MABS
2018, VOL. 10, NO. 6, 815–826
https://doi.org/10.1080/19420862.2018.1489633

Taylor & Francis
Taylor & Francis Group

REVIEW



# Antigen recognition by single-domain antibodies: structural latitudes and constraints

Kevin A. Henry[a] and C. Roger MacKenzie[a,b]

[a]Human Health Therapeutics Research Centre, National Research Council Canada, Ottawa, Ontario, Canada; [b]School of Environmental Sciences, University of Guelph, Guelph, Ontario, Canada

**ABSTRACT**

Single-domain antibodies (**sdAbs**), the autonomous variable domains of heavy chain-only antibodies produced naturally by camelid ungulates and cartilaginous fishes, have evolved to bind antigen using only three complementarity-determining region (**CDR**) loops rather than the six present in conventional $V_H$:$V_L$ antibodies. It has been suggested, based on limited evidence, that sdAbs may adopt paratope structures that predispose them to preferential recognition of recessed protein epitopes, but poor or non-recognition of protuberant epitopes and small molecules. Here, we comprehensively surveyed the evidence in support of this hypothesis. We found some support for a global structural difference in the paratope shapes of sdAbs compared with those of conventional antibodies: sdAb paratopes have smaller molecular surface areas and diameters, more commonly have non-canonical CDR1 and CDR2 structures, and have elongated CDR3 length distributions, but have similar amino acid compositions and are no more extended (interatomic distance measured from CDR base to tip) than conventional antibody paratopes. Comparison of X-ray crystal structures of sdAbs and conventional antibodies in complex with cognate antigens showed that sdAbs and conventional antibodies bury similar solvent-exposed surface areas on proteins and form similar types of non-covalent interactions, although these are more concentrated in the compact sdAb paratope. Thus, sdAbs likely have privileged access to distinct antigenic regions on proteins, but only owing to their small molecular size and not to general differences in molecular recognition mechanism. The evidence surrounding the purported inability of sdAbs to bind small molecules was less clear. The available data provide a structural framework for understanding the evolutionary emergence and function of autonomous heavy chain-only antibodies.

**KEYWORDS**

single-domain antibody; $V_HH$; $V_{NAR}$; molecular recognition; antibody: antigen interaction; paratope; epitope

## Introduction

Single-domain antibodies (**sdAbs**) are the monomeric binding domains of heavy chain-only antibodies that have arisen through convergent evolution at least three times (twice in *Chondrichthyes* and once in *Camelidae*, roughly 220 and 25 million year ago, respectively[1]). The concept of autonomous, antigen binding-competent sdAbs was first described by Ward *et al.* in 1989,[2] and several years later, naturally-occurring antibodies lacking light chains were discovered in dromedary camels[3] and nurse sharks.[4] The ~12–15 kDa variable domains of these antibodies ($V_HH$s and $V_{NAR}$s, respectively; Figure 1) can be produced recombinantly and can recognize antigen in the absence of the remainder of the antibody heavy chain. The modular nature of $V_HH$s and $V_{NAR}$s has been widely and productively exploited in the development of antibody-based drugs (reviewed in Ref.[5]).

Structural studies of the first $V_HH$s and $V_{NAR}$s isolated[6,7] provided an early indication that these molecules might interact with antigens using mechanisms distinct from those of conventional antibodies. With hindsight, the notion that sdAbs might preferentially target particular types of antigenic structures may not seem totally unexpected, given their recombination from distinct repertoires of V, D and J genes (see **Box 1**),[8] their potential ontogeny from separate B-cell precursors,[9] and for camelid $V_HH$s, their specialized constant regions bearing very long hinge regions.[10] However, the specific mechanisms of sdAb antigen recognition (*e.g.*, the tertiary structures and physicochemical properties of sdAb:antigen interfaces, which may differ fundamentally from those of conventional antibody:antigen interfaces) remain unclear, although several studies have suggested protein cleft recognition as a general function for both $V_HH$s[11] and $V_{NAR}$s.[12] Over time, the idea that sdAbs can target 'cryptic' epitopes (so-called because they are inaccessible to conventional antibodies, either for steric reasons or due to their fundamental antigenic properties) has become entrenched, and although several case studies have supported it, its generality and implications are questionable. Several excellent recent reviews and opinion pieces have alluded to the nature of sdAb paratopes and their interactions with antigens, but have either not been rigorous in their approach or have incompletely addressed the topic, analyzing the properties of sdAb paratopes only, with no comparison to those of conventional antibodies.[13–17] Thus, the aim of this review was to comprehensively investigate whether and how sdAb:antigen interactions differ from conventional antibody:antigen

**CONTACT** Kevin A. Henry Ph.D. kevin.henry@nrc-cnrc.gc.ca Human Health Therapeutics Research Centre, National Research Council Canada, 100 Sussex Drive, Ottawa, ON K1A 0R6, Canada

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/kmab.

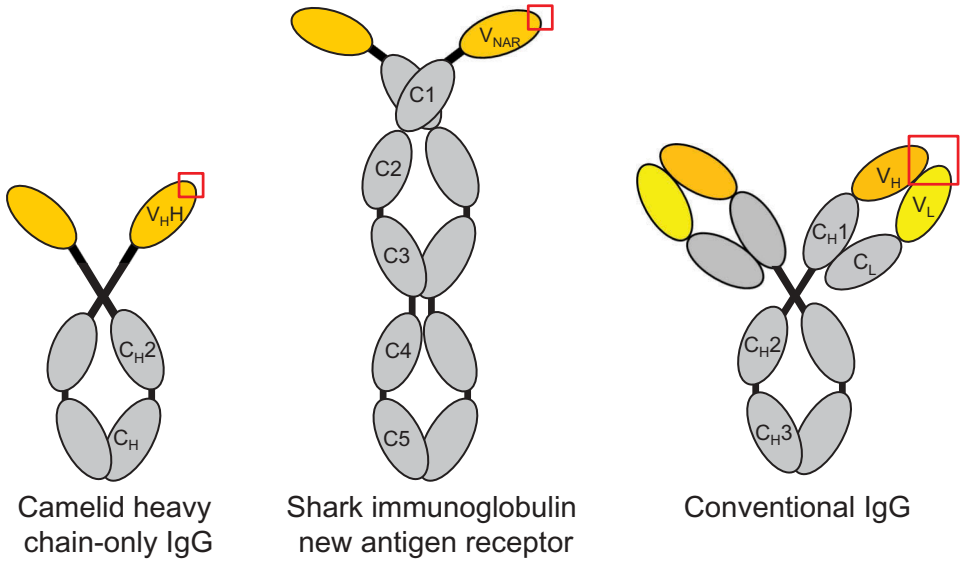


**Figure 1.** Domain structures of camelid heavy chain-only IgG, shark immunoglobulin new antigen receptor (IgNAR) and conventional vertebrate tetrameric IgG. The variable domain(s) of each antibody molecule are shown in yellow and the antigen-combining site is indicated by a red box.

**Box 1.** Immunogenetics of sdAbs

$V_HH$s, the variable domains of camelid heavy chain-only antibodies, are recombined during B-cell development from a unique set of germline V genes and common D and J genes (shared with the $V_H$ domains of conventional tetrameric antibodies) located within the *igh* locus on chromosome 4.[8] Most camelid $V_HH$ and $V_H$ genes[18] are homologous to human IGHV3-family genes (~75–90% identity) and encode distinctive solubilizing residues in FR2 (Phe/Tyr42, Glu49, Arg50 and Gly 52 using IMGT numbering; these positions map to the $V_H$:$V_L$ interface in conventional antibodies), although functional $V_HH$s lacking this consensus have been isolated.[19,20] Some camelid V genes may 'promiscuously' recombine with both heavy chain-only and conventional antibody constant region genes.[19] $V_HH$ domains bear unusually long CDR3 loops in comparison with human and murine conventional antibodies,[21,22] probably reflecting increased non-templated nucleotide addition, although this may be a feature of only a subset of $V_HH$s;[21] in some $V_HH$s, the long CDR3 loop serves a dual purpose, folding over the former $V_L$ interface as well as interacting with cognate antigen. The rearranged $V_HH$ exon is thought to undergo elevated rates of somatic hypermutation of both CDRs and FRs (*e.g.*, FR1-encoding sequences immediately flanking CDR1;[23–25] FR2-encoding sequences which may play a role in structuring the CDR3 loop;[20,25] FR3-encoding sequences that form a β-turn which can make contact with antigen, sometimes called CDR4[24]). $V_HH$s may also acquire somatic insertions and deletions at higher rates than conventional antibodies,[24] and may under some circumstances undergo secondary rearrangement events using a cryptic recombination signal sequence in FR3.[24] Some $V_HH$ genes encode non-canonical disulfide linkages formed between cysteine residue pairs (CDR1-CDR3, FR2-CDR3, CDR2-CDR3 or CDR3-CDR3; see Box 2).

$V_{NAR}$s, the variable domains of cartilaginous fish Ig new antigen receptors, share sequence homology with T-cell receptor and Ig light chain genes[4] and may be descended from Ig-superfamily cell-surface receptors.[26] Compared with Ig $V_H$ domains, $V_{NAR}$s lack two β strands (C′ and C″) and consequently CDR2 is absent, although loops connecting the C-D and D-E strands (HV2 and HV4, respectively) can make contact with antigen. During B-cell development, $V_{NAR}$ domains are rearranged from a small number of loci (perhaps only three) distinct from those encoding other types of Ig molecules detectable in serum (IgM, IgW). Each locus contains one V gene, two or three D genes and one J gene and thus primary repertoire diversity is almost entirely CDR3-based:[4] since $V_{NAR}$ CDR3 loops are formed through either three or four independent rearrangement events, these tend to be long.[27] Unlike the $V_H$ domains of IgMs and IgWs, $V_{NAR}$s accrue somatic hypermutations upon encounter with antigen primarily in CDR1 and CDR3 but also in HV2 and HV4.[27,28]

interactions, and to assess whether $V_HH$s and $V_{NAR}$s share any similarities in this respect despite their evolutionary divergence. The answer to this question has direct relevance for the 'druggable' target space available to sdAbs *vs.* conventional antibodies.

## Single-domain antibodies directed against folded proteins

As with conventional antibodies, the bulk of sdAbs studied have been directed against folded proteins. Certain regions and epitopes on folded proteins are inherently more immunogenic than others, a concept known as immunodominance. The immunological mechanisms underlying B-cell immunodominance are poorly understood, and patterns of immunodominance probably are not completely conserved across species.[29]

The first indication that sdAbs might preferentially target different sets of epitopes compared with conventional antibodies came from studies of anti-enzyme sdAbs (Table 1). Conventional antibodies can act as enzyme inhibitors, most commonly by inducing allosteric conformational changes or by sterically blocking substrate access to the active site.[65] It was recognized from early structural studies of anti-lysozyme $V_HH$s[6] and $V_{NAR}$s[7] that these molecules interacted with the enzyme in unusual fashion, probing deeply into its active site using extended complementarity-determining region (CDR)3 loops. These results were later replicated independently using additional $V_H$s,[51] $V_HH$s[11,47,48] and $V_{NAR}$s[12] directed against the active site of lysozyme, as well as with active site-binding $V_HH$s against α-amylase,[31,32] carbonic anhydrase,[32] and urokinase.[62,63] Inhibition of α-amylase was achieved by one $V_HH$ through penetration of the active site cleft with its CDR2 loop,[31]

demonstrating that CDR3-centric binding is not the only mechanism of competitive enzyme inhibition by sdAbs. Competitive inhibition of these enzymes by conventional antibodies targeting their active sites has not been described despite intensive study, especially of murine antibodies against lysozyme. Naturally-occurring competitive inhibitors of protease enzymes are convex, and this appears to be a difficult geometry for the paratopes of conventional antibodies to achieve (see below): even in cases of near-true competitive inhibition, conventional antibodies use a flat or concave $V_H/V_L$ interface to bind protruding regions on enzymes and partially insert one or more CDRs into the active site cleft in a non-substrate-like manner.[66,67] This hypothesis is supported by experiments using purified polyclonal immunoglobulin (Ig)Gs from enzyme-immunized dromedaries showing that competitive inhibition was a feature of heavy chain-only IgGs, but not of conventional IgGs.[11,32] It remains unclear why immunization with some enzymes yields mostly sdAbs with planar paratopes and bind outside the active site, achieving allosteric or no inhibition, although tolerance mechanisms may play a role.

**Table 1.** Single-domain antibodies as enzyme inhibitors.

| Enzyme | Type of sdAb(s) | Inhibition | Mechanism(s) of Inhibition | Reference(s) |
|---|---|---|---|---|
| Aldolase | $V_HH$ | ND | NA | 30 |
| α-amylase | $V_HHs$ | +/- | Competitive[1], allosteric[1], NA | 31,32 |
| ART2.2 | $V_HHs$ | +/- | ND, NA | 33 |
| Aurora-A kinase | $V_{NAR}$ | + | Allosteric[1] | 34 |
| β-lactamase | $V_HHs$ | +/- | Allosteric[2,3], NA | 35 |
| | $V_HHs$ | +/- | ND, NA | 36 |
| Botulinum toxin | $V_HHs$ | +/- | Steric exclusion of substrate[1] | 37 |
| | $V_HHs$ | +/- | Competitive[2], NA | 38 |
| Carbonic anhydrase | $V_HHs$ | +/- | Competitive[2], NA | 32,39 |
| CD38 | $V_HHs$ | ND | NA | 40 |
| | $V_HHs$ | +/- | Allosteric[2], NA | 41 |
| CDT binary toxin | $V_HHs$ | + | ND | 42 |
| DHFR | $V_HH$ | + | Allosteric[1,3] | 43 |
| Furin | $V_HHs$ | +/- | Steric exclusion of substrate[1,3], NA | 44,45 |
| Lysozyme | $V_HHs$ | + | Competitive[1,2] | 11 |
| | $V_HH$ | + | Competitive[1] | 6,46 |
| | $V_HH$ | + | Competitive[2] | 47 |
| | $V_HHs$ | + | Competitive[1] | 48 |
| | $V_HH$ | ND | NA[4] | 49 |
| | $V_HH$ | ND | NA[4] | 50 |
| | $V_{NAR}$ | + | Competitive[1] | 7 |
| | $V_{NAR}$ | + | Competitive[1] | 12 |
| | $V_HS$ | +, ND | Competitive[1,2], NA | 51 |
| NOR | $V_HHs$ | +/- | ND, NA | 52 |
| HCV NS3 protease | $V_HS$ | +/- | Competitive[3], NA | 53 |
| PglK flippase | $V_HHs$ | +/- | Allosteric[1], NA | 54 |
| Ricin toxin A | $V_HHs$ | ND | NA | 55 |
| RNase A | $V_HH$ | + | Steric exclusion of substrate[1] | 56 |
| SBE-A | $V_HHs$ | +/- | ND, NA | 57 |
| TAFI | $V_HHs$ | +/- | Competitive[2], NA | 58 |
| | $V_HHs$ | +/- | ND, NA | 59 |
| Trans-sialidase | $V_HHs$ | +/- | Competitive[2], NA | 60 |
| Urease | $V_HH$ | + | ND | 61 |
| Urokinase | $V_HHs$ | + | Competitive[1], allosteric[1] | 62 |
| | $V_HHs$ | + | Competitive[1,2] | 63 |
| | $V_HHs$ | +/- | Allosteric[2], NA | 64 |

[1]Mechanism inferred from antibody:enzyme X-ray co-crystal structures.
[2]Specificity for active site or non-active site regions demonstrated through epitope mapping experiments.
[3]Mechanism inferred from studies of enzyme kinetics.
[4]Inhibition was not assessed, but structural studies showed that the antibody did not target the active site.
Abbreviations used: ART2.2, ecto-ADP-ribosyltransferase 2.2; CDT, *Clostridium difficile* transferase; DHFR, dihydrofolate reductase; HCV NS3, hepatitis C virus non-structural protein 3; NA, not applicable; ND, not determined; NOR, nitric oxide reductase; SBE-A, starch branching enzyme A.

A second line of evidence clearly supporting distinct specificities of sdAbs *vs.* conventional antibodies can be found in studies of sdAbs against pathogenic microorganisms. Stijlemans *et al.*[68] hypothesized that the ability of a dromedary $V_HH$, cAb-An33, to target a cryptic glycopeptide epitope conserved across all variant surface glycoprotein classes of *Trypanosoma brucei* was due to the $V_HH$'s small size as well as, potentially, the nature of this epitope. This hypothesis was supported by the inability of rabbit and dromedary polyclonal conventional antibodies as well as a ~90-kDa lectin to access this site. Henderson *et al.*[69] suggested that recognition of a conserved hydrophobic cleft on *Plasmodium* AMA1 by a $V_{NAR}$ (12Y-2 and its affinity-matured variants) reflected a novel binding mode; although the epitope of a murine conventional antibody (1F9) substantially overlapped that of $V_{NAR}$ 12Y-2, 1F9 binding depended to a greater degree on polymorphic loop residues surrounding the hydrophobic trough. Likewise, Ditlev *et al.*[70] attributed the binding of a panel of alpaca $V_HHs$ to multiple domains of the malarial VAR2CSA protein to an inherent ability of $V_HHs$ to recognize subdominant epitopes, although limited understanding of the human conventional antibody response against VAR2CSA as well as irreproducibility of these reactivity patterns by llama $V_HHs$[71] complicated this assessment.

Probably the clearest examples of epitopes that are more favorable for binding by sdAbs than conventional antibodies can be found in the envelope glycoprotein trimer of HIV-1: heterologous cross-strain neutralization is extraordinarily difficult to achieve by conventional antibodies, requiring months of chronic infection and multiple rounds of somatic mutation and selection, yet cross-neutralizing camelid heavy chain-only antibodies directed against the CD4-binding site[72-75] and CD4-induced sites[76-78] can be easily elicited by routine immunizations with recombinant protein antigens. Similar examples can be found for other viral pathogens. Serotype cross-neutralizing antibodies targeting the CD155-binding 'canyon' of the poliovirus capsid are rarely produced by the murine or human humoral immune systems,[79,80] but are apparently common in llama heavy-chain only responses.[81] Likewise, $V_HHs$ targeting the HBGA-binding pocket of norovirus VP1 neutralized a broad range of genotypes,[82] while larger conventional antibodies also made contact with antigenically variable residues surrounding the HBGA pocket and were thus strain-specific.[83]

Finally, compared with conventional antibodies, sdAbs have been implied to have privileged access to recessed sites on membrane proteins,[84] such as ion channels and G protein-coupled receptors (**GPCRs**). While this is an intriguing hypothesis, it has yet to be substantiated by any data. Camelid $V_HHs$ generated against the Kv1.3 ion channel targeted extracellular loops, not the channel cavity,[85] and the epitopes of $V_HHs$ against the P2X7 ion channel were not defined.[86] Similarly, camelid $V_HHs$ developed as potential therapeutics against the chemokine receptors CXCR4,[84] CXCR7[87] and ChemR23,[88] as well as $V_HHs$ used as crystallization chaperones for several GPCRs, channels and

transporters,[89–95] all appear to bind solvent-exposed extracellular or intracellular loops of these receptors in a manner similar to conventional antibodies and their fragments. By contrast, a synthetic CXCR4-binding "i-body" engineered from an Ig-like NCAM domain was found to penetrate deep into the receptor's ligand-binding pocket to occupy a truly cryptic, partially transmembrane epitope.[96] Thus, there is at least some reason to believe that the small size of sdAbs may grant them access to recessed regions on pores and channels, although experimental evidence is still lacking.

Overall, the evidence is compelling that camelid $V_HHs$, at least, can interact with recessed epitopes on proteins that are poorly available for binding by conventional antibodies. Additional examples of binding to recessed epitopes on proteins (clefts, cavities, crevices or grooves) can be found for sdAbs against lactococcal siphophage,[97] *Plasmodium falciparum* MTIP,[98] epidermal growth factor receptor,[99] and respiratory syncytial virus fusion protein,[100] although in these cases it is less clear that these sites are inaccessible to conventional antibodies. While it is possible that $V_{NAR}s$ may share similar cleft-binding proclivities, such claims are based on very limited published data (three structures[7,12,69]). Moreover, it should be noted that there are many examples (not covered in this review) of partial or complete overlap between the epitopes of sdAbs and conventional antibodies, and thus the degree to which sdAbs bind cryptic epitopes *vs.* conventional antibody-accessible epitopes, as well as whether the magnitude of this difference exceeds more general species-to-species reactivity differences of conventional antibodies, remain unknown.

## Single-domain antibodies direct against linear protein epitopes

It is generally recognized that the majority of conventional antibodies raised against folded proteins are directed against conformational epitopes (≥90%[101]), although this may depend to some extent on the nature of the antigen. Several authors have suggested that $V_HHs$, at least, are even less likely than conventional antibodies to bind linear peptides with high affinity.[102,103] Although this is a plausible hypothesis based on the typical structures of sdAb paratopes (see below), it has not yet been substantiated by any data. Moreover, the relatively large number of studies reporting sdAb reactivity by western blotting suggests that sdAbs directed against continuous epitopes are probably not vanishingly rare.

## Single-domain antibody paratope structures

The paratopes of conventional antibodies directed against folded proteins tend to be flat or concave;[104] convex binding sites are difficult to achieve, at least by murine and human conventional antibodies, although synthetic conventional antibodies can be engineered to adopt such geometries.[105] By contrast, sdAb paratopes can clearly adopt both flat[106,107] and convex[11] topologies, although possibly only inefficiently adopt concave ones. The CDR1 and CDR2 loops of $V_HHs$ depart from the typical canonical structures of conventional antibodies (Figure 2A), potentially through somatic mutation since germline human $V_H$ and camelid $V_HH$ repertoires appear to have similar canonical structures.[18] Only a handful of $V_{NAR}s$ have been crystallized, and several showed a structural class of CDR1 (H1-13–9) that is more common in $V_HHs$ than in conventional antibodies, although others had CDR1 canonical structures closer to those of $V_L$ domains. The CDR3 length distributions of both $V_HHs$ and $V_{NAR}s$ (Figure 2B) are broader than those of conventional antibodies and biased towards longer lengths; the long CDR loops of sdAbs may be structurally constrained by non-canonical disulfide linkages (see Box 2). Despite potentially elevated somatic mutation rates (at least of $V_HHs$), the paratopes of $V_HHs$, $V_{NAR}s$ and conventional antibodies have similar amino acid contents, all being enriched for Gly, Ser and Tyr, and their CDR sequences bear no obvious patterns of sequence homology (Figure 2C, D). Both $V_HH$ and $V_{NAR}$ paratopes have smaller molecular surface areas and smaller diameters than conventional antibodies (Figure 2E, F). However, sdAb paratopes as a group are not more globally extended than those of conventional antibodies, as reflected by the maximum interatomic distance between the tips and the bases of any CDR loop (Figure 2G).

## Single-domain antibody:antigen interactions

The footprints of sdAbs on antigens are smaller than those of conventional antibodies, given that the paratopes of the former molecules are roughly half the size of the latter ones. Using only three CDR loops (two CDR loops and potentially two HV loops for $V_{NAR}s$), sdAbs can bury similar solvent-accessible surface areas on proteins compared with conventional antibodies (Figure 3A). This is made possible by a number of molecular contacts (hydrogen bonds, salt bridges) that is slightly lower for sdAbs than in conventional antibodies, but higher on a per-chain basis (Figure 3B, 3C). Moreover, the surface complementarity of sdAb:protein interfaces is on the high end for antibody:antigen interactions (Figure 3D). Thus, sdAbs and conventional antibodies bind protein antigens through similar types of non-covalent interactions, but these are more concentrated in the smaller paratopes of sdAbs.

## Single-domain antibodies directed against small molecules

The dominant mechanism by which conventional antibodies interact with haptens, small-molecule lipids and oligosaccharides is by forming a binding pocket at the interface between the $V_H$ and $V_L$ domains, typically involving the bases of the CDR-H3 and CDR-L3 loops.[113–115] Similarly, conventional antibodies tend to accommodate short linear peptides and nucleic acid polymers within grooves formed from both heavy- and light-chain CDRs.[104] Four studies have reported structures of camelid $V_HHs$ in complex with haptens and peptides (Table 2); the recognition mechanism of all but one (a methotrexate-specific $V_HH$ with a non-canonical binding site involving framework region (FR)3 residues located below CDR1[113]) was basically similar to that of conventional antibodies, with the hapten-binding pocket formed from two or more CDRs and extending in some instances into the former $V_L$ interface. Notably, three of these $V_HHs$ have non-

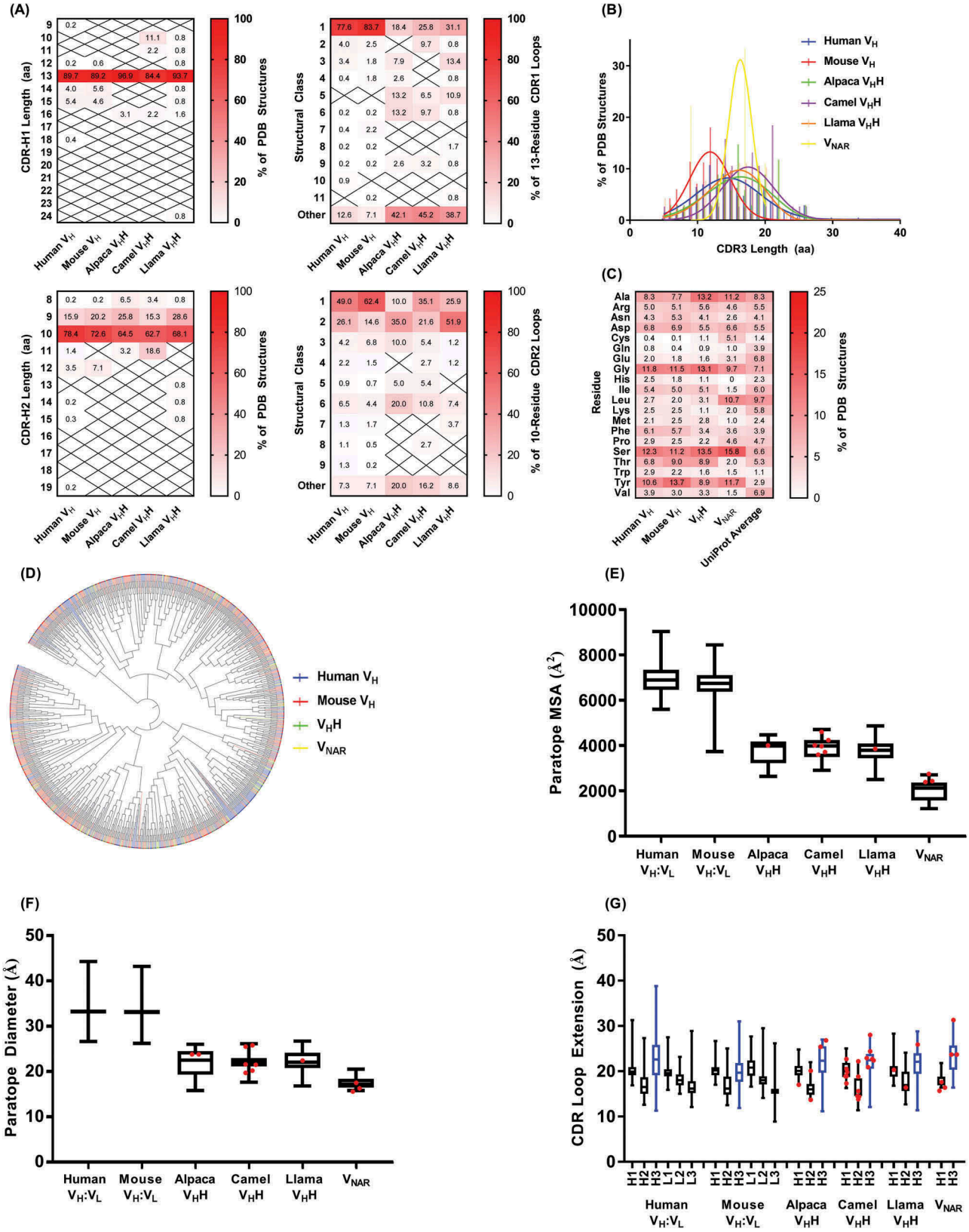


**Fig 2.** Properties of sdAb *vs.* conventional antibody paratopes. **(A)** Structural classification of CDR1 and CDR2 according to PyIgClassify.[108] **(B)** CDR3 length distributions. **(C)** Amino acid compositions of conventional antibody ($V_H$ domain) and sdAb paratopes. For $V_HH$s, sequences of CDR1, CDR2 and CDR3 (Honegger-Plückthun numbering) were used and for $V_{NAR}$s, sequences of CDR1 and CDR3 only were used. **(D)** Relatedness of conventional antibody ($V_H$ domain) and sdAb CDR3 sequences. The phylogenetic tree was produced using neighbor-joining methods in ClustalW2 and the cladogram was visualized using iTOL[109] with CDR3s colored according to species origin as in part B. **(E)** Molecular surface areas of conventional antibody ($V_H$:$V_L$) and sdAb paratopes. Areas were calculated for merged CDR sequences (Honegger-Plückthun numbering) using PyMol. **(F)** Diameters of conventional antibody ($V_H$:$V_L$) and sdAb paratopes. Diameters were calculated as the maximum interatomic distance between any two FR-CDR boundary residues (Honegger-Plückthun numbering). **(G)** Extension of conventional antibody ($V_H$:$V_L$) and sdAb paratopes. Extension was calculated as the maximum interatomic distance between the CDR base (first or last residue according to Honegger-Plückthun numbering) and the CDR tip. The CDR(H)3 loop is shown in blue. In parts **(E)** – **(G)**, boxplot lines represent medians, the box boundaries represent quartiles and the box whiskers represent ranges. Red dots indicate sdAbs targeting cryptic epitopes discussed in the main text. Data are representative of all complete antibody structures available in the Protein Data Bank and indexed in PyIgClassify as of January 2018.

**Box 2.** Non-canonical disulfide linkages of sdAbs

Some but not all camelid $V_HH$s bear paired cysteine residues, resulting in formation of a second intradomain disulfide linkage in addition to the conserved Cys23-Cys104 linkage (IMGT numbering) present in all Ig domains. Non-canonical disulfide linkages most commonly bridge Cys residues in CDR1 and CDR3,[20,21,24] but can also link FR2 and CDR3,[21,22] CDR2 and CDR3,[23] or two positions within the CDR3 loop.[19] The Cys residues in CDR1 are encoded by germline $V_HH$ genes that are frequently used in the repertoires of dromedary camels, and B cells using these genes presumably acquire a partner Cys during receptor rearrangement. Two hypotheses have been invoked to explain the presence of non-canonical disulfide linkages in $V_HH$ domains: they may impart greater stability to the $V_HH$ fold and/or restrict the conformational flexibility of long CDR3 loops, potentially minimizing entropic penalties for antigen binding. However, mutagenesis studies have showed that Cys residues forming non-canonical disulfide linkages can be replaced with a spectrum of other residues with only modest impairment of antigen binding affinity and thermal stability.[110]

Most cartilaginous fish $V_{NAR}$s bear an additional non-canonical disulfide linkage spanning either FR2-CDR3 (type I) or CDR1-CDR3 (types II and III[16]). In addition, type I $V_{NAR}$s also bear a CDR3-FR4 disulfide linkage and, sometimes, an intra-CDR3 disulfide linkage (three or four intradomain disulfide linkages in total[27]). A minority of $V_{NAR}$s (type IV) bear only the single canonical disulfide linkage. As for $V_HH$s, most $V_{NAR}$ Cys residues in CDR1, FR2 and FR4 are probably encoded in the germline and non-canonical disulfide linkages are formed during primary repertoire development[4,27,28]

Although the precise roles of non-canonical disulfide linkages in sdAb structure and function remain unclear, these linkages very likely influence sdAb paratope structure, since patterns of antigen-driven somatic hypermutation appear to vary depending on their presence and location.[27]

canonical structures of either CDR1 or CDR2 that have not been observed in structures of other $V_HH$s and may not be germline-encoded.

Multiple studies have reported the isolation of hapten-specific $V_HH$s without investigating their structures,[119] although several also reported weaker and inconsistent serum heavy chain-only IgG titers compared with conventional IgG titers against the hapten. No studies have reported hapten-specific $V_{NAR}$s, and only one study has described a carbohydrate-specific $V_HH$ directed against *Neisseira meningitidis* lipopolysaccharide;[120] at least two camelid $V_HH$s have been described that bind to glycopeptide epitopes.[68,76] No sdAbs of any type have been described that convincingly bind lipids or nucleic acids. Together, the consensus of the data is that it is probably difficult, but not impossible, for sdAb paratopes to accommodate haptens and that three CDRs are sufficient to form the binding pockets and grooves required for such interactions, although potential involvement of solubility-enhancing FR2 residues at the former $V_L$ interface in pocket formation may impose restrictions on hapten-binding specificities.

## Synthetic single-domain antibodies and non-antibody scaffolds

Fully synthetic sdAbs, derived from $V_HH$s, $V_{NAR}$s or from rare human and murine $V_H$ and $V_L$ domains that remain stable and soluble outside the context of the natural $V_H$:$V_L$ pairing, can be engineered to bind antigens using *in vitro* methods (*e.g.*, phage display). More recently, technologies have been developed for generating semi-synthetic sdAbs using engineered cell lines capable of inducible V(D)J recombination[121] and transgenic mice bearing either hybrid llama-human or fully human *igh* loci;[122] in both cases, a limited set of $V_H$, D and $J_H$ genes (some of which are in non-germline configurations to promote autonomous folding) are rearranged in a foreign cellular or *in vivo* system. Limited numbers of synthetic sdAbs have been described and fewer still have been studied structurally in complex with antigens. Nevertheless, the available data suggests that some synthetic sdAbs have cleft-binding properties akin to those of $V_HH$s and $V_{NAR}$s[51] while others employ unusual mechanisms to interact with planar protein epitopes (*e.g.*, dramatic CDR3 restructuring of a MDM4-specific $V_H$ domain to accommodate packing against a hydrophobic helix;[123] significant involvement of FRs in binding of $V_H$s to vascular endothelial growth factor[124,125] and CD40[126] using distinct mechanisms). Even less is known regarding the paratope structures and binding modes of non-Ig-based antibodies such as variable lymphocyte receptors[127] and non-antibody scaffolds (based on monomeric non-Ig domains such as fibronectin type III and SRC homology 3 domains), and their synthetic origin may imply that they follow no general patterns. If so, restrictions on the binding specificities of naturally-occurring sdAbs may not equally affect synthetic sdAbs and non-antibody scaffolds, although fundamental structural constraints on the amino acid sequences that can be tolerated by stable Ig folds would still apply.

## Conclusions and perspectives

Recent work on unusual antibodies produced by unorthodox model organisms (*e.g.*, cows, chickens) has spurred renewed interest in the comparative immunology of antibody responses. Some 'cryptic' regions on proteins (*e.g.*, enzyme active sites, recessed regions of viral glycoproteins) are clearly more accessible to sdAbs than to conventional antibodies. More generally, we surmise that the major advantage of sdAb recognition is the ability to target conserved cleft and pocket regions (typically binding sites) on hypervariable pathogens without making ancillary contact with the easily mutable perimeters of these sites. Why and how pathogen selection produced two evolutionarily-unrelated sdAb systems in sharks and camelids, but not in other organisms, remains to be clarified. In the case of sdAbs, privileged access is conferred by their compact paratope diameters (in the absence of a paired $V_L$ domain) rather than any global difference in paratope shape or structure. Similar non-covalent interactions mediate the binding of conventional antibodies and sdAbs, although these are more efficiently concentrated in the compact paratopes of sdAbs to produce high-affinity interactions. Although it is likely that sdAb paratopes have difficulty adopting concave geometries and recognizing small molecules, it remains unclear whether such paratope restrictions disfavor interaction with certain types of protein epitopes as well.

Future studies will need to rigorously assess the degree of separation and overlap in the protein epitope space

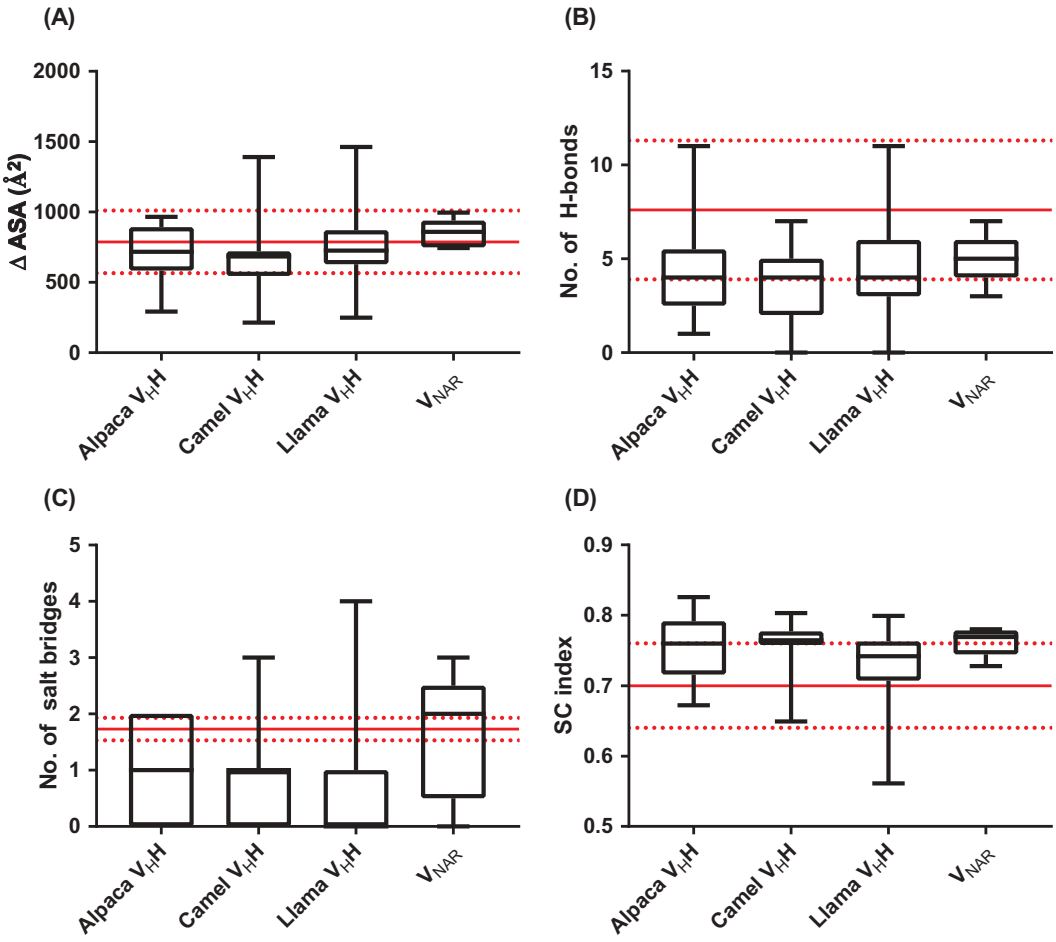


**Figure 3.** Properties of sdAb:antigen and conventional antibody:antigen interfaces. **(A)** Change in solvent-accessible surface area on proteins upon binding by conventional antibodies or sdAbs. **(B)** Number of hydrogen bonds and **(C)** number of salt bridges in conventional antibody:antigen and sdAb:antigen interfaces. **(D)** Shape complementarity index of conventional antibody:protein and sdAb:protein interfaces. Results in parts **(A)** – **(C)** were calculated using the PISA server, and in part **(D)** using the SC algorithm implemented in CCP4. Boxplot lines represent medians, the box boundaries represent quartiles and the box whiskers represent ranges. Red lines indicate the means and standard deviations for conventional antibodies.[111,112] Data are representative of all complete antibody:antigen co-crystal structures available in the Protein Data Bank and indexed in PyIgClassify as of January 2018.

**Table 2.** Structural features of anti-hapten sdAb paratopes.

| Antigen | sdAb Type | CDR3 Length (aa) | CDR1/2 Canonical Structures | Paratope MSA (Å$^2$) | Paratope Diameter (Å) | CDR Loop Extension (Å) | Reference |
|---|---|---|---|---|---|---|---|
| Reactive Red 6 | $V_HH$ | 17 | H1-13–1, H2-15–1 | 4380 | 14.7 | 17.8 (CDR1), 22.8 (CDR2), 22.3 (CDR3) | [116] |
| Reactive Red 1 | $V_HH$ | 18 | H1-16–1, H2-10–2 | 4242 | 15.0 | 21.8 (CDR1), 22.3 (CDR2), 22.9 (CDR3) | [117] |
| Methotrexate | $V_HH$ | 17 | H1-13–11, H2-10–2 | 3866 | 14.7 | 22.4 (CDR1), 17.6 (CDR2), 21.0 (CDR3) | [113] |
| DYEPEA peptide | $V_HH$ | 18 | H1-13–11, H2-11-* | 3698 | 14.8 | 16.3 (CDR1), 16.6 (CDR2), 21.8 (CDR3) | [118] |

targeted by sdAbs *vs.* conventional antibodies, and to explore whether sdAb-accessible (and inaccessible) epitopes can be predicted *in silico*. Basic studies of the immunological functions of conventional *vs.* heavy chain-only antibodies in host defense (*e.g.*, neutralization; opsonization; antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity) would also be highly valuable. Given the apparent sufficiency of sdAb paratopes to mediate high-affinity interactions with proteins, both the evolutionary forces responsible for shaping the more complex paired $V_H$:$V_L$ antibody system in vertebrates, as well as the overall functions of light chains, are open questions.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

Funding for this work was provided by the National Research Council Canada.

## Abbreviations

CDR complementarity-determining region
FR framework region
GPCR G protein-coupled receptor

| | |
|---|---|
| HV | hypervariable |
| Ig | immunoglobulin |
| sdAb | single-domain antibody |
| $V_H$ | variable heavy chain domain of conventional antibody |
| $V_HH$ | variable heavy chain domain of camelid heavy chain-only antibody |
| $V_L$ | variable light chain domain of conventional antibody |
| $V_{NAR}$ | variable domain of shark immunoglobulin new antigen receptor. |

## References


1. Flajnik MF, Deschacht N, Muyldermans S. A case of convergence: why did a simple alternative to canonical antibodies arise in sharks and camels? PLoS Biol. 2011;9:e1001120. doi:10.1371/journal.pbio.1001120.
2. Ward ES, Gussow D, Griffiths AD, Jones PT, Winter G. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature. 1989;341:544–546. doi:10.1038/341544a0.
3. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, Bendahman N, Hamers R. Naturally occurring antibodies devoid of light chains. Nature. 1993;363:446–448. doi:10.1038/363446a0.
4. Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature. 1995;374:168–173. doi:10.1038/374168a0.
5. Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. Nat Biotechnol. 2005;23:1126–1136. doi:10.1038/nbt1142.
6. Desmyter A, Transue TR, Ghahroudi MA, Thi MH, Poortmans F, Hamers R, Muyldermans S, Wyns L. Crystal structure of a camel single-domain $V_H$ antibody fragment in complex with lysozyme. Nat Struct Biol. 1996;3:803–811.
7. Stanfield RL, Dooley H, Flajnik MF, Wilson IA. Crystal structure of a shark single-domain antibody V region in complex with lysozyme. Science. 2004;305:1770–1773. doi:10.1126/science.1101148.
8. Achour I, Cavelier P, Tichit M, Bouchier C, Lafaye P, Rougeon F. Tetrameric and homodimeric camelid IgGs originate from the same *igh* locus. J Immunol. 2008;181:2001–2009.
9. Eason DD, Litman RT, Luer CA, Kerr W, Litman GW. Expression of individual immunoglobulin genes occurs in an unusual system consisting of multiple independent loci. Eur J Immunol. 2004;34:2551–2558. doi:10.1002/eji.200425224.
10. Griffin LM, Snowden JR, Lawson AD, Wernery U, Kinne J, Baker TS. Analysis of heavy and light chain sequences of conventional camelid antibodies from *Camelus dromedarius* and *Camelus bactrianus* species. J Immunol Methods. 2014;405:35–46. doi:10.1016/j.jim.2014.01.003.
11. De Genst E, Silence K, Decanniere K, Conrath K, Loris R, Kinne J, Muyldermans S, Wyns L. Molecular basis for the preferential cleft recognition by dromedary heavy-chain antibodies. Proc Natl Acad Sci U S A. 2006;103:4586–4591. doi:10.1073/pnas.0505379103.
12. Stanfield RL, Dooley H, Verdino P, Flajnik MF, Wilson IA. Maturation of shark single-domain (IgNAR) antibodies: evidence for induced-fit binding. J Mol Biol. 2007;367:358–372. doi:10.1016/j.jmb.2006.12.045.
13. Al Qaraghuli MM, Ferro VA. Analysis of the binding loops configuration and surface adaptation of different crystallized single-domain antibodies in response to various antigens. J Mol Recognit. 2017;30:e2592. doi:10.1002/jmr.2592.
14. Konning D, Zielonka S, Grzeschik J, Empting M, Valldorf B, Krah S, Schroter C, Sellmann C, Hock B, Kolmar H. Camelid and shark single domain antibodies: structural features and therapeutic potential. Curr Opin Struct Biol. 2017;45:10–16. doi:10.1016/j.sbi.2016.10.019.
15. Muyldermans S, Smider VV. Distinct antibody species: structural differences creating therapeutic opportunities. Curr Opin Immunol. 2016;40:7–13. doi:10.1016/j.coi.2016.02.003.
16. Zielonka S, Empting M, Grzeschik J, Konning D, Barelle CJ, Kolmar H. Structural insights and biomedical potential of IgNAR scaffolds from sharks. MAbs. 2015;7:15–25. doi:10.4161/19420862.2015.989032.
17. Desmyter A, Spinelli S, Roussel A, Cambillau C. Camelid nanobodies: killing two birds with one stone. Curr Opin Struct Biol. 2015;32:1–8. doi:10.1016/j.sbi.2015.01.001.
18. Klarenbeek A, El Mazouari K, Desmyter A, Blanchetot C, Hultberg A, De Jonge N, Roovers RC, Cambillau C, Spinelli S, Del-Favero J, et al. Camelid Ig V genes reveal significant human homology not seen in therapeutic target genes, providing for a powerful therapeutic antibody platform. MAbs. 2015;7:693–706. doi:10.1080/19420862.2015.1046648.
19. Deschacht N, De Groeve K, Vincke C, Raes G, De Baetselier P, Muyldermans S. A novel promiscuous class of camelid single-domain antibody contributes to the antigen-binding repertoire. J Immunol. 2010;184:5696–5704. doi:10.4049/jimmunol.0903722.
20. Li X, Duan X, Yang K, Zhang W, Zhang C, Fu L, Ren Z, Wang C, Wu J, Lu R, et al. Comparative analysis of immune repertoires between bactrian camel's conventional and heavy-chain antibodies. PLoS One. 2016;11:e0161801. doi:10.1371/journal.pone.0161801.
21. Harmsen MM, Ruuls RC, Nijman IJ, Niewold TA, Frenken LG, De Geus B. Llama heavy-chain V regions consist of at least four distinct subfamilies revealing novel sequence features. Mol Immunol. 2000;37:579–590.
22. Muyldermans S, Atarhouch T, Saldanha J, Barbosa JA, Hamers R. Sequence and structure of $V_H$ domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. Protein Eng. 1994;7:1129–1135.
23. Vu KB, Ghahroudi MA, Wyns L, Muyldermans S. Comparison of llama $V_H$ sequences from conventional and heavy chain antibodies. Mol Immunol. 1997;34:1121–1131.
24. Nguyen VK, Hamers R, Wyns L, Muyldermans S. Camel heavy-chain antibodies: diverse germline $V_HH$ and specific mechanisms enlarge the antigen-binding repertoire. EMBO J. 2000;19:921–930. doi:10.1093/emboj/19.5.921.
25. Nguyen VK, Su C, Muyldermans S. van der Loo W. Heavy-chain antibodies in *Camelidae*; a case of evolutionary innovation. Immunogenetics. 2002;54:39–47. doi:10.1007/s00251-002-0433-0.
26. Streltsov VA, Varghese JN, Carmichael JA, Irving RA, Hudson PJ, Nuttall SD. Structural evidence for evolution of shark Ig new antigen receptor variable domain antibodies from a cell-surface receptor. Proc Natl Acad Sci U S A. 2004;101:12444–12449. doi:10.1073/pnas.0403509101.
27. Diaz M, Stanfield RL, Greenberg AS, Flajnik MF. Structural analysis, selection, and ontogeny of the shark new antigen receptor (IgNAR): identification of a new locus preferentially expressed in early development. Immunogenetics. 2002;54:501–512. doi:10.1007/s00251-002-0479-z.
28. Diaz M, Greenberg AS, Flajnik MF. Somatic hypermutation of the new antigen receptor gene (NAR) in the nurse shark does not generate the repertoire: possible role in antigen-driven reactions in the absence of germinal centers. Proc Natl Acad Sci U S A. 1998;95:14343–14348.
29. Abdiche YN, Harriman R, Deng X, Yeung YA, Miles A, Morishige W, Boustany L, Zhu L, Izquierdo SM, Harriman W. Assessing kinetic and epitopic diversity across orthogonal monoclonal antibody generation platforms. MAbs. 2016;8:264–277. doi:10.1080/19420862.2015.1118596.
30. Pinto J, Odongo S, Lee F, Gaspariunaite V, Muyldermans S, Magez S, Sterckx YG. Structural basis for the high specificity of a Trypanosoma congolense immunoassay targeting glycosomal aldolase. PLoS Negl Trop Dis. 2017;11:e0005932. doi:10.1371/journal.pntd.0005932.
31. Desmyter A, Spinelli S, Payan F, Lauwereys M, Wyns L, Muyldermans S, Cambillau C. Three camelid $V_HH$ domains in complex with porcine pancreatic alpha-amylase. Inhibition and

versatility of binding topology. J Biol Chem. 2002;277:23645–23650. doi:10.1074/jbc.M202327200.
32. Lauwereys M, Arbabi Ghahroudi M, Desmyter A, Kinne J, Holzer W, De Genst E, Wyns L, Muyldermans S. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. EMBO J. 1998;17:3512–3520. doi:10.1093/emboj/17.13.3512.
33. Koch-Nolte F, Reyelt J, Schossow B, Schwarz N, Scheuplein F, Rothenburg S, Haag F, Alzogaray V, Cauerhff A, Goldbaum FA. Single domain antibodies from llama effectively and specifically block T cell ecto-ADP-ribosyltransferase ART2.2 in vivo. FASEB J. 2007;21:3490–3498. doi:10.1096/fj.07-8661com.
34. Burgess SG, Oleksy A, Cavazza T, Richards MW, Vernos I, Matthews D, Bayliss R. Allosteric inhibition of Aurora-A kinase by a synthetic $V_{NAR}$ domain. Open Biol. 2016;6:160089. doi:10.1098/rsob.160089.
35. Sohier JS, Laurent C, Chevigne A, Pardon E, Srinivasan V, Wernery U, Lassaux P, Steyaert J, Galleni M. Allosteric inhibition of VIM metallo-β-lactamases by a camelid nanobody. Biochem J. 2013;450:477–486. doi:10.1042/BJ20121305.
36. Conrath KE, Lauwereys M, Galleni M, Matagne A, Frere JM, Kinne J, Wyns L, Muyldermans S. β-lactamase inhibitors derived from single-domain antibody fragments elicited in the *Camelidae*. Antimicrob Agents Chemother. 2001;45:2807–2812. doi:10.1128/AAC.45.10.2807-2812.2001.
37. Dong J, Thompson AA, Fan Y, Lou J, Conrad F, Ho M, Pires-Alves M, Wilson BA, Stevens RC, Marks JD. A single-domain llama antibody potently inhibits the enzymatic activity of botulinum neurotoxin by binding to the non-catalytic alpha-exosite binding region. J Mol Biol. 2010;397:1106–1118. doi:10.1016/j.jmb.2010.01.070.
38. Thanongsaksrikul J, Srimanote P, Maneewatch S, Choowongkomon K, Tapchaisri P, Makino S, Kurazono H, Chaicumpa W. A $V_HH$ that neutralizes the zinc metalloproteinase activity of botulinum neurotoxin type A. J Biol Chem. 2010;285:9657–9666. doi:10.1074/jbc.M109.073163.
39. Desmyter A, Decanniere K, Muyldermans S, Wyns L. Antigen specificity and high affinity binding provided by one single loop of a camel single-domain antibody. J Biol Chem. 2001;276:26285–26290. doi:10.1074/jbc.M102107200.
40. Li T, Qi S, Unger M, Hou YN, Deng QW, Liu J, Lam CM, Wang XW, Xin D, Zhang P, et al. Immuno-targeting the multifunctional CD38 using nanobody. Sci Rep. 2016;6:27055. doi:10.1038/srep27055.
41. Fumey W, Koenigsdorf J, Kunick V, Menzel S, Schutze K, Unger M, Schriewer L, Haag F, Adam G, Oberle A, et al. Nanobodies effectively modulate the enzymatic activity of CD38 and allow specific imaging of $CD38^+$ tumors in mouse models *in vivo*. Sci Rep. 2017;7:14289. doi:10.1038/s41598-017-14112-6.
42. Unger M, Eichhoff AM, Schumacher L, Strysio M, Menzel S, Schwan C, Alzogaray V, Zylberman V, Seman M, Brandner J, et al. Selection of nanobodies that block the enzymatic and cytotoxic activities of the binary *Clostridium difficile* toxin CDT. Sci Rep. 2015;5:7850. doi:10.1038/srep07850.
43. Oyen D, Srinivasan V, Steyaert J, Barlow JN. Constraining enzyme conformational change by an antibody leads to hyperbolic inhibition. J Mol Biol. 2011;407:138–148. doi:10.1016/j.jmb.2011.01.017.
44. Zhu J, Declercq J, Roucourt B, Ghassabeh GH, Meulemans S, Kinne J, David G, Vermorken AJ, Van de Ven WJ, Lindberg I, et al. Generation and characterization of non-competitive furin-inhibiting nanobodies. Biochem J. 2012;448:73–82. doi:10.1042/BJ20120537.
45. Dahms SO, Creemers JW, Schaub Y, Bourenkov GP, Zogg T, Brandstetter H, Than ME. The structure of a furin-antibody complex explains non-competitive inhibition by steric exclusion of substrate conformers. Sci Rep. 2016;6:34303. doi:10.1038/srep34303.
46. Transue TR, De Genst E, Ghahroudi MA, Wyns L, Muyldermans S. Camel single-domain antibody inhibits enzyme by mimicking carbohydrate substrate. Proteins. 1998;32:515–522.
47. Chan PH, Pardon E, Menzer L, De Genst E, Kumita JR, Christodoulou J, Saerens D, Brans A, Bouillenne F, Archer DB, et al. Engineering a camelid antibody fragment that binds to the active site of human lysozyme and inhibits its conversion into amyloid fibrils. Biochemistry. 2008;47:11041–11054. doi:10.1021/bi8005797.
48. De Genst E, Silence K, Ghahroudi MA, Decanniere K, Loris R, Kinne J, Wyns L, Muyldermans S. Strong *in vivo* maturation compensates for structurally restricted H3 loops in antibody repertoires. J Biol Chem. 2005;280:14114–14121. doi:10.1074/jbc.M413011200.
49. De Genst E, Chan PH, Pardon E, Hsu SD, Kumita JR, Christodoulou J, Menzer L, Chirgadze DY, Robinson CV, Muyldermans S, et al. A nanobody binding to non-amyloidogenic regions of the protein human lysozyme enhances partial unfolding but inhibits amyloid fibril formation. J Phys Chem B. 2013;117:13245–13258. doi:10.1021/jp403425z.
50. Dumoulin M, Last AM, Desmyter A, Decanniere K, Canet D, Larsson G, Spencer A, Archer DB, Sasse J, Muyldermans S, et al. A camelid antibody fragment inhibits the formation of amyloid fibrils by human lysozyme. Nature. 2003;424:783–788. doi:10.1038/nature01870.
51. Rouet R, Dudgeon K, Christie M, Langley D, Christ D. Fully human $V_H$ single domains that rival the stability and cleft recognition of camelid antibodies. J Biol Chem. 2015;290:11905–11917. doi:10.1074/jbc.M114.614842.
52. Conrath K, Pereira AS, Martins CE, Timoteo CG, Tavares P, Spinelli S, Kinne J, Flaudrops C, Cambillau C, Muyldermans S, et al. Camelid nanobodies raised against an integral membrane enzyme, nitric oxide reductase. Protein Sci. 2009;18:619–628. doi:10.1002/pro.69.
53. Martin F, Volpari C, Steinkuhler C, Dimasi N, Brunetti M, Biasiol G, Altamura S, Cortese R, De Francesco R, Sollazzo M. Affinity selection of a camelized $V_H$ domain antibody inhibitor of hepatitis C virus NS3 protease. Protein Eng. 1997;10:607–614.
54. Perez C, Kohler M, Janser D, Pardon E, Steyaert J, Zenobi R, Locher KP. Structural basis of inhibition of lipid-linked oligosaccharide flippase PglK by a conformational nanobody. Sci Rep. 2017;7:46641. doi:10.1038/srep46641.
55. Rudolph MJ, Vance DJ, Cheung J, Franklin MC, Burshteyn F, Cassidy MS, Gary EN, Herrera C, Shoemaker CB, Mantis NJ. Crystal structures of ricin toxin's enzymatic subunit (RTA) in complex with neutralizing and non-neutralizing single-chain antibodies. J Mol Biol. 2014;426:3057–3068. doi:10.1016/j.jmb.2014.05.026.
56. Decanniere K, Desmyter A, Lauwereys M, Ghahroudi MA, Muyldermans S, Wyns L. A single-domain antibody fragment in complex with RNase A: non-canonical loop structures and nanomolar affinity using two CDR loops. Structure. 1999;7:361–370.
57. Jobling SA, Jarman C, Teh MM, Holmberg N, Blake C, Verhoeyen ME. Immunomodulation of enzyme function in plants by single-domain antibody fragments. Nat Biotechnol. 2003;21:77–80. doi:10.1038/nbt772.
58. Hendrickx ML, A DEW, Buelens K, Compernolle G, Hassanzadeh-Ghassabeh G, Muyldermans S, Gils A, Declerck PJ. TAFIa inhibiting nanobodies as profibrinolytic tools and discovery of a new TAFIa conformation. J Thromb Haemost. 2011;9:2268–2277. doi:10.1111/j.1538-7836.2011.04495.x.
59. Buelens K, Hassanzadeh-Ghassabeh G, Muyldermans S, Gils A, Declerck PJ. Generation and characterization of inhibitory nanobodies towards thrombin activatable fibrinolysis inhibitor. J Thromb Haemost. 2010;8:1302–1312. doi:10.1111/j.1538-7836.2010.03816.x.
60. Ratier L, Urrutia M, Paris G, Zarebski L, Frasch AC, Goldbaum FA. Relevance of the diversity among members of the *Trypanosoma cruzi* trans-sialidase family analyzed with camelids single-domain antibodies. PLoS One. 2008;3:e3524. doi:10.1371/journal.pone.0003524.
61. Ardekani LS, Gargari SL, Rasooli I, Bazl MR, Mohammadi M, Ebrahimizadeh W, Bakherad H, Zare H. A novel nanobody against urease activity of *Helicobacter pylori*. Int J Infect Dis. 2013;17:e723–8. doi:10.1016/j.ijid.2013.02.015.

62. Kromann-Hansen T, Louise Lange E, Peter Sorensen H, Hassanzadeh-Ghassabeh G, Huang M, Jensen JK, Muyldermans S, Declerck PJ, Komives EA, Andreasen PA. Discovery of a novel conformational equilibrium in urokinase-type plasminogen activator. Sci Rep. 2017;7:3385. doi:10.1038/s41598-017-03457-7.
63. Chan PH, Pardon E, Menzer L, De Genst E, Kumita JR, Christodoulou J, Saerens D, Brans A, Bouillenne F, Archer DB. A camelid-derived antibody fragment targeting the active site of a serine protease balances between inhibitor and substrate behavior. J Biol Chem. 2016;291:15156–15168. doi:10.1074/jbc.M116.732503.
64. Kaczmarek JZ, Skottrup PD. Selection and characterization of camelid nanobodies towards urokinase-type plasminogen activator. Mol Immunol. 2015;65:384–390. doi:10.1016/j.molimm.2015.02.011.
65. Arnon R. Enzyme inhibition by antibodies. Acta Endocrinol Suppl (Copenh). 1975;194:133–153.
66. Farady CJ, Egea PF, Schneider EL, Darragh MR, Craik CS. Structure of an Fab-protease complex reveals a highly specific non-canonical mechanism of inhibition. J Mol Biol. 2008;380:351–360. doi:10.1016/j.jmb.2008.05.009.
67. Wu Y, Eigenbrot C, Liang WC, Stawicki S, Shia S, Fan B, Ganesan R, Lipari MT, Kirchhofer D. Structural insight into distinct mechanisms of protease inhibition by antibodies. Proc Natl Acad Sci U S A. 2007;104:19784–19789. doi:10.1073/pnas.0708251104.
68. Stijlemans B, Conrath K, Cortez-Retamozo V, Van Xong H, Wyns L, Senter P, Revets H, De Baetselier P, Muyldermans S, Magez S. Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies: African trypanosomes as paradigm. J Biol Chem. 2004;279:1256–1261. doi:10.1074/jbc.M307341200.
69. Henderson KA, Streltsov VA, Coley AM, Dolezal O, Hudson PJ, Batchelor AH, Gupta A, Bai T, Murphy VJ, Anders RF, et al. Structure of an IgNAR-AMA1 complex: targeting a conserved hydrophobic cleft broadens malarial strain recognition. Structure. 2007;15:1452–1466. doi:10.1016/j.str.2007.09.011.
70. Ditlev SB, Florea R, Nielsen MA, Theander TG, Magez S, Boeuf P, Salanti A.. Utilizing nanobody technology to target non-immunodominant domains of VAR2CSA. PLoS One. 2014;9:e84981. doi:10.1371/journal.pone.0084981.
71. Nunes-Silva S, Gangnard S, Vidal M, Vuchelen A, Dechavanne S, Chan S, Pardon E, Steyaert J, Ramboarina S, Chene A, et al. Llama immunization with full-length VAR2CSA generates cross-reactive and inhibitory single-domain antibodies against the DBL1X domain. Sci Rep. 2014;4:7373. doi:10.1038/srep07373.
72. Forsman A, Beirnaert E, Aasa-Chapman MM, Hoorelbeke B, Hijazi K, Koh W, Tack V, Szynol A, Kelly C, McKnight A, et al. Llama antibody fragments with cross-subtype human immunodeficiency virus type 1 (HIV-1)-neutralizing properties and high affinity for HIV-1 gp120. J Virol. 2008;82:12069–12081.
73. McCoy LE, Quigley AF, Strokappe NM, Bulmer-Thomas B, Seaman MS, Mortier D, Rutten L, Chander N, Edwards CJ, Ketteler R, et al. Potent and broad neutralization of HIV-1 by a llama antibody elicited by immunization. J Exp Med. 2012;209:1091–1103. doi:10.1084/jem.20112655.
74. Strokappe N, Szynol A, Aasa-Chapman M, Gorlani A, Forsman Quigley A, Hulsik DL, Chen L, Weiss R, de Haard H, Verrips T. Llama antibody fragments recognizing various epitopes of the CD4bs neutralize a broad range of HIV-1 subtypes A, B and C. PLoS One. 2012;7:e33298. doi:10.1371/journal.pone.0033298.
75. McCoy LE, Rutten L, Frampton D, Anderson I, Granger L, Bashford-Rogers R, Dekkers G, Strokappe NM, Seaman MS, Koh W, et al. Molecular evolution of broadly neutralizing Llama antibodies to the CD4-binding site of HIV-1. PLoS Pathog. 2014;10:e1004552. doi:10.1371/journal.ppat.1004552.
76. Acharya P, Luongo TS, Georgiev IS, Matz J, Schmidt SD, Louder MK, Kessler P, Yang Y, McKee K, O'Dell S, et al. Heavy chain-only IgG2b llama antibody effects near-pan HIV-1 neutralization by recognizing a CD4-induced epitope that includes elements of coreceptor- and CD4-binding sites. J Virol. 2013;87:10173–10181. doi:10.1128/JVI.01332-13.
77. Matz J, Kessler P, Bouchet J, Combes O, Ramos OH, Barin F, Baty D, Martin L, Benichou S, Chames P. Straightforward selection of broadly neutralizing single-domain antibodies targeting the conserved CD4 and coreceptor binding sites of HIV-1 gp120. J Virol. 2013;87:1137–1149. doi:10.1128/JVI.00461-12.
78. Chen W, Zhu Z, Feng Y, Dimitrov DS. Human domain antibodies to conserved sterically restricted regions on gp120 as exceptionally potent cross-reactive HIV-1 neutralizers. Proc Natl Acad Sci U S A. 2008;105:17121–17126. doi:10.1073/pnas.0805297105.
79. Chen Z, Fischer ER, Kouiavskaia D, Hansen BT, Ludtke SJ, Bidzhieva B, Makiya M, Agulto L, Purcell RH, Chumakov K. Cross-neutralizing human anti-poliovirus antibodies bind the recognition site for cellular receptor. Proc Natl Acad Sci U S A. 2013;110:20242–20247. doi:10.1073/pnas.1320041110.
80. Puligedda RD, Kouiavskaia D, Adekar SP, Sharma R, Devi Kattala C, Rezapkin G, Bidzhieva B, Dessain SK, Chumakov K. Human monoclonal antibodies that neutralize vaccine and wild-type poliovirus strains. Antiviral Res. 2014;108:36–43. doi:10.1016/j.antiviral.2014.05.005.
81. Strauss M, Schotte L, Thys B, Filman DJ, Hogle JM. Five of five $V_HHs$ neutralizing poliovirus bind the receptor-binding site. J Virol. 2016;90:3496–3505. doi:10.1128/JVI.03017-15.
82. Koromyslova AD, Hansman GS. Nanobodies targeting norovirus capsid reveal functional epitopes and potential mechanisms of neutralization. PLoS Pathog. 2017;13:e1006636. doi:10.1371/journal.ppat.1006636.
83. Shanker S, Czako R, Sapparapu G, Alvarado G, Viskovska M, Sankaran B, Atmar RL, Crowe JE, Jr., Estes MK, Prasad BV. Structural basis for norovirus neutralization by an HBGA blocking human IgA antibody. Proc Natl Acad Sci U S A. 2016;113:E5830–E7. doi:10.1073/pnas.1609990113.
84. Jahnichen S, Blanchetot C, Maussang D, Gonzalez-Pajuelo M, Chow KY, Bosch L, De Vrieze S, Serruys B, Ulrichts H, Vandevelde W, et al. CXCR4 nanobodies ($V_HH$-based single variable domains) potently inhibit chemotaxis and HIV-1 replication and mobilize stem cells. Proc Natl Acad Sci U S A. 2010;107:20565–20570. doi:10.1073/pnas.1012865107.
85. Delanote V, Janssen D, Van Hoorick D. Characterization of anti-Kv1.3 Nanobodies® and activity in inflammatory model systems. Poster presented at 12th Annual Ion Channel Retreat. Vancouver, British Columbia, Canada, 2014.
86. Danquah W, Meyer-Schwesinger C, Rissiek B, Pinto C, Serracant-Prat A, Amadi M, Iacenda D, Knop JH, Hammel A, Bergmann P, et al. Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. Sci Transl Med. 2016;8:366ra162. doi:10.1126/scitranslmed.aaf0746.
87. Maussang D, Mujic-Delic A, Descamps FJ, Stortelers C, Vanlandschoot P, Stigter-van Walsum M, Vischer HF, van Roy M, Vosjan M, Gonzalez-Pajuelo M, et al. Llama-derived single variable domains (nanobodies) directed against chemokine receptor CXCR7 reduce head and neck cancer cell growth *in vivo*. J Biol Chem. 2013;288:29562–29572. doi:10.1074/jbc.M113.498436.
88. Peyrassol X, Laeremans T, Gouwy M, Lahura V, Debulpaep M, Van Damme J, Steyaert J, Parmentier M, Langer I. Development by genetic immunization of monovalent antibodies (nanobodies) behaving as antagonists of the human ChemR23 receptor. J Immunol. 2016;196:2893–2901. doi:10.4049/jimmunol.1500888.
89. Burg JS, Ingram JR, Venkatakrishnan AJ, Jude KM, Dukkipati A, Feinberg EN, Angelini A, Waghray D, Dror RO, Ploegh HL, et al. Structural biology. Structural basis for chemokine recognition and activation of a viral G protein-coupled receptor. Science. 2015;347:1113–1117. doi:10.1126/science.aaa5026.
90. Huang W, Manglik A, Venkatakrishnan AJ, Laeremans T, Feinberg EN, Sanborn AL, Kato HE, Livingston KE, Thorsen TS, Kling RC, et al. Structural insights into μ-opioid

receptor activation. Nature. 2015;524:315–321. doi:10.1038/nature14886.
91. Hassaine G, Deluz C, Grasso L, Wyss R, Tol MB, Hovius R, Graff A, Stahlberg H, Tomizaki T, Desmyter A, et al. X-ray structure of the mouse serotonin 5-HT3 receptor. Nature. 2014;512:276–281. doi:10.1038/nature13552.
92. Ehrnstorfer IA, Geertsma ER, Pardon E, Steyaert J, Dutzler R. Crystal structure of a SLC11 (NRAMP) transporter reveals the basis for transition-metal ion transport. Nat Struct Mol Biol. 2014;21:990–996. doi:10.1038/nsmb.2904.
93. Kruse AC, Ring AM, Manglik A, Hu J, Hu K, Eitel K, Hubner H, Pardon E, Valant C, Sexton PM, et al. Activation and allosteric modulation of a muscarinic acetylcholine receptor. Nature. 2013;504:101–106. doi:10.1038/nature12735.
94. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, et al. Crystal structure of the $\beta_2$ adrenergic receptor-Gs protein complex. Nature. 2011;477:549–555. doi:10.1038/nature10361.
95. Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, et al. Structure of a nanobody-stabilized active state of the $\beta_2$ adrenoceptor. Nature. 2011;469:175–180. doi:10.1038/nature09648.
96. Griffiths K, Dolezal O, Cao B, Nilsson SK, See HB, Pfleger KD, Roche M, Gorry PR, Pow A, Viduka K, et al. i-bodies, human single domain antibodies that antagonize chemokine receptor CXCR4. J Biol Chem. 2016;291:12641–12657. doi:10.1074/jbc.M116.721050.
97. Desmyter A, Farenc C, Mahony J, Spinelli S, Bebeacua C, Blangy S, Veesler D, van Sinderen D, Cambillau C. Viral infection modulation and neutralization by camelid nanobodies. Proc Natl Acad Sci U S A. 2013;110:E1371–9. doi:10.1073/pnas.1301336110.
98. Khamrui S, Turley S, Pardon E, Steyaert J, Fan E, Verlinde CL, Bergman LW, Hol WG. The structure of the D3 domain of *Plasmodium falciparum* myosin tail interacting protein MTIP in complex with a nanobody. Mol Biochem Parasitol. 2013;190:87–91. doi:10.1016/j.molbiopara.2013.06.003.
99. Schmitz KR, Bagchi A, Roovers RC, Van Bergen En Henegouwen PM, Km F. Structural evaluation of EGFR inhibition mechanisms for nanobodies/$V_HH$ domains. Structure. 2013;21:1214–1224. doi:10.1016/j.str.2013.05.008.
100. Rossey I, Gilman MS, Kabeche SC, Sedeyn K, Wrapp D, Kanekiyo M, Chen M, Mas V, Spitaels J, Melero JA, et al. Potent single-domain antibodies that arrest respiratory syncytial virus fusion protein in its prefusion state. Nat Commun. 2017;8:14158. doi:10.1038/ncomms14158.
101. Rubinstein ND, Mayrose I, Halperin D, Yekutieli D, Gershoni JM, Pupko T. Computational characterization of B-cell epitopes. Mol Immunol. 2008;45:3477–3489. doi:10.1016/j.molimm.2007.10.016.
102. Dmitriev OY, Lutsenko S, Muyldermans S. Nanobodies as Probes for Protein Dynamics in Vitro and in Cells. J Biol Chem. 2016;291:3767–3775. doi:10.1074/jbc.R115.679811.
103. Pardon E, Laeremans T, Triest S, Rasmussen SG, Wohlkonig A, Ruf A, Muyldermans S, Hol WG, Kobilka BK, Steyaert J. A general protocol for the generation of nanobodies for structural biology. Nat Protoc. 2014;9:674–693. doi:10.1038/nprot.2014.039.
104. MacCallum RM, Martin AC, Thornton JM. Antibody-antigen interactions: contact analysis and binding site topography. J Mol Biol. 1996;262:732–745. doi:10.1006/jmbi.1996.0548.
105. Nam DH, Rodriguez C, Remacle AG, Strongin AY, Ge X. Active-site MMP-selective antibody inhibitors discovered from convex paratope synthetic libraries. Proc Natl Acad Sci U S A. 2016;113:14970–14975. doi:10.1073/pnas.1609375114.
106. Kirchhofer A, Helma J, Schmidthals K, Frauer C, Cui S, Karcher A, Pellis M, Muyldermans S, Casas-Delucchi CS, Cardoso MC, et al. Modulation of protein properties in living cells using nanobodies. Nat Struct Mol Biol. 2010;17:133–138. doi:10.1038/nsmb.1727.
107. Chaikuad A, Keates T, Vincke C, Kaufholz M, Zenn M, Zimmermann B, Gutierrez C, Zhang RG, Hatzos-Skintges C, Joachimiak A, et al. Structure of cyclin G-associated kinase (GAK) trapped in different conformations using nanobodies. Biochem J. 2014;459:59–69. doi:10.1042/BJ20131399.
108. Adolf-Bryfogle J, Xu Q, North B, Lehmann A, Dunbrack RL Jr. PyIgClassify: a database of antibody CDR structural classifications. Nucleic Acids Res. 2015;43:D432–8. doi:10.1093/nar/gku1106.
109. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics. 2007;23:127–128. doi:10.1093/bioinformatics/btl529.
110. Govaert J, Pellis M, Deschacht N, Vincke C, Conrath K, Muyldermans S, Saerens D. Dual beneficial effect of interloop disulfide bond for single domain antibody fragments. J Biol Chem. 2012;287:1970–1979. doi:10.1074/jbc.M111.242818.
111. MacRaild CA, Richards JS, Anders RF, Norton RS. Antibody recognition of disordered antigens. Structure. 2016;24:148–157. doi:10.1016/j.str.2015.10.028.
112. Kuroda D, Gray JJ. Shape complementarity and hydrogen bond preferences in protein-protein interfaces: implications for antibody modeling and protein-protein docking. Bioinformatics. 2016;32:2451–2456. doi:10.1093/bioinformatics/btw197.
113. Fanning SW, Horn JR. An anti-hapten camelid antibody reveals a cryptic binding site with significant energetic contributions from a nonhypervariable loop. Protein Sci. 2011;20:1196–1207. doi:10.1002/pro.648.
114. Haji-Ghassemi O, Blackler RJ, Martin Young N, Evans SV. Antibody recognition of carbohydrate epitopes. Glycobiology. 2015;25:920–952. doi:10.1093/glycob/cwv037.
115. Fleming JK, Wojciak JM, Campbell MA, Huxford T. Biochemical and structural characterization of lysophosphatidic acid binding by a humanized monoclonal antibody. J Mol Biol. 2011;408:462–476. doi:10.1016/j.jmb.2011.02.061.
116. Spinelli S, Frenken LG, Hermans P, Verrips T, Brown K, Tegoni M, Cambillau C. Camelid heavy-chain variable domains provide efficient combining sites to haptens. Biochemistry. 2000;39:1217–1222.
117. Spinelli S, Tegoni M, Frenken L, Van Vliet C, Cambillau C. Lateral recognition of a dye hapten by a llama $V_HH$ domain. J Mol Biol. 2001;311:123–129. doi:10.1006/jmbi.2001.4856.
118. De Genst EJ, Guilliams T, Wellens J, O'Day EM, Waudby CA, Meehan S, Dumoulin M, Hsu ST, Cremades N, Verschueren KH, et al. Structure and properties of a complex of α-synuclein and a single-domain camelid antibody. J Mol Biol. 2010;402:326–343. doi:10.1016/j.jmb.2010.07.001.
119. Bever CS, Dong JX, Vasylieva N, Barnych B, Cui Y, Xu ZL, Hammock BD, Gee SJ. $V_HH$ antibodies: emerging reagents for the analysis of environmental chemicals. Anal Bioanal Chem. 2016;408:5985–6002. doi:10.1007/s00216-016-9585-x.
120. El Khattabi M, Adams H, Heezius E, Hermans P, Detmers F, Maassen B, van der Ley P, Tommassen J, Verrips T, Stam J. Llama single-chain antibody that blocks lipopolysaccharide binding and signaling: prospects for therapeutic applications. Clin Vaccine Immunol. 2006;13:1079–1086. doi:10.1128/CVI.00107-06.
121. Gallo M, Kang JS, Pigott CR, Inventors; Innovative Targeting Solutions, Inc., Assignee. Sequence diversity generation in immunoglobulins. 2011. United States Patent US 8012714 B2.
122. Drabek D, Janssens R, de Boer E, Rademaker R, Kloess J, Skehel J, Grosveld F. Expression cloning and production of human heavy-chain-only antibodies from murine transgenic plasma cells. Front Immunol. 2016;7:619. doi:10.3389/fimmu.2016.00619.
123. Yu GW, Vaysburd M, Allen MD, Settanni G, Fersht AR. Structure of human MDM4 N-terminal domain bound to a single-domain antibody. J Mol Biol. 2009;385:1578–1589. doi:10.1016/j.jmb.2008.11.043.
124. Ma X, Barthelemy PA, Rouge L, Wiesmann C, Sidhu SS. Design of synthetic autonomous $V_H$ domain libraries and structural analysis

of a $V_H$ domain bound to vascular endothelial growth factor. J Mol Biol. 2013;425:2247–2259. doi:10.1016/j.jmb.2013.03.020.

125. Walker A, Chung CW, Neu M, Burman M, Batuwangala T, Jones G, Tang CM, Steward M, Mullin M, Tournier N, et al. Novel interaction mechanism of a domain antibody-based inhibitor of human vascular endothelial growth factor with greater potency than ranibizumab and bevacizumab and improved capacity over aflibercept. J Biol Chem. 2016;291:5500–5511. doi:10.1074/jbc.M115.691162.
126. Yamniuk AP, Suri A, Krystek SR, Tamura J, Ramamurthy V, Kuhn R, Carroll K, Fleener C, Ryseck R, Cheng L, et al. Functional antagonism of human CD40 achieved by targeting a unique species-specific epitope. J Mol Biol. 2016;428:2860–2879. doi:10.1016/j.jmb.2016.05.014.
127. Gunn RJ, Herrin BR, Acharya S, Cooper MD, Wilson IA. VLR recognition of TLR5 expands the molecular characterization of protein antigen binding by non-Ig-based antibodies. J Mol Biol. 2018;430:1350–1367. doi:10.1016/j.jmb.2018.03.016.