Antiviral Research 87 (2010) 257–264

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

In vitro antiviral activity of single domain antibody fragments against poliovirus

Bert Thys[a], Lise Schotte[a], Serge Muyldermans[b,c], Ulrich Wernery[d], Gholamreza Hassanzadeh-Ghassabeh[b,c], Bart Rombaut[a,*]

[a] Department of Pharmaceutical Biotechnology and Molecular Biology, Centre for Pharmaceutical Research, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium
[b] Laboratory of Cellular and Molecular Immunology, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium
[c] Department of Molecular and Cellular Interactions, VIB, Pleinlaan 2, B-1050 Brussels, Belgium
[d] Central Veterinary Research Laboratory, P.O. Box 597, Dubai, United Arab Emirates

ARTICLE INFO

Article history:
Received 18 March 2010
Received in revised form 19 May 2010
Accepted 31 May 2010

Keywords:
Anti-poliovirus compound
Poliovirus
Antiviral
VHH
Nanobody

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.
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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.

* Corresponding author. Tel.: +32 2 477 44 97, fax: +32 2 477 44 95.
E-mail addresses: bert.thys@vub.ac.be (B. Thys), lschotte@vub.ac.be (L. Schotte), svmuylde@vub.ac.be (S. Muyldermans), ghassanz@vub.ac.be (G. Hassanzadeh-Ghassabeh), brombaut@vub.ac.be (B. Rombaut).

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doi:10.1016/j.antiviral.2010.05.012

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

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