colonies were randomly picked, and expression of soluble periplasmic VHH was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant VHH extracted from the periplasm was tested for antigen recognition in a solid-phase binding ELISA (Skerra and Plückthun, 1988).

2.7. Expression and purification of antibody fragments (Conrath et al., 2001a)

The VHH genes from the selected clones were re-cloned into the expression vector pHEN6(c) using the restriction enzymes NcoI and BstEII. The plasmid constructs were transformed into *E. coli* WK6 cells. Large-scale production of recombinant VHH was performed in shaker flasks by growing the bacteria in Terrific Broth supplemented with 0.1% glucose and ampicillin until an OD_{600} between 0.6 and 0.9 was reached. VHH expression was then induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 16 h at 28 °C. After pelleting the cells, the periplasmic proteins were extracted by osmotic shock (Skerra and Plückthun, 1988). This periplasmic extract was loaded onto a Ni-nitrilotriacetic acid (Ni-NTA) superflow Sepharose column (Qiagen), and after washing, the bound proteins were eluted with 500 mM imidazole. The eluted fraction was concentrated on Pierce concentrators (Thermo Scientific) with a molecular mass cut-off of 9 kDa and loaded on preplaced Sephadex G-25 colums (Pharmacia Biotech). The purity of the protein was evaluated in a Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). VHHs are present as a single band of ca. 14 kDa. No contaminants or degradation products were detected. The final yield was determined from the UV absorption at 280 nm, and the theoretical extinction coefficient (A_{280} [0.1%, 1 cm]) of the VHH was calculated for its amino acid content. The yield varied from 0.1 mg to 14 mg/l of the overnight bacterial culture, depending on VHH (data not shown).

2.8. Standard neutralization assay (STAN)

Equal volumes of virus and VHH in Tris-buffer were mixed and incubated for 1 h at 37 °C. HeLa cell monolayers were infected with 0.2 ml of the mixtures and allowed to stand for 30 min at 37 °C. Five milliliters of overlay, which consisted of modified Eagle's medium, 1.1% agar and 5% newborn calf serum, was added. After the petri dishes were incubated for 3 days at 37 °C, the cells were fixated with 10% formaldehyde, stained with 0.5% crystal violet and the plaque number was counted. The number of plaques was 50–100 in controls without VHH.

2.9. Cytotoxicity assay

HeLa cell monolayers grown to 50% confluency in 96-well cell culture plates were incubated with three-fold dilutions of the test compounds diluted in MEM-medium for 72 h at 37 °C and 5% CO_2. The cell viability was monitored daily by reading fluorescence (560EX/590EM) using the CellTiter-Blue® Cell Viability Assay (Promega, The Netherlands) according to the instruction manual. The percentage of cell survival was calculated as the ratio of cells incubated with test compound (treated)/cells incubated without test compound (untreated). The values were plotted against the concentrations of the test compound. The concentration needed to cause 50% cytotoxicity (CC_{50}) was determined graphically. Each sample was tested in triplicate.

The HeLa cells used here were from the same passage as those used in the antiviral assays.

2.10. Cytopathic effect reduction assay

Cytopathic effect reduction assay was performed as described previously (Thys et al., 2008). The CellTiter-Blue® Cell Viability Assay (Promega, The Netherlands) was used to monitor cell viability of infected cells in order to calculate cytopathic effect reduction. For cytopathic effect reduction values, HeLa cells (4×10^4 cells) were infected with 100 PFU of poliovirus type 1 and allowed to stand for 30 min at 37 °C. After this time, three-fold dilutions of the test compound diluted in MEM were added to the cells. The cultures were incubated for 3 days until complete cytopathic effect was observed in untreated infected cells (control cultures). After removal of the medium, CellTiter-Blue® Reagent was added to each well and the cells were incubated for 2 h at 37 °C prior to recording fluorescence (560EX/590EM). Data are expressed as percentage of viability compared to uninfected and untreated cell cultures. Each sample was tested in triplicate. Nb1, a VHH specific for the transcription factor Ss-LrpB of *Sulfolobus sulfatarius*, was used as negative control.

2.11. Infectious viral yield reduction assay

Quantification of poliovirus type 1 inhibition was performed by an infectious virus yield reduction assay according to the procedure reported by Thys et al. (2008). HeLa cells (4×10^4 cells) were infected with 100 PFU of poliovirus type 1 and allowed to stand at 37 °C. After 30 min incubation three-fold dilutions of the test compound diluted in MEM were added to the cells. The cultures were incubated for 3 days until a complete cytopathic effect was observed in untreated infected cells (control cultures). Nb1, a VHH specific for the transcription factor Ss-LrpB of *Sulfolobus sulfataricus*, was used as negative control. Virus load of the supernatant was measured by a standard plaque assay (Verlinden et al., 2002). Data are expressed as the log reduction in virus yield, as compared to control cultures.

2.12. Generation of neutralization-escape virus mutants

Neutralization-escape type 1 poliovirus variants were generated in HeLa cell monolayers. 10^6 to 10^8 PFU of Sabin type 1 poliovirus were first mixed with 7.1 μM compound and incubated for 1 h at 37 °C. HeLa cell monolayers were infected with 0.2 ml of the mixtures and allowed to stand for 30 min at 37 °C. Five milliliters of overlay, which consisted of modified Eagle's medium, 1.1% agar and 5% newborn calf serum, was added. After the petri dishes were incubated for 2 days at 37 °C, a second overlay of 0.1% neutral red was added and further incubated for 1 day at 37 °C. The plaque number was counted.

3. Results

3.1. Humoral response of the dromedary after immunization with poliovirus type 1 Sabin

One dromedary was injected six times with poliovirus type 1 Sabin. From the plasma, the IgG-subclasses were fractionated into the conventional immunoglobulins IgG1 and the heavy-chain immunoglobulins IgG2 and IgG3 to evaluate the humoral response within each subclass. As shown by solid-phase ELISA, an antigen-specific response existing of conventional antibodies and IgG2 immunoglobulins was elicited. However, there was no IgG3 response above the detection limit of the technique used (Fig. 1).

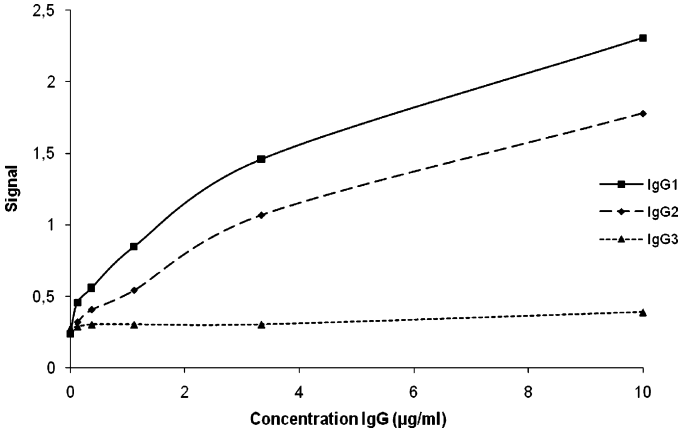

Fig. 1. Analysis of antigen-specific antibodies. Microtiter plates were coated with poliovirus type 1 Sabin at a concentration of 1 μg/ml. Serial dilutions of the immunoglobulin subclasses, isolated from plasma after immunization, were added to the coated plates. Bound IgG1, IgG2 and IgG3 were subsequently detected with a rabbit anti-dromedary IgG antiserum and anti-rabbit IgG-alkaline phosphatase conjugate. OD_{405} was measured after 25 min.

3.2. Selection and sequence analysis of poliovirus type 1-specific VHHs

From the lymphocytes prepared from anti-coagulated blood of an immunized dromedary, cDNA was prepared and used as template to amplify genes coding for the variable domains of the heavy-chain antibodies by PCR. The PCR fragments were ligated into pHEN4 phagemid vector and transformed into *E. coli* TG1 cells. A VHH library of about 10^7 independent transformants was obtained. The percentage of the clones within the library containing a vector with a VHH gene insert of the proper size was about 60% as determined by PCR. The VHH repertoires of the library were displayed on phages after infection with helper phages. The library was then subjected to three rounds of panning against poliovirus type 1 Sabin strain as bait. After three rounds of selection, a clear enrichment for poliovirus type 1 Sabin-specific phage particles was observed. Ninety-four colonies were randomly chosen after the third round of panning and the expression of their VHHs as soluble proteins was induced with IPTG. These soluble VHHs were then screened by ELISA for their antigen specificity and several of them proved to be specific for poliovirus type 1 Sabin (Fig. 2A). A threshold value of three times the background value (the signal obtained with only-blocked wells) was taken as the lower limit of specificity for poliovirus type 1. Fifteen ELISA reactive VHH clones were selected for further study.

The predicted amino acid sequences of the VHHs are shown in Fig. 2B. The amino acid sequence analysis of these binders revealed eight distinct binders based on their complementarity determining region (CDR) 3 sequence. All binders are derived from the heavy-chain antibody specific VHH germline genes (Nguyen et al., 2000), as they contain the hallmark amino acid substitutions in framework 2. The eight different groups varied significantly in amino acid composition, not only within the CDR3 region, but also within the other CDR regions, suggesting they target different epitopes.

3.3. Neutralization activity of poliovirus type 1 binding VHHs

The fifteen VHHs were examined for neutralizing activity against the three types of poliovirus. In case of poliovirus type 1, we included besides the vaccine strain also one wild-type strain (Mahoney). While no neutralizing activity against poliovirus type 2 and 3 was detected, PVSP6A, PVSS8A, PVSP19B, PVSS21E, and PVSP29F from five different CDR3 groups neutralized both the vaccine and the wild-type strain of poliovirus type 1. Other VHHs did not show any neutralizing activity (Table 1).

3.4. Cytopathic reduction effect and cytotoxicity of neutralizing VHHs

All six VHHs tested in this assay showed no cytotoxic effect on HeLa cells after 72 h incubation at concentrations up to 35 μM (Table 2).

To evaluate their antiviral activity, the effect of increasing concentrations of VHH to inhibit the induced cytopathic effect in HeLa cells by poliovirus type 1 was examined and compared with two potent anti-poliovirus compounds. We used the neutralizing anti-poliovirus type 1 monoclonal antibody 35-1f4 as a first reference compound and the capsid-binding pyridazinamine analogue R75761 with anti-poliovirus activity as a second reference compound (Brioen et al., 1982; Thys et al., 2008). All tested "neutralizing" VHHs, i.e., PVSP6A, PVSS8A, PVSP19B, PVSS21E, and PVSP29F, the mAb 35-1f4 and the synthetic compound R75761 demonstrated a dose-dependent inhibitory effect on the virus induced cytopathic effect in HeLa cells (Fig. 3A). PVSP6A and PVSP29F VHHs could fully protect the cells from a cytopathic effect induced by poliovirus type 1 infection at concentrations lower than

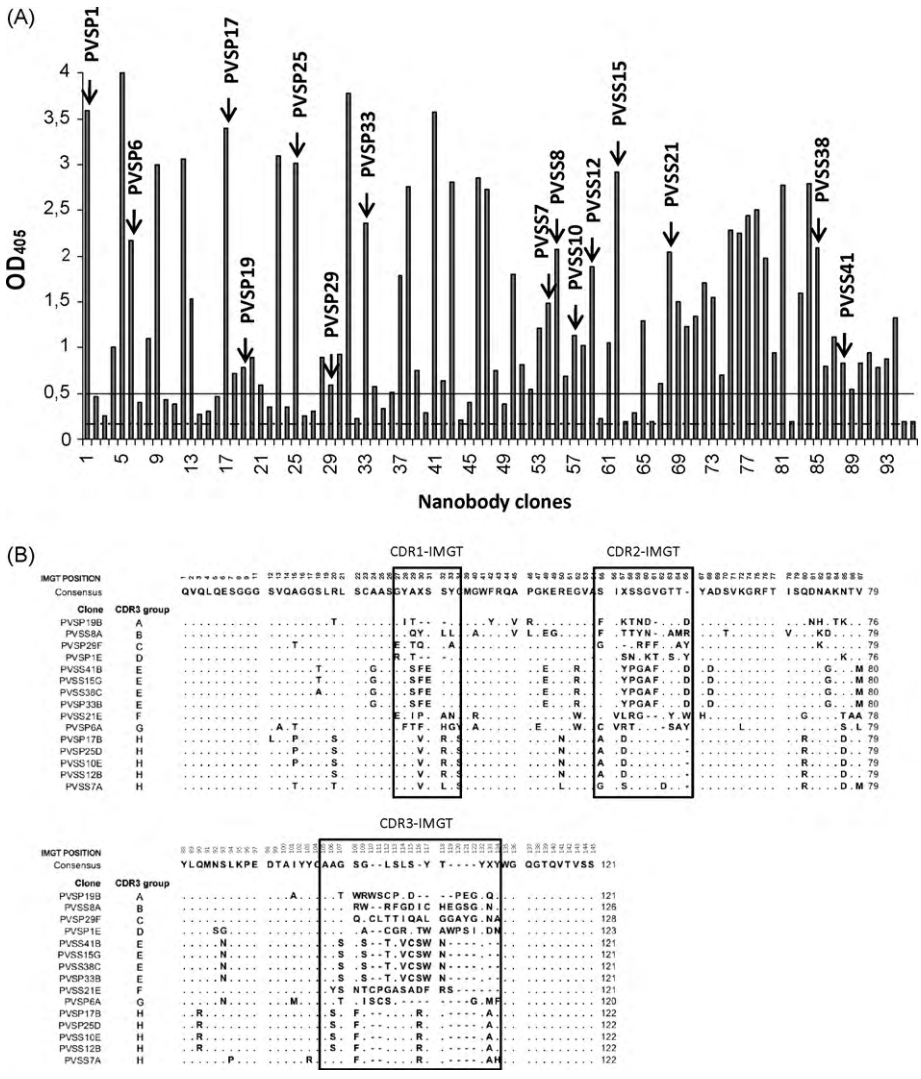

Fig. 2. (A) Screening of VHH clones for their specificity for poliovirus type1 Sabin. The periplasmic extracts of IPTG-induced individual colonies selected after panning against poliovirus type 1 Sabin were assayed for poliovirus type 1 specificity. The chosen clones for further studies are shown with arrows. The dashed line represents the background value of the OD value when no antigen was coated on the microtiter plate. The full line represents the threshold value where the OD value exceeds three times the background value. VHHs with OD value above the threshold value are considered to be specific binders for the antigen. (B) Multiple amino acid sequence alignment of poliovirus type 1 binding VHHs. Dashes indicate gaps introduced for sequence alignment. Residual identical to the consensus sequence are shown by dots. An X in the consensus sequence is depicted as the ambiguous symbol for residues without consensus. Amino acid numbering and CDR definitions are according to the IMGT numbering system (Lefranc, 2004). The VHHs are grouped according to their CDR3 sequence.

those of the other VHHs and the synthetic compound R75761. This protective activity of PVSP6A and PVSP29F VHHs was comparable to that of the neutralizing mAb 35-1f4. An irrelevant VHH (Nb1) that was used as negative control did not show any antiviral activity (Fig. 3A and Table 2). PVSP6A and PVSP29F VHHs proved to be the most potent inhibitors with EC_{50} values of, respectively, 7 and 16 nM, whereas the EC_{50} values of the other neutralizing VHHs ranged between 439 and 692 nM. The EC_{90} values ranged between 17 nM for PVSP6A VHH and 1770 nM for PVSS21E VHH (Table 2). The selectivity index values could not be accurately calculated because of the lack of cytotoxicity at the highest concentration tested but they are at least ranging from more than 51 for PVSS21E to more than 5071 for PVSP6A (Table 2).

3.5. Antiviral activity measured by reduction in virus yield

To further confirm and define the antiviral activity observed with the cytopathic effect reduction assay, we performed an infectious virus yield reduction assay on poliovirus type 1-infected cell cultures treated with increasing amounts of VHHs. As depicted in Fig. 3B, PVSP6A and PVSP29F, the most potent inhibitors from the cytopathic effect reduction assay, were also in this assay the most potent antiviral compounds deduced from the dose-dependent relationship of the VHH and the infectious virus yield reduction. Also in this assay, the antiviral activity of these VHHs is comparable to the antiviral activity of the mAb 35-1f4. Remarkable is the complete abolishment of virus replication in cell culture treated with certain concentrations of PVSP6A and PVSP29F VHHs, whereas still a minute amount of infectious virus (ca. 10^2 PFU/ml) was detected in infected HeLa cells treated with the same concentration of mAb 35-1f4. PVSP6A VHH was even able to completely inhibit the formation of infectious virus at a concentration of 1.2 μM. This in contrast with untreated infected HeLa cell cultures were virus titer of 8.3 $\log_{10}$ PFU/ml were detected. PVSP6A VHH could further reduce the virus yield by 10^2- to 10^5-fold at concentrations between 15 and 394 nM, respectively, whereas PVSP29F inhibited virus production by 10^2- to10^5-fold at concentrations of 16 and 3944 nM, respectively. PVSP19B and PVSS21E showed also antiviral activity as virus titers started to drop significantly in infected cell cultures treated with concentrations higher than 0.4 μM with a

Table 1
In vitro neutralization activity of poliovirus type 1 binding VHHs (100 μg/ml) against different poliovirus types.

VHH	CDR group	Poliovirus			
		Type 1 Sabin	Type 1 Mahoney	Type 2 Sabin	Type 3 Sabin
PVSP19B	A	+++	+++	–	–
PVSS8A	B	+	+	–	–
PVSP29F	C	+++	+++	–	–
PVSP1E	D	–	–	–	–
PVSS41B	E	–	–	–	–
PVSS15G	E	–	–	–	–
PVSS38C	E	–	–	–	–
PVSP33B	E	–	–	–	–
PVSS21E	F	+++	++	–	–
PVSP6A	G	+++	+++	–	–
PVSP17B	H	–	–	–	–
PVSP25D	H	–	–	–	–
PVSS10E	H	–	–	–	–
PVSS12B	H	–	–	–	–
PVSS7A	H	–	–	–	–

Equal volumes of virus and VHH in Tris-buffer were mixed and incubated for 1 h at 37 °C. HeLa cell monolayers were infected with 0.2 ml of the mixtures and incubated for 0.5 h at 37 °C. Five milliliters of overlay, which consisted of modified Eagle's medium and 1.1% agar, was added. After the petri dishes were incubated for 3 days at 37 °C, the cells were fixated with 10% formaldehyde and stained with 0.2% crystal violet. The plaques were counted. The number of plaques was between 50 and 100 in the controls without VHH [–, no plaque reduction; +, plaques reduction between 50% and 90%; ++, plaque reduction between 90 and 100%; +++, 100% plaque reduction].

maximum reduction of 6 $\log_{10}$ PFU/ml in virus yield at a concentration of 3.6 μM for PVSP19B and 8 $\log_{10}$ PFU/ml at a concentration of 35.5 μM for PVSS21E. Conversely, PVSS8A is a very weak inhibitor of virus production, although it has an EC_{50} value similar to those of PVSP19B and PVSS21E measured by the cytopathic effect reduction assay. Almost no decrease in virus yield (less than two-fold) was noted for poliovirus-infected cell cultures treated with PVSS8A. Nb1, an irrelevant VHH showed also no antiviral activity in this assay.

3.6. Generation of neutralization-escape virus mutants

To evaluate the generation of neutralization-escape virus mutants against the VHHs, we mixed 10^6 PFU of poliovirus type 1 with VHH and tried to recover resistant clones following agar overlay of cells infected with this mixture. For PVSP6A and PVSP29F plaque formation was completely inhibited (Table 3) and no resistant virus could be isolated. In case of the other VHHs, ca. 100 plaques were noted which could not be neutralized. This number

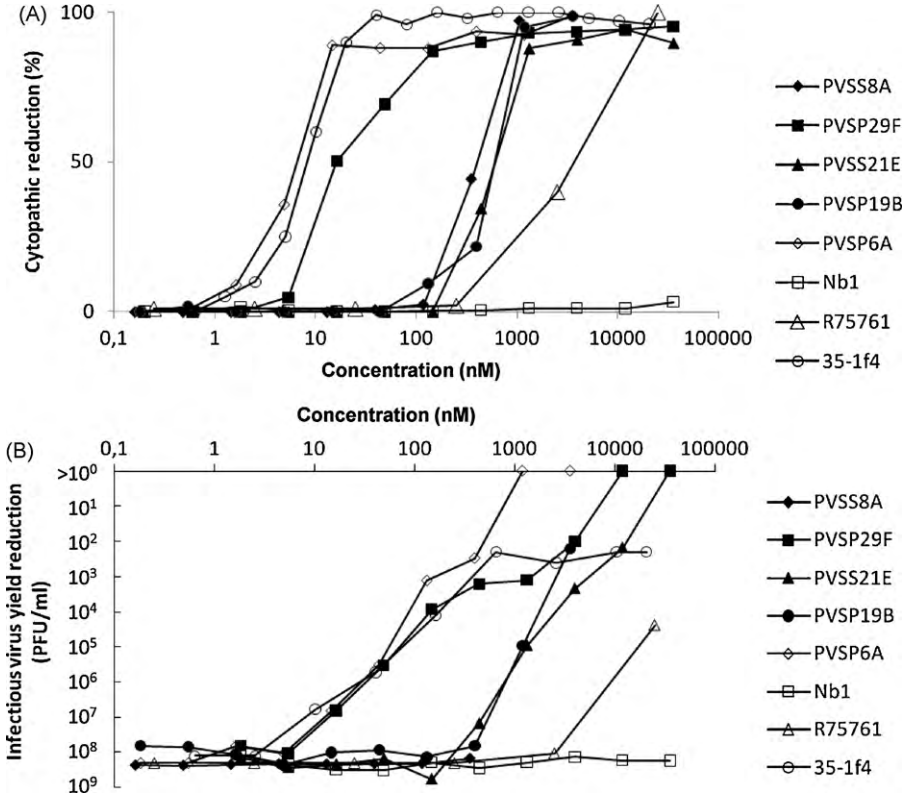

Fig. 3. Dose–response relationship of increasing concentrations of compound on the induced cytopathic effect by poliovirus type 1 on HeLa cells (A) and the inhibitory effect on the virus yield of HeLa cells (B). 4×10^4 HeLa cells were infected with 100 PFU poliovirus type 1 and incubated for 3 days at 37 °C until complete cytopathic effect was observed in untreated infected cells. The reduction of the cytopathic effect was calculated as percentage viability, as compared to uninfected untreated cell cultures. Virus yield was determined by a standard plaque assay and data are expressed as the reduction in infectious virus yield compared to untreated infected cells. The data reported on the horizontal (A and B) and vertical (B) axes are expressed in $\log_{10}$ units.

Table 2
Antiviral effect of VHHs against poliovirus type 1, as monitored by the cytopathic reduction assay.

VHH	CC_{50}[a] (μM)	EC_{50}[b] (μM)	EC_{90}[c] (μM)	SI[d]
PVSS8A	>35.5	0.439	1.032	>80
PVSS21E	>35.5	0.692	1.770	>51
PVSP19B	>35.5	0.680	1.083	>52
PVSP29F	>35.5	0.016	0.215	>2218
PVSP6A	>35.5	0.007	0.017	>5071
Nb1	>35.5	>35.5	>35.5	–
R75761	>250	3.715	16.298	>67
35-1f4	>35.5	0.008	0.019	>4438

[a] CC_{50}: concentration of the compound which reduces cell viability by 50%.
[b] EC_{50}: concentration of the compound which reduces by 50% the cytopathic effect induced by poliovirus type 1 Mahoney.
[c] EC_{90}: concentration of the compound which reduces by 90% the cytopathic effect induced by poliovirus type 1 Mahoney.
[d] SI: selectivity index (CC_{50}/EC_{50}).

Table 3
Generation of neutralization-escape virus mutants against neutralizing VHHs.

VHH	Number of PFU[a]
PVSP6A	0
PVSS8A	105
PVSP19	98
BPVSS21	110
EPVSP29	0
F35-1f4	95

[a] 10^6 PFU of poliovirus Sabin type1 were treated with 7.1 μM compound. HeLa cell monolayers were infected with this mixture and overlay was added. Plaques were counted after 3 days at 37 °C.

of plaques was equal to the expected number of plaques escaping the neutralization by a neutralizing monoclonal antibody.

4. Discussion

In this study, a VHH phage display library, derived from an immunized dromedary, was screened against poliovirus type 1 Sabin with the aim to obtain specific VHH molecules able to neutralize infectious poliovirus particles. Fifteen VHHs with different binding values (Fig. 2A) were randomly chosen among the binders.

This panel of VHHs was expressed as soluble proteins, purified and further examined for their ability to neutralize different strains of poliovirus. Five VHHs neutralized a poliovirus type 1 vaccine strain as well as a neurovirulent strain, albeit to different degrees (Table 1). There was no correlation between the binding profiles of VHHs in ELISA (data not shown) and their neutralization activity against poliovirus type 1: none of the VHHs with the highest binding values neutralized poliovirus, while some VHHs with a low binding value did so (Table 1). It should be noted that the most potent neutralizing VHH fragments gave low to moderate signals in ELISA screening and would have been missed if priority was given to a limited number of ELISA-positive clones to be analyzed for their neutralization activity.

Unlike conventional antibodies, VHHs have the tendency to bind into cavities and clefts (Conrath et al., 2001b). One such cavity or cleft on the capsid of poliovirus corresponds to the canyon. Poliovirus uses this "hidden" epitope on the capsid for docking into the viral receptor on the host cells (He et al., 2000, 2003). Our idea was that VHHs could bind into the canyon, thereby blocking the site of cellular attachment and preventing the virus from entering the cell. The canyon region seems to be a rather conserved structure reflected by the recognition of a common cellular receptor by all three types of poliovirus for cell attachment and entry (He et al., 2000; Belnap et al., 2000). We hoped to select a VHH binding to the canyon and, therefore, able to neutralize different types of poliovirus. However, the VHHs which showed neutralization activity against poliovirus type 1, did not neutralize poliovirus type 2 and type 3. We are convinced, however, that by modifying the immunization protocol and the panning procedure, it would be possible to select VHHs with a broader spectrum against polioviruses and even against other enteroviruses including rhinoviruses. For example, if each immunization boost and/or each panning round are (is) carried out with a different poliovirus strain, the possibility of generation and isolation of pan-reactive VHHs neutralizing different poliovirus strains is enhanced.

The antiviral activity of PVSP6A and PVSP29F VHHs are very promising in terms of cytopathic effect reduction and infectious virus yield reduction. When HeLa cells were infected with a low MOI and incubated for multiple replication rounds during 72 h post-infection, no infectious virus could be detected at certain concentrations (Fig. 3B). Even, when the VHHs were challenged with a high MOI (up to 1000), HeLa cells were protected against viral infection (data not shown). The antiviral activities of PVSP6A and PVSP29F VHHs have been in the nanomolar range, which is comparable to the neutralizing efficiencies of monoclonal antibodies against poliovirus.

The antigenic sites of poliovirus were previously mapped into at least four antigenic sites, denominated sites 1, 2, 3A, and 3B, by the induction of escape mutants and cross-neutralization (Minor et al., 1986). Due to their nature, mAbs are able to neutralize virions by aggregating pathogen particles or the destabilization of virion particles (reviewed by Reading and Dimmock, 2007). In order to determine the antigenic sites for VHHs, we attempted to generate escape variants against the neutralizing VHHs but failed for two out of five VHH (Table 3). Interestingly, these two VHHs were PVSP6A and PVSP29F, the two VHHs with the most potent antiviral activity. These data suggest that these antibody fragments may recognize hidden epitopes and that these epitopes could even be a unique epitope for both of them and different from the described antigenic sites for mAbs. This can be of extreme importance when these VHHs would be used as antiviral therapy together with other available tools such as IPV vaccination to control poliovirus outbreaks. If VHHs bind to epitopes other than those which are antigenic sites of mAbs, these sites will still be accessible for defensive antibodies and VHHs will not interfere with virus particles (both infectious particles and killed particles as found in IPV) in terms of induction of host antibody response.

The occurrence of escape variants against other VHHs studied here varied between 1 per 10^4 to 1 per 10^5 virions as expected for antiviral compounds and mAbs against positive RNA-stranded viruses (Ahmad et al., 1987; Heinz et al., 1989). Work is underway to map the antigenic binding site of the VHHs on the capsid. For those VHHs which are not generating escape variants, the antigenic binding site will be determined via crystallography while for the other VHHs, the work will be done by sequencing the genome of the escape variants in order to map the mutations to possible antigenic sites.

In conclusion, this study demonstrates the feasibility of using VHHs as a novel class of antiviral drugs and provides, at least two neutralizing VHHs, for the development of a potentially efficacious antiviral drug for the treatment and prophylaxis of poliomyelitis induced by poliovirus type 1 at a low, economic cost per dose. Efforts are underway to widen the breadth of protection against the three poliovirus types and to increase the potency of the VHHs against poliovirus. In a next study, we hope to demonstrate the antiviral potency of our VHHs in a relevant animal model.

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