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MINIREVIEW

The art of blocking ADP-ribosyltransferases (ARTs): Nanobodies as experimental and therapeutic tools to block mammalian and toxin ARTs

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In 1901, the first Nobel Prize in Physiology or Medicine was awarded to Emil von Behring for his ground-breaking discovery of serum therapy: serum from horses vaccinated with toxin-containing culture medium of *Corynebacterium diphtheriae* contained life-saving 'antitoxins'. The molecular nature of the ADP-ribosylating toxin and the neutralizing antibodies were unraveled only 50 years later. Today, von Behring's antibody therapy is being refined with a new generation of recombinant antibodies and antibody fragments. Nanobodies, which are single-domain antibodies derived from the peculiar heavy-chain antibodies of llamas and other camelids, are emerging as a promising new class of highly specific enzyme inhibitors. In this review, we illustrate the potential of nanobodies as tools to block extracellular and intracellular ADP-ribosyltransferases (ARTs), using the toxin-related membrane-bound mammalian ecto-enzyme ARTC2 and the actin-ADP-ribosylating *Salmonella* virulence plasmid factor B toxin of *Salmonella enterica* as examples.

Diphtheria antiserum and the advent of therapeutic antibodies

The first Nobel Prize in Physiology or Medicine was awarded to Emil von Behring for 'his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths' (Fig. 1A) [1]. Emil von Behring noticed that the pathogenicity of diphtheria was mediated by a soluble factor ('toxin') contained in the supernatant of *Corynebacterium diphtheriae* cultures. He further discovered that the serum of animals that survived injections of toxin-containing culture supernatants contained a soluble factor ('antitoxin') that neutralized the pathogenic principle in the bacterial culture supernatants. Moreover, diphtheria patients showed rapid recovery after injections of antitoxin-containing animal sera.

It took another half century for scientists to unravel the molecular structure and function of the toxin and antitoxin. Diphtheria toxin (DT) is a large protein composed of three distinct domains: an N-terminal receptor-binding domain, a central translocation domain, and a C-terminal ADP-ribosyltransferase (ART) domain (Fig. 1B) [2]. Subsequent to its translocation to the mammalian cell cytosol, the catalytic domain catalyzes transfer of the ADP-ribose moiety from NAD^+ to a specific amino acid in elongation

Abbreviations
ART, ADP-ribosyltransferase; CDR, complementarity-determining region; EF2, elongation factor 2; hcAb, heavy-chain antibody; NICD, NAD^+-induced cell death; SpvB, *Salmonella* virulence plasmid factor B; VHH, variable domain of camelid heavy-chain antibody.

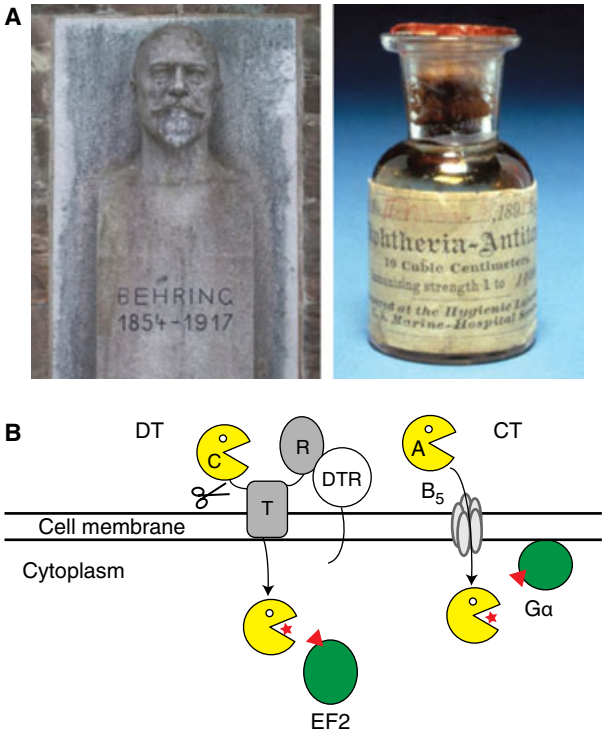

Fig. 1. Mode of action of diphtheria and cholera toxins. (A) The first Nobel Prize in Medicine or Physiology was awarded to Emil von Behring for his discovery of serum therapy. Diphtheria antiserum obtained from horses immunized with toxin-containing supernatants of *C. diphtheriae* saved thousands of lives. (B) Half a century after von Behring's discovery, DT was identified as an ART. Subsequently, *V. cholerae* and many other bacteria were discovered to secrete pathogenic ADP-ribosylating toxins, e.g. CT. DT is a three-domain holotoxin, consisting of catalytic (C), translocation (T) and receptor-binding (R) domains. The last of these binds to the DT receptor (DTR). CT is a binary A-B5 toxin. The pentameric B domain mediates binding to the host cell and translocation of the catalytic domain (A) to the cytosol. The target protein of DT – EF2 – and that of CT – the α-subunit of heterotrimeric G-proteins (Gα) – are indicated in green. The ART catalytic domain is symbolized by a pacman in reference to two core β-sheets that form the upper and lower jaws of a deep NAD^+-binding crevice. NAD^+ is symbolized by a star, and ADP-ribose is symbolized by a triangle. The photographs in (A) are reprinted with kind permission of Florian Manz, kollektiv25.de and by a Creative Commons license (commons.wikimedia.org/wiki/File: Antitoxin_diptheria – A short history of the National Institutes of Health).

factor 2 (EF2), i.e. diphthamide 715, a modified histidine [3,4]. The bulky ADP-ribose moiety attached to diphthamide prevents interaction of EF2 with other proteins of the translational machinery, thereby halting protein synthesis [5]. Diphtheria antitoxin is a polyclonal mixture of antibodies that opsonize DT, thereby inhibiting its binding to the host cell receptor and facilitating its phagocytic clearance.

Other bacteria (e.g. *Vibrio cholerae*, *Bordetella pertussis*, *Clostridium difficile*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) were subsequently discovered to produce toxins that similarly catalyze ADP-ribosylation of key cellular proteins, causing diseases such as diarrhea and whooping cough. Neutralizing these ADP-ribosylating toxins with antibodies constitutes a potential therapeutic strategy for these diseases.

Today, von Behring's discovery of antibodies as therapeutics is recapitulated in an ever-expanding arsenal of antibodies. This arsenal includes classic polyclonal hyperimmune sera, conventional mAbs, and a new generation of antibody-based recombinant proteins [6]. Nanobodies, which are single-domain antibodies derived from camelids, hold promise as a new generation of enzyme inhibitors [7,8]. Here, we illustrate the potential use of nanobodies as highly specific inhibitors of ADP-ribosylating toxins and toxin-related mammalian ARTs.

The unusual features of nanobodies derived from camelids

Antibodies are typically composed of two heavy and two light chains (Fig. 2A), the variable domains of which form two identical antigen-binding sites. These binding sites are formed by loops in the variable domains designated complementarity-determining regions (CDRs). In addition to such conventional antibodies, camels and llamas also produce peculiar antibodies composed only of heavy chains (Fig. 2) [9,10]. These heavy-chain antibodies (hcAbs) lack light chains and the CH1 domain. Therefore, their antigen-binding site is formed only by a single domain linked directly via a hinge region to the Fc domain. The variable domain of these antibodies is designated VHH, or, when produced as a recombinant protein, also nanobody or single-domain antibody [8,11]. Recombinant nanobodies can be generated by PCR-amplifying and cloning the coding region for VHH from blood cells of immunized llamas into a phage-display vector (Fig. 2B). Specific phages can then be selected by panning on immobilized antigen.

The immune system of camelids seems to possess an inherent propensity for forming enzyme-blocking hcAbs [7,12]. Conventional antibodies typically bind enzymes with a flat interface outside the active site. Nanobodies, in contrast, often bind and penetrate into the active sites of enzymes, thereby effectively blocking their catalytic activity [12,13]. The CDR3s of VHHs are often longer than those of conventional VH domains, e.g. 16–23 residues in the case of the

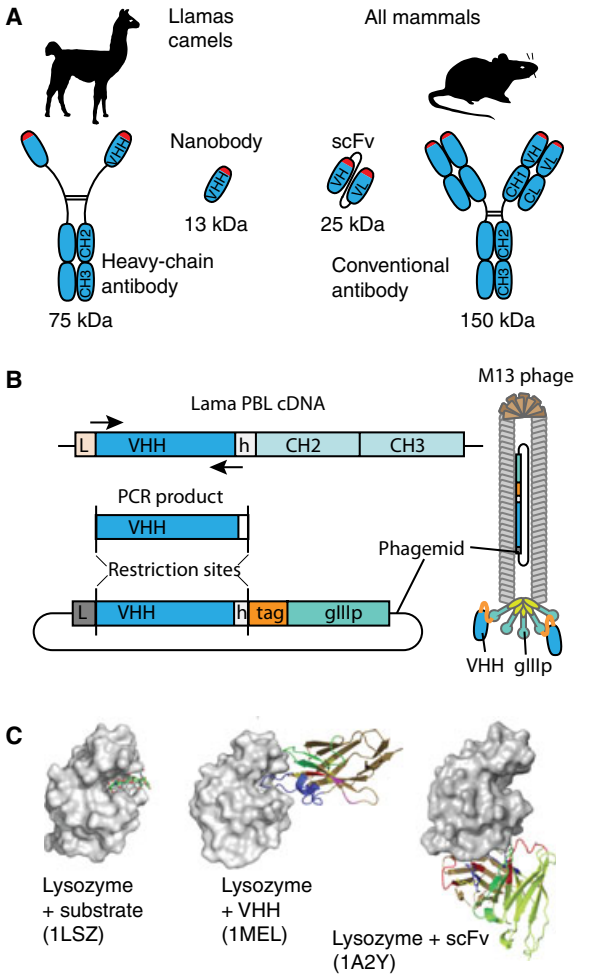

Fig. 2. Structural features of camelid hcAbs and nanobodies. (A) Camelid hcAbs lack the light chains and the CH1 domain of conventional antibodies. The antigen-binding paratope (red) of hcAbs is formed by a single variable domain (VHH). Recombinant VHHs (nanobody) are stable and soluble, whereas VH and VL domains of conventional antibodies are stable and soluble only when produced as genetic fusion proteins [single-chain variable fragments (scFvs)]. (B) Nanobodies are generated by PCR-amplifying and cloning the coding region for VHH from blood cells of immunized llamas into a phagemid vector. Specific phages can then be selected from the phage library by panning on an immobilized target antigen. (C) Nanobodies have an extraordinary propensity to bind and fill crevices on protein surfaces, e.g. the substrate-binding site of hen egg lysozyme. In contrast, conventional antibodies usually bind with a flat interface away from the active site. Images were generated with PYMOL [48]. (D) Amino acid sequence alignment of a conventional VH domain and camelid VHHs. Sequences are from a conventional anti-lysozyme VH (1a2y), the lysozyme-blocking nanobody Cablys3 (1mel), the ARTC2-blocking nanobody s+16a, and the SpvB-blocking nanobody VHH5. The high solubility of nanobodies is attributed to hydrophilic amino acids in framework region (FR)2 (in pink). (B, C) The three CDRs are color-coded as follows: CDR1, red; CDR2, green; and CDR3, blue. Disulfide bonds are depicted in yellow.

nanobodies directed against ARTC2, *Salmonella* virulence plasmid factor B (SpvB) and lysozyme versus seven residues in the case of the VH domain from a mouse mAb against lysozyme (Fig. 2C) [14,15]. This extended CDR3 is sometimes stabilized by an additional disulfide bond connecting it to an adjacent CDR loop (Fig. 2). The high solubility of nanobodies is attributed to hydrophilic amino acids in framework region 2, corresponding to the hydrophobic interface of conventional VH and VL domains [8,16].

Nanobodies are usually generated by PCR cloning of the VHH repertoire from lymphocyte cDNA from immunized llamas into a phagemid vector. Antigen-specific nanobodies are selected by panning phage libraries on immobilized antigen [15,17,18]. The high affinity of nanobodies from immune libraries is attributed to the natural selection of variant nanobodies during the clonal expansion of B cells in the lymphoid organs of the immunized animals. Nanobodies can readily be produced in *Escherichia coli*, yeast, plants, and mammalian cells [15].

Nanobodies have several advantages over conventional antibodies and single-chain variable fragments derived from such antibodies (Table 1) [8,16]. Their high stability, high refolding capacity, good tissue penetration *in vivo* and ready conversion into bispecific reagents make nanobodies ideally suited for various biotechnological and therapeutic applications [8,19,20]. Tandem cloning to a nanobody with specificity for serum albumin, for example, can be used to increase the *in vivo* half-life [21]. A bivalent hcAb can be reconstituted by genetic fusion to the Fc domain of any conventional antibody, e.g. mouse or human IgG_1. In transfected cells, nanobodies can be targeted to the cytosol by removing the leader peptide [22,23], or to vesicular compartments such as chloroplasts [24] or the endoplasmic reticulum [25] by genetic fusion with suitable targeting sequences. To date, a number of nanobodies targeting different proteins, including cytokines, blood clotting factors, and viruses, have been administered in clinical trials to > 700 subjects, without any adverse off-target effects [26].

Table 1. Comparison of the properties of nanobodies and conventional antibodies.

Property	Nanobodies	Antibodies
Molecular mass (kDa)	17	150
Size (amino acids)	110	1300
Size (nm)	2 × 3	10 × 30
Domains	Single domain	12 domains
Affinity	High	High
Specificity	High	High
Reformatting	Easy	Difficult
Enzyme inhibition	Excellent	Poor
Production costs	Low	High
Tissue penetration[a]	Good	Moderate
Renal excretion[a]	Fast	Slow
In vivo half-life[a]	Short	Long
Toxicity	Low	Low

[a] The presence and isotype of an Fc domain can influence tissue penetration and renal excretion, and thereby the half-life *in vivo*.

Extracellular and intracellular ARTs

ADP-ribosylation was originally discovered as the post-translational protein modification by which DT inactivates EF2, an essential component of the translational machinery [3]. Many other toxins, including cholera toxin (CT) and botulinum C2 toxin, were subsequently shown to use the same mechanism for inactivating G-protein and actin, respectively [27]. These toxins are secreted by *C. diphtheriae*, *V. cholerae* and *Clostridium botulinum* as multidomain (DT) or binary (CT and C2) proteins harboring a catalytic ART domain. Following translocation across cellular membranes to the cytosol of mammalian cells, the ART domain of these toxins transfers ADP-ribose from NAD^+ to specific amino acids in specific target proteins, i.e. to diphthamide 715 of EF2 (DT), arginine 187 of the α-subunit of G-proteins (CT), and arginine 177 of actin (C2) (Fig. 1B).

Molecular cloning and 3D structure analyses have revealed related enzymes in all kingdoms of life [28]. Biochemical analyses have revealed ARTs that ADP-ribosylate amino acids other than diphthamide and arginine, including cysteine, asparagine, threonine, glutamine, lysine, and glutamate, as well as ARTs that ADP-ribosylate nucleotides or antibiotics [29]. Remarkably, all of these enzymes fall into two major subclasses that are distinguished by conserved structural motifs: HYE in the ARTD family (related to DT); and RSE in the ARTC family (related to C2 and C3 clostridial toxins) [28]. In mammals, all known members of the ARTC family are extracellular, membrane-bound or secretory proteins, whereas all known members of the three ARTD subfamilies are made as intracellular proteins. The results of elegant *in silico* analyses indicate that endogenous ARTs in plants and animals have been acquired by lateral gene transfer of polymorphic prokaryotic biological conflict systems [30,31].

The crystal structures of ARTs show a deep active site crevice that seems to be suitable for targeting by nanobodies (Fig. 3). The following two examples suggest that this is indeed the case, and underscore the potential of nanobodies as ART inhibitors.

Nanobodies directed against a mammalian ecto-ARTC

Membrane-bound ARTs expressed by leukocytes (ARTC1 and ARTC2, also named CD296) ADP-ribosylate other cell surface proteins and secretory proteins in response to NAD^+ released from cells during inflammation, thereby affecting cell migration, cell communication, and apoptosis [32–35]. On T cells, ARTC2 catalyzes ADP-ribosylation of arginine 125 of the P2X7 purinergic receptor [36]. This causes gating of the P2X7 ion channel. The subsequent influx of calcium ions and efflux of potassium ions initiates a cascade of events that rapidly alter the composition and function of the cell surface, including externalization of phosphatidylserine, and metalloprotease-mediated shedding of the homing receptor L-selectin (CD62L) (Fig. 3A) [36–38]. Chronic activation of P2X7 causes cell death, a process called NAD-induced cell death (NICD) [37,39]. Regulatory T-cell subsets (Tregs and iNKT cells) are particularly sensitive to NICD [34,35,40]. As an ecto-enzyme, ARTC2 should be readily accessible to antibodies and nanobodies *in vivo*.

In a proof-of-principle study, we selected nanobodies from an immunized llama by absorption of nanobody-displaying phages from a phage display library on ARTC2-expressing cells [41]. Three of four distinct ARTC2-specific nanobodies were found to block the enzymatic activity of ARTC2, but not that of its closest paralog, ARTC1. Following intravenous injection, the monovalent nanobody s+16a completely blocked ARTC2 enzymatic activity and NAD-induced shedding of CD62L by T cells in peripheral lymphatic organs [41]. This blockade was effective within 10 min after injection. Injection of nanobody s+16a in a bivalent format, i.e. after fusion to the Fc domain of mouse IgG_1, achieved complete blockade of ARTC2 on lymph node cells within 2 h after injection [40]. This somewhat slower inhibition by the Fc-fusion protein than by monovalent nanobodies probably reflects the slower tissue penetration capacity of the larger Fc-fusion protein. The blockade of ARTC2 by s+16a–Fc was effective for > 7 days, reflecting the

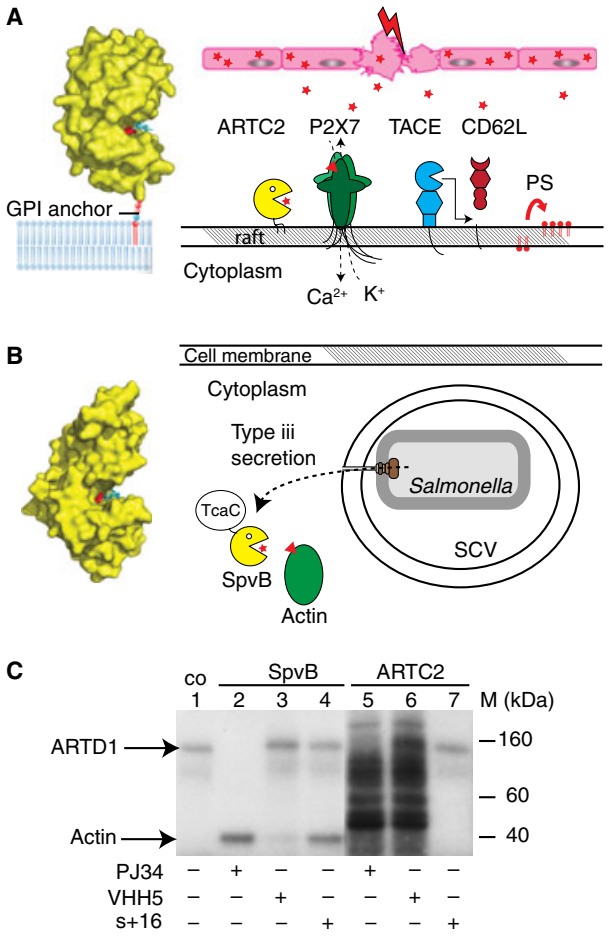

Fig. 3. Nanobodies effectively and specifically block ARTC2 and SpvB. (A) The mouse ecto-ART ARTC2 consists of an isolated catalytic domain that is attached to the cell membrane by covalent linkage of the C-terminal amino acid to glycosylphosphatidylinositol (GPI). The GPI-anchor restricts the local distribution of ARTC2 in the plasma membrane to cholesterol-rich microdomains, designated lipid rafts. Inflammation causes release of NAD^+ from cells by lytic and nonlytic mechanisms (red asterisks). On T cells, ARTC2 ADP-ribosylates the P2X7 purinergic receptor on arginine 125 when exposed to extracellular NAD^+. ADP-ribosylation of P2X7 activates its cation channel function. Gating of P2X7 by ADP-ribosylation induces influx of calcium ions and efflux of potassium ions. This, in turn, induces externalization of phosphatidylserine (PS) and activation of the metalloprotease TACE by an ill-defined mechanism, resulting in ecto-domain shedding of the homing receptor CD62L. (B) The *Salmonella* ADP-ribosylating SpvB toxin contains a C-terminal ARTC domain with a deep NAD-binding crevice fused to a 'TcaC' domain of unknown function, which is structurally related to insecticidal toxins. Expression of SpvB is induced only after endocytic uptake of bacteria and formation of the *Salmonella*-containing vacuole (SCV). SpvB is injected into the host cell cytosol through the endosomal membrane via a type III secretion system. In the host cell cytosol, SpvB ADP-ribosylates actin on arginine 177. This modification sterically blocks the interaction of monomeric actin with filamentous actin, resulting in actin depolymerization and alteration of the cytoskeleton. (C) HEK cell lysates were incubated with [^{32}P] NAD^+ in the absence or presence of SpvB or ARTC2, as indicated at the top, and the inhibitors indicated below. The small-molecule ARTD antagonist PJ34 blocks auto-ADP-ribosylation of ARTD1/PARP1 (but not ADP-ribosylation by SpvB or ARTC2), nanobody VHH5 blocks ADP-ribosylation of actin by SpvB (but not ADP-ribosylation by ARTD1 or ARTC2), and nanobody s+16a blocks ADP-ribosylation of multiple proteins by ARTC2 (but not ADP-ribosylation by ARTD1 or SpvB).

higher *in vivo* half-life resulting from slower elimination via the kidney. Systemic injection of s+16a–Fc effectively protected Tregs and iNKT cells from NICD *in vivo*, and showed clinical benefit in a mouse model of autoimmune diabetes [35,40]. These results indicate that nanobodies are effective tools for specifically blocking individual members of the ARTC family of ecto-ARTs.

Secreted toxin ARTs should be similarly accessible to antibodies in the extracellular environment. Their excellent solubility, stability and high capacity to diffuse through the extracellular compartment suggest that nanobodies also have promise as therapeutics against secreted toxin ARTs.

Targeting nanobodies to a toxin ARTC in the cytosol

The cell membrane is impermeable to proteins, and extracellular antibodies therefore usually cannot reach intracellular antigens. Two experimental strategies have been pursued to target antibodies to intracellular antigens: transfection of cells with cDNA constructs encoding intracellular antibodies; and the fusion of antibodies to peptides mediating translocation across the cell membrane [42]. Nanobodies appear to be particularly suited for these strategies, as they are small and can readily (re)fold in different environments, including the cytosol, nucleus, and chloroplasts [23,24,43].

In contrast to many other bacteria, salmonellae do not secrete their actin-ADP-ribosylating SpvB toxin into the extracellular space. Instead, expression of SpvB is induced only after salmonellae have been endocytosed and the endosomes have been converted into specialized vesicles, the so-called *Salmonella*-containing vacuoles. SpvB is injected via a needle-like type III secretion system through the vacuole membrane into the host cell cytosol (Fig. 3B). SpvB then ADP-ribosylates actin on arginine 177, thereby blocking actin polymerization and disturbing cytoskeletal functions [44–47]. From an immunized llama, we

isolated SpvB-specific nanobodies by absorption of nanobody-displaying phages from a phage-display library on biotinylated SpvB immobilized on streptavidin beads. Three of four SpvB-specific nanobodies blocked SpvB-catalyzed ADP-ribosylation of actin at a 1 : 1 molar ratio [23].

An ADP-ribosylation assay with HEK cell lysates and [^{32}P]NAD$^+$ illustrates the high specificity and effectivity of the nanobodies directed against SpvB and ARTC2 (Fig. 3C). The prominent radiolabeled band at 120 kDa corresponds to auto-ADP-ribosylation of endogenous ARTD1 (PARP1). Addition of the monospecific SpvB results in radiolabeling of an additional band at 40 kDa, corresponding to actin. Addition of the promiscuous ARTC2 results in radiolabeling of several bands. VHH5 specifically blocks ADP-ribosylation of actin but not auto-ADP-ribosylation of ARTC1, and s+16a specifically blocks ADP-ribosylation of multiple proteins but not auto-ADP-ribosylation of ARTD1.

When expressed as an intrabody in transfected cells, nanobody VHH5 also effectively inhibited SpvB-mediated disruption of the cellular cytoskeleton [23]. These results highlight the potential of nanobodies as blockers of other cytosolic toxin ARTs, and possibly also as specific inhibitors of individual members of the mammalian ARTD family of intracellular ARTs. A transfection-based strategy to express such ART-blocking nanobodies in the cytosol seems to be applicable to cell lines *in vitro* as well as to transgenic animals. Direct *in vivo* transfection has been achieved for skin cells, with ballistic DNA immunization, and for the liver with hydrodynamic transfection by intravenous bolus injections of DNA in large volumes.

Conclusions and perspectives

In the 20 years since the serendipitous discovery of camelid hcAbs in a practical laboratory course, numerous studies have underscored the diagnostic and therapeutic potential of recombinant nanobodies derived from these hcAbs. *In vivo*, nanobodies have favorable biodistribution properties, including deep penetration into dense tissues and rapid elimination via the kidney. Their unusual propensity to block enzymes makes nanobodies useful for neutralizing membrane-bound and secretory ecto-enzymes, including mammalian ecto-ARTC enzymes and secreted ADP-ribosylating bacterial toxins. To neutralize ADP-ribosylating toxins in the skin (*S. aureus* toxins), the lung (*P. aeruginosa* toxins), or the intestine (*V. cholerae* and *C. difficile* toxins), nanobodies could be administered in ointments, aerosols, or capsules. To neutralize ART toxins systemically, and to modulate inflammatory reactions by blocking ARTC2-mediated cytotoxicity, nanobodies could be administered by intravenous or subcutaneous injections. For intracellular targets, e.g. mammalian ARTD enzymes or bacterial toxins such as SpvB that bypass the extracellular environment via injection into the cytosol, there still is a need for more efficient tools to deliver nanobodies to the cytosol and other intracellular compartments.

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