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Teaser This review provides an extensive overview of the current state of all the possible therapeutic and diagnostic uses of nanobodies.

Nanobodies as therapeutics: big opportunities for small antibodies

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Members of the Camelidae (including camels and llamas) produce, in addition to conventional antibodies (Ab), a unique type of Ab that lacks light chains. The variable antigen-binding domains derived from these Ab are named 'nanobodies' (Nbs). Nbs exert high specificity and affinity and, when properly selected, are more stable than conventional Ab. Furthermore, their toxicity and immunogenicity are both low. They are easy to produce and their modularity makes them amenable for the generation of multivalent complexes. In this review, we discuss how Nbs are being explored as therapeutics in many fields of medicine, including oncology, inflammatory, infectious and neurological diseases, and imaging. In addition, we highlight their potential for use in the diagnosis and monitoring of diseases. Finally, we provide an extended overview of Nbs that are, or have been, involved in clinical trials.

Introduction

Approximately 30% of drugs in development are biologics, most of which are Ab-based proteins used as treatment for inflammatory diseases, cancer, and allergies [1]. The success of the anti-tumor necrosis factor (TNF) Abs has boosted the search for other Abs. In 2013, 22% of the sales of large pharmaceutical companies were biologics and this figure is expected to rise still further [2].

Monoclonal Abs (mAbs) have become indispensable therapeutic and research tools. Given that they are difficult and expensive to produce, they impose a heavy burden on healthcare and research budgets. Moreover, they are not suitable for some applications. First, they are large molecules (150 kDa), which limits their tissue and/or tumor penetration and biodistribution. Second, they can elicit immune reactions that neutralize their activities, which sometimes limits the long-term use of chimeric and humanized Abs available on the market. Third, mAbs typically have a half-life of several days and this limits their use in molecular imaging because of the intense background signal [3,4]. Since many of the disadvantages of Abs are related to their large size, efforts have been made to trim them down and to improve their pharmacological properties

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Claude Libert is a group leader at the VIB in Ghent. He has a MSc and obtained his PhD at the University of Ghent under the guidance of Walter Fiers. After a post-doc in Italy, he became a group leader at VIB in 1997 and a professor at the University of Ghent in 2003. His major research interest is acute inflammation and the crosstalk between several important players in inflammation, including TNF, interferon (IFN), MMPs, and glucocorticoids.

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GLOSSARY

Affibodies antibody mimetics comprising three α-helices based on the Z-domain of protein A found in the cell wall of Staphylococcus aureus.
Bionanocapsules hollow NPs that comprise the L-protein of hepatitis B virus, which allows targeted drug or gene delivery to hepatocytes. Linking them to Nbs makes it possible to deliver them to other organs.
Chromobodies® Nbs that are genetically fused to a fluorescent protein, such as TagGFR or TagRFP. They can be used for real-time visualization of endogenous cellular structures or cellular processes.
Immunocytokines mutant nontoxic cytokines linked to Nbs to actively target them and activate the cytokines again by accumulation (revitalize the clinical potency of the Nbs).
Immunoliposomes liposomes that are decorated with biologicals, such as Nbs, to actively target liposomes.
Immunotoxin toxins that are linked to a targeting moiety, such as Abs or Nbs, to actively target them to toxins to limit toxicity.
Intrabodies Abs or Ab fragments, such as Nbs, that are intracellularly expressed and directly bind to intracellular proteins; mainly useful to treat neurodegenerative diseases and fight intracellular pathogens.
Lactobodies Ab fragments, such as Nbs, that are produced by genetically engineered probiotic Lactobacilli and that can be directed against gastrointestinal pathogens, such as Rotavirus; can be used as prophylactics or therapeutics.
Liposomes spherical vesicles with an aqueous solution core that is surrounded by a lipid bilayer structure comprising mainly phospholipids; can be used as vehicle to deliver drugs.
Micelles aggregates of surfactant molecules that comprise monolayers consisting of hydrophilic heads, sequestering the hydrophobic single-tail regions in the center.
Microbubbles and nanobubbles structures comprising a gas-filled shell of lipids or proteins; can be used as contrast agents for ultrasound imaging. Microbubbles typically range in size between 0.5 and 10 mm diameter, while nanobubbles range from a few hundred nm to 100 nm.
Pentabodies Nbs that are pentamerized by fusing them to homopentamerizing proteins, such as the nontoxic verotoxin B.
Photoimmunotherapy a type of molecular-targeted cancer therapy that uses PS that are conjugated to biologicals, such as Nbs. Irradiation of those complexes with NIR induces cell death of the tumors.
Photosensitizers (PS) molecules that rapidly destroy cells via the production of reactive oxygen species when exposed to light at a specific wavelength.
Polymersomes highly stable hollow spherical vesicles with a hydrophilic aqueous core that is surrounded by a membrane comprising amphiphilic synthetic block copolymers. The vesicles generally have radii ranging from 50 nm to 5 Mm.
Radionuclides isotopes of elements with an atom nucleus that have an excess of nuclear energy, making it unstable. This excess of energy will emit new radiation from the nucleus or will create a new particle.
Transbodies Nbs that are molecularly linked to a cell-penetrating moiety, such as the peptide penetratin or the bacterial type III secretion system; thereby, they are capable of crossing the cell membrane to function intracellularly.

[5]. This led to the development of the antigen-binding fragments (Fab fragments), variable fragments (Fv fragments), and single-chain variable fragments (scFv fragments) [6]. Nevertheless, the stability of these recombinant proteins remains inadequate and their activity is sometimes suboptimal compared with conventional Abs because of lower interaction possibilities with antigenic epitopes (i.e., lower avidity) [5].

During the early 1990s, a new type of Ab was serendipitously discovered in members of the Camelidae by Hamers-Casterman and her team. In addition to conventional immunoglobulin G (IgG) Abs, camelids were found to express another type of Ab devoid of light chains, called 'heavy-chain-only antibodies' (HcAbs). These comprise two constant domains (CH2 and CH3), a hinge region, and an antigen-binding or variable heavy chain domain (VHH) called the Nb, which retains full antigen-binding capacity [7]. VHHs comprise four conserved sequence stretches (the framework regions) surrounding three hypervariable complementarity-determining regions (CDR), which might participate in antigen recognition and binding [8]. CDR3 is the major contributor to antigen binding; at least 60–80% of the contact with the antigen is through this region [9,10]. Nbs are small (15 kDa) and can have a long protruding CDR3 loop. Their prolate shape exposes a convex paratope and both these features allow them to access more easily receptor clefts or binding pockets that are inaccessible to Abs. They can also reach 'hidden', cryptic, and concave epitopes. The selection, identification, and production procedure of the antigen-specific VHHs were described by Ghahroudi *et al.*, and the other molecular and biochemical properties of Nbs have been extensively reviewed by Muyldermans [8,11–14].

Furthermore, Nbs have other advantages over conventional Abs, such as their unique physical and chemical robustness. When the appropriate selection strategies are performed, Nbs can be selected with a remarkable stability under certain harsh conditions, including extreme temperatures, the presence of proteases, high pressure, or low pH [15–19]. This can also result in molecules that are suitable for administration routes other than the parental route. Additionally, following *in vivo* administration, they rapidly diffuse throughout the body and have good tissue penetration [20]. Unfortunately, their small size is below the renal cut-off, leading to rapid renal clearance. This is particularly undesirable in chronic therapies. Consequently, strategies to extend their half-life have been described extensively. These include polyethylene glycol (PEG)-ylation, conjugation to the Fc domain of conventional Ab, and coupling to abundant serum proteins, such as human serum albumin (HSA) or IgG via the Nbs that target them [21–23]. Linking to an antialbumin Nb creates an additional advantage: the complex will be targeted to regions where albumin accumulates, mostly inflamed areas [24].

Although they do not originate from humans, Nbs have a low immunogenicity because of a large sequence identity with the human VH gene family III [11], making them suitable for chronic indications. However, strategies to humanize Nbs are useful and have been described by Vincke *et al.* [25]. Importantly, binding specificity, stability, and solubility need to be preserved after this procedure. Vincke *et al.* designed a humanized scaffold Nb onto which the antigen-binding loops (CDRs) of specific Nbs can be grafted. Humanization is desirable before clinical application, and is now done routinely [20]. In nine clinical studies with Nbs, the

incidence of antidrug antibodies was low (3%) and their presence mainly transient. In addition, they did not influence the safety or efficacy of the Nbs [26]. Nevertheless, other groups have reported important immune adverse events that even led to discontinuation of clinical trials [27,28].

Given their single-domain and hydrophilic nature, Nbs are well expressed in economic production systems, such as bacteria (*Escherichia coli*) and yeast (*Pichia pastoris* and *Saccharomyces cerevisiae*), yielding high batch-to-batch consistency. Nbs are well also expressed in probiotic bacteria and, when they are expressed in *Lactobacillus paracasei* or *Lactococcus lactis*, they are called 'lactobodies'. These are useful for the delivery of Nbs against pathogenic enteric bacteria [29]. Additionally, because they are encoded by only a single gene (*Vhh*) comprising (approximately) 360 base pairs, Nbs are very modular and can be easily covalently linked to other molecules or prodrugs. Fusion of Nbs that bind different epitopes or have different modes of action allows the creation of multivalent molecules with high affinity or potency [30,31] (see Fig. 1a for all Nb formats). It has also been reported that optimization or formatting of the lead component (e.g., the generation of multivalent constructs) does not substantially influence the manufacturing yield. Multivalent Nbs can be formulated at high concentrations with limited impact on viscosity or syringeability [32].

As a result of their single-domain nature and intracellular robustness, Nbs are suitable for expression as intracellular proteins. These so-called 'intrabodies' (Fig. 1a) can interact with intracellular targets, making it possible to target proteins that are otherwise inaccessible. This approach is particularly attractive because Nbs can readily fold in different reducing intracellular environments (e.g., the nucleus or cytosol) [33]. Additionally, Nbs retain antigen-binding capacity in the absence of disulfide bonds, which are not formed inside cells [34]. Chromobodies® (Fig. 1a) are a special type of intrabody, comprising Nbs against cellular proteins genetically fused to fluorescent proteins [35–37]. Those tagged Nbs are valuable for real-time visualization of endogenous cellular processes, such as the cell cycle, or for tracking structural proteins, such as integrins *in vivo*. Chromobodies are commercially available for research purposes, such as actin- or lamin-specific Chromobodies [35]. Nano-Boosters® are also commercially available, but here the Nbs are directed against endogenously expressed GFP or RFP and carry a superior fluorescent dye to boost the initial signal (www.chromotek.com) (Fig. 1a).

Since its invention, the Nb technology has expanded rapidly, evidenced by the explosion in the number publications. Reviewing all the applications of Nbs in all fields is impossible. Here, we mainly focus on the recent advances made towards possible clinical applications and more particularly on the possibilities of using Nbs in treatment and diagnosis (Table 1). Nbs are valuable tools in human medicine, as illustrated by current clinical trials, and, although not explicitly mentioned, Nbs also have much to offer as a research tool, because they are inexpensive to produce in a short period of time, which could make them accessibly to the scientific community worldwide. Table 2 provides an overview of the major companies that make Nbs or Nb-derived products.

Treatment of cancer

Antibodies that target tumor-associated antigens have been successfully introduced in the fight against cancer, with 25 US Food and Drug Administration (FDA)-approved monoclonal Abs (mAbs)

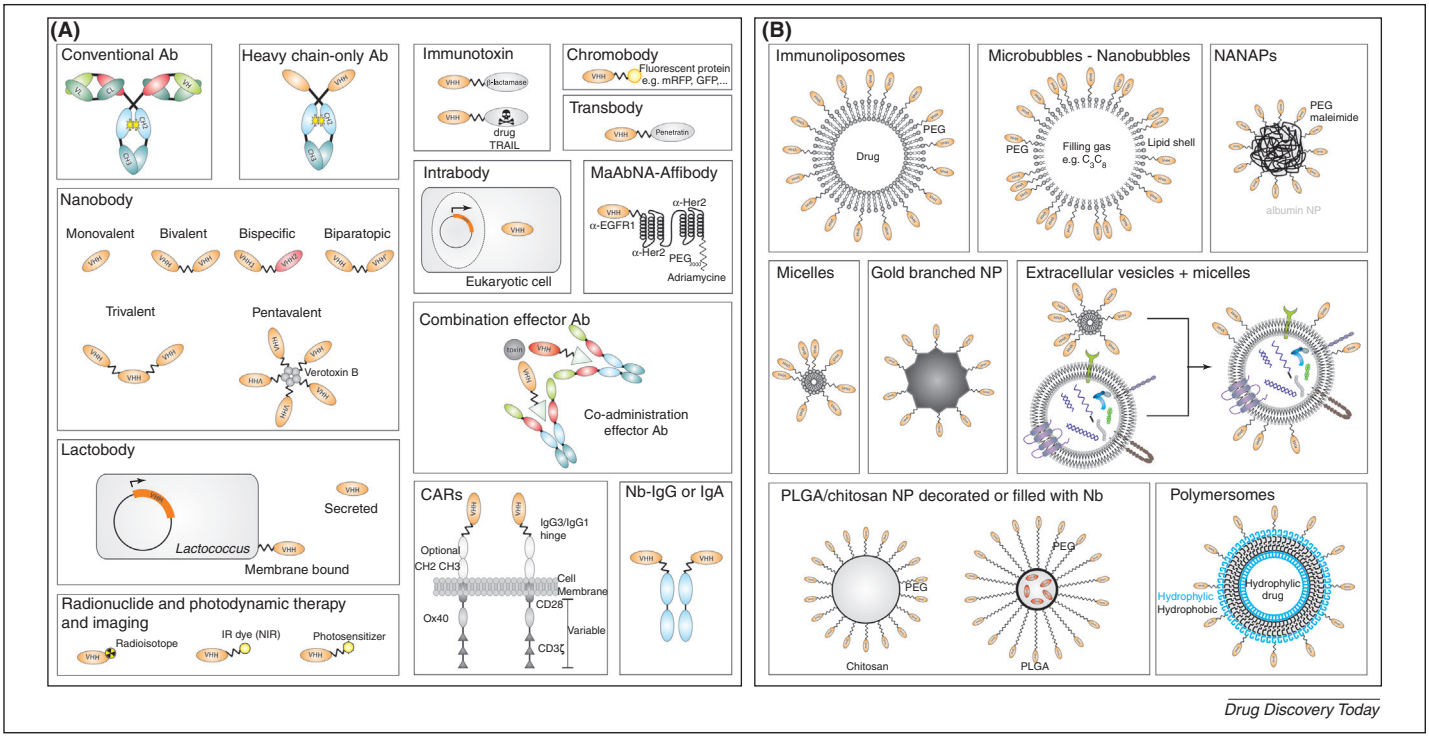

FIGURE 1

Overview of the different nanobody formats and their use in drug delivery systems. **(a)** Schematic representation of antibodies (Abs) and all possible monovalent and multivalent nanobody (Nb) formats and their application as targeting moiety of toxins, cytokines, radionuclides, near infra-red (NIR) dyes, and photosensitizers (PS). **(b)** Schematic representation of Nbs incorporated in drug delivery systems for targeted drug delivery.

TABLE 1

Overview of the use of Nbs in multiple therapeutic areas: hematology, oncology, *in vivo* imaging, and infectious, neurological, and inflammatory diseases[a]

Product name	Target	Disease	Evidence		Comment	Refs; clinical trial identifier
			Proof-of concept	Model		
Hematology						
Caplacizumab	A1 domain of vWF	TTP	Phase II; now in Phase III	HERCULES study in patients with acquired TTP	Bivalent Nb; first in class; first Nb to enter Phase III clinical study	[324] NCT01151423; NCT02553317
Oncology						
ALX-0651	CXCR4	Multiple myeloma and non-Hodgkin's lymphoma	Phase I	Interventional study in healthy male volunteers	Development not pursued beyond Phase I	[317]
TAS266	DR5	Solid tumors	*In vivo*; Phase I	Mice xenografted with multiple tumors; interventional study in patients with advanced solid tumors	Agonistic tetrameric Nb; Phase I clinical trial stopped prematurely	[318,319]
Antagonistic Nbs						
Ia1; CONAN-1	EGFR	Solid EGFR$^+$ tumors	*In vivo*	A431-xenografted mice	Ia1: monovalent Nb; CONAN-1: trivalent, biparatopic Nb	[45,47]
OA-cb6	EGFR	Solid EGFR$^+$ tumors	*In vitro*	A431, SK-BR-3, and MCF-7 cells		[48]
4NC2	EGFR	Gastric cancer	*In vivo*	Mice injected with BGC-823 gastric cancer cells	Nb fused to tumor-penetrating and cell-internalizing peptide iRGD, with or without co-administration of paclitaxel	[51,52]
1E2-Alb8; 6E10-Alb8	HGF	Solid HGF$^+$ tumors	*In vivo*	U87 MG xenografted mice	Bivalent, linked to Alb8 (anti-alb Nb)	[48]
Anti-c-MET Nanobody®	c-MET	Multiple myeloma	*In vitro*	Adhesion and migration assays in ANBL-6 MM and INA-6 MM cells; osteoblast differentiation assay in hMSCs		[49]
NB 4; NB 5	CXCR7	Head and neck cancer	*In vivo*	22A and Fadu cell-xenografted mice	NB 4: biparatopic, trivalent linked to Alb8 (anti-alb Nb); NB 5: bivalent NB 2 coupled to Alb8 (anti-alb Nb)	[58]
CAPNb2	CapG	Breast cancer metastasis	*In vivo*	Orthotopic xenografts and tail-vein models of metastasis	Transbody coupled to T3s signal sequence of the *Escherichia coli*-secreted protein F injected into cancer cells by exploiting the T3SS system of bacteria	[53]
nAb-C3; nAb-C8	uPA	Inhibition metastasis	*In vitro*	uPA activity assay		[57]
3VGR19 (VEGFR2); ZFR-5; VA12 (VEGF-A)	VEGF-A and VEGFR2	Inhibition tumor metastasis and neovascularization	*In vitro*; *in vivo*	HUVEC cells; *in vivo* chorioallantoic membrane assay	3VGR19: bivalent; ZFR-5; VA12: monovalent	[59–61]
	VEGF and Ang2	Inhibition angiogenesis	*In vivo*	SW620 tumor-bearing mice	Humanized trivalent, bispecific Nb coupled to anti-HSA Nb	[62]
Nb 2.17	Leptin receptor	Melanoma	*In vivo*	Melanoma B16-xenografted mice	Nb 2.17: bivalent, linked to mouse serum albumin; selective peripheral activity required	[65,66]
K24	CAIX	Metastasis	*In vitro*	HeLa cells		[63]
C21; C28	Fc-γ-RIIIa (CD16) on NK and PMN cells	Fc-γ-RIIIa engagement to destroy tumor cells	*In vitro*	Binding assay and IL-2/IFN-γ production assay in Jurkat T cells, NK cells, and PMN cells	Recruitment of Fc-γ-RIII killer cells to target and destroy tumor cells; selective engagement of Fc-γ-RIIIa on cells strongly induced IL-2 and IFN-γ production	[67]

TABLE 1 (*Continued*)

Product name	Target	Disease	Evidence		Comment	Refs; clinical trial identifier
			Proof-of concept	Model		
BsFab C21; BsFab C28	CEA/Fc-γ-RIIIa	Colon cancer	*In vivo*	Non-obese diabetic/severe combined immunodeficient gamma CEA^+ tumor-xenografted mice	Fab-like bispecific antibodies comprising Nb C21/C28 (Fc-γ-RIIIa) and Nb C17 (CEA), fused to human Cκ and CH1 IgG1 domains used as heterodimerization motif; simultaneous binding to target the tumor-associated antigen and Fc-γ-RIII	[68]
Nbs linked to effector moieties						
cAb-CEA5-β-lactamase	CEA	Colon cancer	*In vivo*	LS174T-xenografted mice	Nb coupled to β-lactamase activates cephalosporin nitrogen mustard prodrug 7-(4-carboxybutanamido) cephalosporin mustard at CEA-expressing tumor surfaces	[352]
7D12/38G7; 7D12/9G8	EGFR	Glioblastoma multiforme	*In vivo*	U87-mCherry-FLuc xenografted mice	Expressed and secreted by NSCs transfected with lentivirus virions that co-express secretable cytotoxic TRAIL recombinant protein	[72]
3VGR19-PE	VEGFR2	Inhibited tumor metastasis and angiogenesis	*In vitro*	Thymidine proliferation and MTT assay in 293KDR cells	Immunotoxin: Nb conjugated to bacterial toxin *Pseudomonas* exotoxin A	[70]
7D12 Nb coupled to two ZHER2:4 affibodies	EGFR and HER2	$EGFR1^+$ and/or $HER2^+$ tumor	*In vivo*	549, MDA-MB-231 and MCF-7-xenografted mice	MaAbNA coupled via PEG2000 to anticancer drug adriamycin	[50]
RR-B_2-PIC fused to CD28-CD3ζ chimera	MUC-1-specific CARs	Adoptive T cell transfer with CARs in MUC-1 overexpressing tumors	*In vitro*	Human breast ductal carcinoma T47D and human breast adenocarcinoma MCF7 and SKBR3 cells	Jurkat T cell expression; used PhiC31 integrase for high and stable CAR gene expression in T cell lines	[78]
N13 fused to CD28-OX40-CD3ζ chimera	TAG-72-specific CARs	Adoptive T cell transfer with CARs in TAG-72 overexpressing tumors	*In vitro*	IL2 production and cytotoxicity assays in LS-174T and MCF7 cells	Expression as chimeric T cell receptor	[76]
VHH_{RR10}-1HOX, VHH_{RR14}-1HOX and VHH_{RR16}-2HOX fused to CD28-CD3ζ and CD28-OX40-CD3ζ chimera	HER2-specific CARs	Adoptive T cell transfer with CARs in HER2 overexpressing tumors	*In vitro*	IL2 production and cytotoxicity assays in SK-BR-3 and NIH 3T3$HER2^+$ cells	Oligoclonal Jurkat T cell expression	[79]
Nb GPA7 fused to CD28- CD3ζ chimera (GPA7-28z)	Gp100-specific CARs	Adoptive T cell transfer with CARs in melanoma	*In vitro*; *ex vivo*; *in vivo*	Cytotoxicity assays in T2 cells; lysis of isolated primary human melanoma cells; Malme-3m ($HLA\text{-}A2^+$ melanoma cells)-xenografted mice	Expression by transgenic human primary T cells	[80]
VHH7	Class II MHC	Class II MHC^+ cells in tumor tissue	*In vivo*	Mice xenografted with A20 tumor cells	Conjugated to DM1, a potent microtubule polymerization inhibitor	[71,116]

Nbs encoated to drug delivery systems						
EGa1	Ectodomain	$EGFR^+$ epithelial tumors	*In vivo*	Mice engrafted with ^{14}C human head and neck squamous carcinoma cells	Coupled to PEG liposomes	[82]
				EGFR-(over)expressing UM-SCC-^{14}C tumor cells	Coupled to PEG liposomes filled with anti-IGF-1R kinase inhibitor AG538	[83]
				Mice engrafted with ^{14}C human head and neck squamous carcinoma cells	Conjugated to micelles filled with covalently trapped doxorubicin	[84]
				BrdU assay in ^{14}C cells	Coupled to albumin NPs (NANAPs) and filled with multikinase inhibitor 17864	[85]
				Mice xenografted with A431 cells	Conjugated to PEG micelles; resulting Nb-PEG-micelles incorporated into membrane of extracellular vesicles	[92]
Ia1	EGFR	$EGFR^+$ epithelial tumors	*In vitro*	Internalization assay using A431 cells	Biotinylated Nb coupled *via* streptavidin to biotinylated bionanocapsules	[86]
G2	Met/HGFR	Met-overexpressing aggressive cancers	*In vitro*	Human ovarian TOV-112D, human lung A549 and human epidermoid squamous A431 carcinoma cell lines	Anti-Met NANAPs	[353]
A12	PlexinD1	Tumor vessels	*In vitro*	Functional characterization	Coupled to polymersomes	[89]
VHH1	HER2	$HER2^+$ breast cancer	*In vitro*	Functional characterization and binding to SKBR3 cells	Coupled to polymersomes	[90]
Nbs in targeted radionuclide therapy and photoimmunotherapy						
2Rs15d	HER2	$HER2^+$ cancers	*In vivo*	SKOV3-LUC xenografted mice	Coupled to ^{177}Lu *via* 1B4M-DTPA chelator; co-infusion with GeloFusin	[94]
5F7GCC	HER2	$HER2^+$ breast cancer	*In vivo*	BT474M1-xenografted mice	Coupled to ^{125}I or ^{131}I	[97,98]
R3B23	M-protein	Multiple myeloma	*In vivo*	Mouse 5T2MM model	Coupled to ^{177}Lu	[96]
2Rb18a	HER2	$HER2^+$ cancer	*In vitro*	SKOV3 cells	Coupled to gold branched NPs used for photothermal therapy	[100]
7D12-R2; 7D12-9G8	EGFR	$EGFR^+$ cancers	*In vitro*	Mouse fibroblast NIH 3T3 2.2 cells, human epithelial carcinoma A431 (CRL-1555) cells and cervical carcinoma cell HeLa (CCL-2) cells	7D12; R2: monovalent Nb; 7D12-G8: biparatopic Nb; coupled to IRDye700DX as PS for photodynamic therapy	[99]
Imaging: theranostics						
2Rs15d	HER2	$HER2^+$ tumors	Phase I	Female patients with $HER2^+$ breast tumors	^{68}Ga-coupled *via* a 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) derivative (PET/CT)	[121,331,354]
	HER2	$HER2^+$ tumors	*In vivo*	$HER2^+$ tumor models	Labeled with ^{99m}TC (SPECT/CT)	[120]
	HER2	$HER2^+$ tumors	*In vivo*	$HER2^+$ tumor models	Site-specifically labeled with ^{111}In	[122]
5F7	HER2	$HER2^+$ tumors	*In vivo*	$Prkdc^{scid}$/J mice implanted with 17-β-estradiol pellets xenografted with BT474M1 tumors	Labeled with ^{125}I, ^{131}I *via* B-Mal-d-GEEEK	[98]
	HER2	$HER2^+$ tumors	*In vivo*	Mice with BT474M1 xenografts	Labeled with ^{18}F *via* 8F-RL-I (micro PET/CT)	[123]
	HER2	$HER2^+$ tumors	*In vitro*	Breast and lung cancer cell lines	Nbs conjugated to CdSe/ZnS quantum dots (multiphoton imaging)	[137]
sdAb 4.43	IGFBP7	IGFBP7-expression brain tumors	*In vitro* and *in vivo*	Small-angle neuron scattering, phantom MRI, and dynamic light scattering; orthotopic U87MG.EGFRvIII glioblastoma xenografted mice	Encoated on sULVs	[131]
1E2; 6E10	HGF	HGF-expressing tumors	*In vivo*	U87 MG glioblastoma xenografted mice	Linked to anti-albumin Nb; labeled with ^{89}Zr (PET)	[48]

TABLE 1 (*Continued*)

Product name	Target	Disease	Evidence		Comment	Refs; clinical trial identifier
			Proof-of concept	Model		
PSMA6; PSMA30	PSMA	Prostate cancer	*In vivo*	LNCaP and PC3 xenografted mice	Labeled with ^{99m}TC (SPECT/micro CT)	[124]
JVZ-007	PSMA	Prostate cancer	*In vivo*	PSMA-positive PC-310 and PSMA-negative PC-3 xenografted mice	Labeled with ^{111}In conjugated with DTPA	[125]
	PSMA	Prostate cancer	*In vivo*	LNCaP, C4-2, and MKN45 xenografted mice	Nb coupled to ultrasound nanobubbles via biotin-streptavidin bridging	[134]
NbCEA5	CEA	Colon carcinoma	*In vivo*	LS174T xenografted mice	Also humanized Nbs; labeled with ^{99m}TC (SPECT/micro CT)	[126]
7C12; 7D12	EGFR	EGFR$^+$ tumors	*In vivo*	A431 (EGFR-positive) and R1M (EGFR-negative) xenografted mice	Labeled with ^{99m}Tc, ^{68}Ga, and IRDye800CW	[109,114,130]
EG2-cys	EGFR and mutant EGFR (EGFRvIII)	Glioblastoma multiforme	*In vivo*	Mice xenografted with orthotopic glioblastoma	Conjugated to NIR quantum dot (Qd800)	[138]
7D12	EGFR	Surgery: resection of EGFR$^+$ tumors	*In vivo*	Mice with orthotopic tongue tumors (OSC-19-luc2-cGFP cells)	Labeled with IRDye800CW	[140]
D10	EGFR	EGFR$^+$ tumors	*In vivo*	Mice with orthotopic human mammary MDA-MB-468 and MDA-MB-231 and subcutaneous human epidermoid A431 carcinoma	Labeled with ^{99m}Tc (SPECT)	[129]
B9	CIAX	Pre-invasive breast cancer	*In vivo*	Mice orthotopically xenografted with MCF10DCIS cells	Labeled with IRDye800CW	[127]
B9 and 11A4	CAIX and HER2	Pre-invasive breast cancer	*In vivo*	Mice orthotopically xenografted with MCF10DCIS cells	Labeled with same (IRDye800CW) or different fluorophores	[132]
s+16a	ART2	T cell imaging	*In vitro*; *in vivo*	Flow cytometry; NIR imaging in WT and ART2$^{-/-}$mice	Labeled with AlexaFluor680	[111]
cAbVCAM1-5	VCAM-1	Tumor vasculature	*In vivo*	MC38 xenografted mice	Nbs coupled to ultrasound microbubbles *via* biotin/streptavidin bridging	[133]
cAbVCAM1-5	VCAM-1	Atherosclerotic plaques	*In vivo*	ApoE$^{-/-}$mice	Labeled with ^{99m}TC	[113,145]
cAbVCAM1-5	VCAM-1	Atherosclerotic plaques	*In vivo*	ApoE$^{-/-}$mice	Labeled with ^{18}F	[146]
LOX-sdAb	LOX-1	Atherosclerotic plaques	*In vivo*	ApoE$^{-/-}$mice	Labeled with ^{99m}TC	[147]
C17	CEA	Colon carcinoma	*Ex vivo*	Human normal and carcinoma colon epithelium	Nbs conjugated to CdSe/ZnS quantum dots (multiphoton imaging); Nbs conjugated to Qd800 (NIR)	[135,136,139]
Nb-DC2.1 and Nb-DC1.8	Myeloid cells; immature bone marrow-derived DCs	Biodistribution-specific immune cells	*In vivo*	Mice	Labeled with ^{99m}TC	[108]
Cl1; Cl3; anti-MMR 3.49	MMR/CD206	Stromal tumor-associated macrophages	*In vivo*	TS/A and 3LL-R xenografted mice	Labeled with ^{99m}TC or ^{18}F (SPECT/PET imaging)	[144]
Cl1; Cl3	MMR/CD206	Inflamed joints in RA	*In vivo*	Mouse CIA model RA	Labeled with ^{99m}TC	[143]
VHH7	Class II MHC	Class II MHC$^+$ cells in tumor tissue	*In vivo*	Mice xenografted with B16, Mel-Juso human melanoma cells, and A20 cells	Labeled with ^{18}F or AF647 *via* a sortase-catalyzed reaction	[71,116]
VHHDC13	CD11b	CD11b$^+$ cells in tumor tissue	*In vivo*	Mice xenografted with B16 or Mel-Juso human melanoma cells	Labeled with ^{18}F *via* a sortase-catalyzed reaction	[116]
NbV4m119	Vsig4: complement receptor of the Ig superfamily	Arthritic lesion	*In vivo*	Mouse CIA model RA	Labeled with ^{99m}TC	[143]

NbV4m119	Vsig4 on Kupffer cells liver	Hepatic inflammation	*In vivo*	Mice undergoing concavalin A-induced hepatitis	Labeled with ^{99m}TC	[355]
ni3A and pa2H	Aβ deposits	AD	*In vivo*	APP/PS1 mice	Labeled with ^{99tm}Tc allows detection of vascular or parenchymal Aβ deposits	[149]
/	YFP	*In vivo* cell tracking in cell-based therapies	*In vivo*	Mice transplanted with YFP/GLuc-expressing cell lines	Labeled with ^{99m}TC: combines sensitivity of bioluminescent imaging and SPECT/CT	[148]
Viruses						
ALX-0171	RSV	RSV infection	Phase IIa	Interventional study in otherwise healthy infants hospitalized with RSV to evaluate safety after inhalation	Trivalent Nb administered via inhalation with a nebulizer	NCT02309320
ARP1	Rhesus monkey RV	RV-induced diarrhea	Phase II/III	Interventional study in healthy children aged 6–12 months to study community-based prevention of RV gastroenteritis with ARP1 as dietary supplement	Rice-based expression system described to allow oral administration of ARP1 via rice	NCT01265355; [246]
VHH1	Rhesus monkey RV serotype G3, strain RRV	RV-induced diarrhea	*In vivo*	Mouse pup model of RV infection	Lactobody as prophylacticum; oral administration; bivalent or tandem co-expression as membrane-bound and soluble Nb	[29,166,168,169]
Nano-85; Nano-25	P domain VP1 capsid protein	Norovirus	*In vitro*	ELISA, X-ray crystallography, EM analysis	Nano-25: GII.10 strain; Nano-85: GII.4, GII.10, GII.12 strains; promotes norovirus particle disassembly	[172]
M6; M7	P domain VP1 capsid protein	Norovirus	*In vitro*	Hemagglutination inhibition assay	M6: multiple strains; M7: GII.4	[171]
H5-VHHb	H5 hemagglutinin	H5N1 influenza	*In vivo*	Intranasal administration Nb, mice infected with NIBRG-14ma virus	Bi- and trivalent constructs	[153]
aHA-7	HA1 hemagglutinin	H5N2 influenza	*In vivo*	Intranasal administration Nb, mice infected with 50 $TCID_{50}$ of influenza virus	Isoleucine zipper domain added	[154]
αNP-VHHs	Nucleoprotein	Influenza A	*In vitro*	MDCK or A549 cells infected with Influenza A	Inhibition nuclear trafficking; intrabody	[159]
Nb6	Nsp9	PRRSV	*In vitro*	MARC-145 cells infected with PRRSV SD16	Inhibition replication; intrabody (cytoplasm)	[160]
Nb D03	HCV E2 glycoprotein	HCV	*In vitro*	HCV-infected producer Huh-7.5 cells	Inhibition of cell–cell transmission	[155]
PEN-V_HH24; PEN-V_HH28; PEN-V_HH41	NS3/4A	HCV genotype 3a	*In vitro*	Human hepatic (Huh7) cells transfected with JFH-1 RNA of HCV genotype 2a	Transbody linked to penetratin	[161]
Nb 190	Rev	HIV-1	*In vitro*	293 T cells infected with pNL4-3	Inhibits viral replication; intrabody	[156–158]
238D2; 238D4 and 238D2-238D4	CXCR4	HIV-1	*In vitro*	Human MT-4, PBMCs or U87 cells infected with HIV-1 strains NL4.3, BaL, and HE	Inhibition of cell entry; inhibition of CXCR4 signaling and chemotaxis	[152]
IH4	Human glycophorin A	HIV	*In vitro*	Autologous red blood cell agglutination assay	Useful as diagnostic; fusion Nb with HIV-1 antigen P24	[230]
sdAb19	HIV-1 Nef	HIV-1	*In vitro*	X-ray crystallization, size exclusion chromatography	Useful for basic research for *in vitro* structural analysis at molecular and cellular level and as tool for small-chemical compound identification	[234]
Multiple	Nucleoprotein	Ebolavirus	*In vitro*	Sandwich ELISA	Useful in diagnostic biothreat assays: MARSA	[226]
KC329705	Nucleoprotein $prN\Delta_{85}$	Hantavirus	*In vitro*	SPR, ELISA	Useful in diagnostic biothreat assay	[227]

TABLE 1 (*Continued*)

Product name	Target	Disease	Evidence		Comment	Refs; clinical trial identifier
			Proof-of concept	Model		
Nb 7; Nb 16; Nb 93	H5N1 influenza	H5N1 influenza	*In vitro*	Double Nb sandwich ELISA	Useful as diagnostic; one Nb: capture Nb; other Nb: detection Nb (HRP)	[356]
Nb 3; Nb 1	H3N2	H3N2 influenza	*In vitro*	Double Nb sandwich ELISA	Useful as diagnostic; Nb 3: capture Nb; Nb 1: detection Nb (HRP)	[357]
4BL	HIV-1 virion infectivity factor (Vif)	HIV monitoring	*In vitro*	Piezoimmunosensors	Useful as diagnostic and as piezoimmunosensor	[231]
Bacteria						
NbFedF6; NbFedF7	Lectin domain F18 fimbriae	ETEC and STEC	*In vitro*	Villous adhesion assay	Prevention of bacterial adhesion	[174,177]
K609; K922	F4 fimbriae	ETEC	*In vitro*; *in vivo*	Small intestinal segment perfusion test; piglets infected with ETEC: *in vivo* proteolytic stability	*In vivo* activity less than *in vitro* activity; DNA shuffling led to proteolytically stable Nb (K922)	[175,176]
V1; V2; V3; V4	FaeGac adhesin of F4 fimbriae	ETEC	*In vivo*	Weaned $F4R^+$ piglets	Bivalent Nbs fused to IgA, produced in *Arabidopsis thaliana* seeds; oral-based passive immunization	[178]
FlagV1; FlagV6	Flagella	*Campylobacter jejuni*	*In vivo*	*C. jejuni*-infected chickens	Pentabody	[31]
FlagV1M and mutants	Flagella	*C. jejuni*	*In vitro*	*C. jejuni* motility assay	Protease-resistant Nbs	[184]
7G; 9D	Flagella	*Pseudomonas aeruginosa*	*In vitro*	Swimming and biofilm formation plate assay in PAO1 cells	Inhibition of biofilm formation	[185]
Multiple	Biofilm-associated protein	*Acinetobacter baumannii*	*In vivo*	Neutralization assay in BALB/C mice injected with live *A. baumannii*	Inhibition of biofilm formation	[186]
S36-VHH	*Streptococcus mutans* strain HG982	*S. mutans*	*In vivo*	Rat-desalivated caries model	Fusion to nonspecific antimicrobial agent glucose-oxidase	[180,181]
Nb 25	TssM protein of type VI secretion system	Gram-negative bacteria	*In vitro*	SCi-dependent antibacterial assay	Prevention of bacterial toxin secretion	[179]
cAbBCII10; cAbTem13	TEM-1 and BcII β-lactamase	β-lactam-resistant bacterial strains	*In vivo*	Ampicillin-resistance *Escherichia coli*	Led to higher ampicillin sensitivity	[188]
Parental and HMR23 VHHs	UreC subunit of urease	*Helicobacter pylori*	*In vitro*	*H. pylori* urease inhibitory test	Inhibition of urease activity: Nb affinity maturation	[159,189]
Parasites and fungi						
Nb An46 (lytic) and Nb An33 (non-lytic)	VSG	*Trypanosoma brucei*	*In vivo*	Mice and rats infected with monomorphic or pleiomorphic *T. b. brucei* AnTat1.1E strain	Active drug targeting: PLGA or chitosan NPs encoated with Nb An33 and loaded with trypanocidal drug pentamidine; Nb An_33 able to cross BBB	[105,191,199]
Nb An46 and Nb An33	VSG	*T. brucei*	*In vitro*; *in vivo*	Expression and secretion by *Sodalis glossinidius* cells, tsetse flies injected with *RecSodalis*	Prevention of tsetse fly transmission via expression by recombinant bacterial symbiont, *S. glossinidius*; *in vivo* expressed at significant levels in tsetse fly and vertical transmission to tsetse fly offspring	[197,198]
Nb An33-Tr-apoL-I	VSG	Human African *Trypanosoma*	*In vivo*	Mouse models of human African trypanosomiasis (*T. b. rhodesiense*)	Immunotoxin: Nb conjugated to truncated apoL-I, a truncated form of a trypanolytic agent effective against resistant *Trypanosoma* strains	[194,195]

Nb 392	Paraflagellar rod protein	Detection of all trypanosome species	*In vitro*	ELISA, immunoprecipitation	Useful as diagnostic	[200]
Multiple	Cell wall protein Malf1	*Malassezia furfur*	*In vitro*	Phage display in shampoos	Added to medicinal shampoos	[18]
Nb 4218	Myosin tail interaction protein	*Plasmodium falciparum*	*In vitro*	X-ray crystallization	Used as crystallization chaperone	[235]
Detoxification						
NbAahI'F12; NbAahII10; NbAahI'22; and NbF12-10	Toxic venom fractions: AahI' and AahII	*Androctonus australis hector* (Aah) scorpion venom	*In vivo*; preclinical studies	Mice ICV injected with 100 LD_{50} of AahI' or 5 LD_{50} crude venom; pharmacodynamic study in rats	NbF12-10: bispecific Nbs; can also offer curative protection	[202,205,206]
F7Nb	HNc	*Hemiscorpius lepturus* scorpion venom	*In vivo*	Mice ICV or s.c. injected with LD_{100} of HNc		[207]
V_HH2-Fc; C2; and C20	α-Cobratoxin	*Naja kaouthia* venom	*In vivo*	Mice i.p. injected with LD_{100} of α-Cobratoxin	Fused to IgG Fc domain; expressed in *Nicotiana benthamiana*	[208]
RTB-B7/RTA-D10; JNA10 (RTA-F6/RTB-B7); JNA11 (RTA-E5/RTB-D12); and JJX21 (RTA-E5/RTB-B7)	RTA/RTB subunits	Ricin	*In vitro*; *in vivo*	Vero cell cytotoxicity assay; mice lethal ricin challenge	RTB-B7-RTA-D10: Bivalent Nb; passive protection only provided with bivalent, heterodimeric Nbs and not with monovalent counterparts or homodimeric Nbs; also useful as crystallization chaperone	[209–212]
A4.2, A5.2, A20.1, and A26.8	CDTa toxin	*Clostridium difficile*	*In vitro*	Human lung fibroblasts		[216]
ABA ('AH3-E3-E3-AA6')	CDTa/CDTb toxin	*C. difficile*	*In vivo*	Mice infected with epidemic 027 strain	Bispecific, tetravalent Nb comprising two CDTa and two CDTb targeting Nb2	[217]
L+8; I+15.1 g	CDTa/CDTb toxin	*C. difficile*	*In vitro*	ADP-ribosylation in HEK cells and cytotoxicity assays of HT29 cells		[215]
VHH 5G	LPS derived from *Neisseria meningitidis*	*N. meningitidis*	*In vitro*; *ex vivo*	ELISA, immunoprecipitation; LPS binding assay to human monocytes		[213]
LSEBg6, LSEBA6, and LSEBe3	Staphylococcal enterotoxin B	Toxin of *Cholera*	*In vitro*	ELISA, SPR		[214]
JKH-C7; JIK-B8; and VNA2-PA	Anthrax	*Bacillus anthracis*	*In vitro*; *in vivo*	Mice challenged with *B. anthracis* spore infection	VNA2-PA: Bispecific Nb comprising JKH-C7 and JIK-B8; JIK-B8: Blocks receptor	[219]
Multiple bivalent Nbs	Shiga toxin 1 and 2	Shiga toxin-producing *Escherichia coli*	*In vivo*	Mice challenged with LD_{100} of Stx1 and Stx2	Co-administration of efAb promoted clearance of toxin–Nb complex	[218]
DF5; DA5; DA2; A18; H7/B5	Botulinum neurotoxin A and E	*Clostridium botulinum*	*In vivo*	Mice 10 x LD_{50} BoNT/A1 or BoNT/E challenge	Also effective using an adenovirus Nb-expressing vector	[220–222]
SpvB	ADP-ribosylating toxin	*Salmonella typhimurium*	*In vitro*	Cytotoxicity assay in Vero cells, RAW 264.7 infection assay	Intrabody	[223]
	Tetanus toxin and CD11b/CD18 (mac-1)	*Clostridium tetani*	*In vivo*	Mouse tetanus toxin toxicity model	Anti-CD11b/CD18 Nbs coupled to anti-tetanus Nb facilitated clearance of toxin–immunocomplex; strategy can easily be applied to all antitoxin Nbs	[224]
Inflammation/immunology						
ALX-0061	IL-6R	RA/SLE	Phase IIb, RA; Phase II, SLE	Interventional monotherapy study in patients with RA; interventional study in patients with moderate to severe active SLE	Monovalent Nb linked to half-life-extending HSA Nb	[305,307] NCT02287922; NCT02437890
ALX-0761	IL17A/IL17F	Psoriasis	Phase Ib	Interventional multiple ascending dose trial in patients with moderate to severe psoriasis	Bispecific Nb linked to HSA	[235]; NCT02156466
Ozoralizumab (ATN-103)	TNF	RA	Phase II	Interventional long-term safety study in subjects with RA	Concluded with compelling results showing DAS28 remission in 38% and EULAR response in 97% of patients at week 48	NCT01063803

TABLE 1 (*Continued*)

Product name	Target	Disease	Evidence		Comment	Refs; clinical trial identifier
			Proof-of concept	Model		
ATN-192	TNF	RA	Phase I	Interventional study in healthy subjects	PEGylated form of ATN-103	NCT01284036
ALX-0962	IgE	Asthma	Preclinical studies		Program stopped beyond preclinical studies because of insufficient differentiation from competitors	[314,315]
MT1; MT1-MT1 (mouse); TR2; TR2-TR2 (human)	Mouse/human TNF	RA, Crohn's disease, and other TNF-mediated diseases	*In vivo*	Mouse CIA model RA, DSS-induced chronic colitis models and colitic $IL10^{-/-}$mice	MT1; TR2: monovalent; MT1-MT1; TR2-TR2: bivalent Nbs linked to anti-alb Nb; expression in bacteria, yeast, *Arabidopsis thaliana*, rice-based lactobodies	[242–244,358]
TROS	Human TNFR1	TNF/TNFR1-mediated diseases	*In vitro*; *ex vivo*; *in vivo*	Biopsies of human inflamed colonic tissue from patients with Crohn's disease; uPA/SCID transgenic mice with partially humanized liver	TROS: biparatopic Nb coupled to anti-albumin Nb	[30]
163E2; 163D2; 2B2; 127D1; 127D1-35GS-163E3	CXCR2	Acute and chronic inflammatory disease	*In vitro*	Binding assays in CHO-CXCR2 cells, human neutrophil whole-blood shape and chemotaxis assay	163E2; 163D2; 2B2; 127D1: monovalent Nbs; 127D1-35GS-163E3: biparatopic Nb linked with $[GGGGS]_7$ linker targeted to completely distinct epitopes; Nbs displayed inverse agonism	[249]
Anti-IgG Nb	IgG	Auto-IgG-mediated diseases (SLE)	*In vitro*; *ex vivo*	SPR, plasma from patients with SLE applied to a anti-IgG Nb Sepharose column	Useful as ligand in plasmapheresis columns used to treat patients with auto-IgG-mediated disease	[250]
Nb 14	MMP8	Sepsis	*In vivo*	LPS-induced endotoxemia	Nb delivered *via* electroporation	[257]
VHH 5G	*N. meningitidis* LPS	Sepsis	*In vitro*; *ex vivo*	ELISA, immunoprecipitation, LPS-binding assay to isolated human monocytes	Useful as ligand in selective plasmapheresis column used to treat patients with sepsis	[213]
Nb 2;Nb 3	Human procalcitonin	Sepsis	*In vitro*	Sandwich ELISA	Sensitive assay to diagnose disease, biomarker; Nb 2: biotinylated capture Nb; Nb 3: detection Nb (HRP)	[252,253]
C21; C28	Fc-γ-RIII (CD16)	Recruitment of Fc-γ-RIII killer cells to boost cytotoxicity of immune cells	*In vitro*	Binding assay and IL-2/IFN-γ production assay in Jurkat T cells, NK cells, and PMN cells	Recruitment of Fc-γ-RIII killer cells to induce cytotoxicity; selective engagement of Fc-γ-RIIa on cells strongly induced IL-2 and IFN-γ production	[67]
Nb 4.11; Nb 96; Nb 122	Murine leptin receptor; human TNFR1 and PD-L2	Multiple applications antiviral/antitumor therapy or regulatory T cell targeting, DC vaccination	*In vitro*; *in vivo*	Leptin-induced proliferation assays (Ba/F3 cells); IFN antiviral assay with HL116 and HL116-mLR10 cells; phospho STAT1 assay in mice	'Activity by targeting' approach; coupled to mutant cytokines such as IFN-type I (R149A) or mutant leptin	[260]
Nb12-12	Kv1.3 ion-channel	Autoimmune and inflammatory diseases	*In vivo*	Rat DNFB sensitization model for allergic dermatitis	Multivalent Nb; Nb with first-in-class potential	[261]
Dano1; Dano2; Dano3	P2X7 ion-channel	Autoimmune and inflammatory diseases and in oncology	*In vivo*	Mouse glomerulonephritis model induced by antipodocyte serum injection and mouse DNFB-sensitization model for atopic dermatitis	Both agonistic and antagonistic Nbs; best-in-class potential; multivalent Nbs	[265]

s+16a	ART2	Restoration/expansion of T cell population; manipulation of immune response in several inflammatory diseases	*In vivo*	Diabetogenic NOD-CD38$^{-/-}$	Prevention of cell death in Tregs and NK cells in presence of α-galactosylceramide	[264]
Nb DC2.1	Myeloid cells; immature bone marrow-derived DCs	Immunization against viral, cancer, and autoimmune antigens	*In vitro*; *In vivo*	Transduction of human DC and macrophages; intranodal injection of Nb DC2.1-displaying LVs that encode FLuc	*In vivo* bioluminescence imaging demonstrated *in situ* transduction of LN cells; immune response mediated by transduction of DCs upon LV transduction	[267,359]
Neurology						
Biparatopic Nb BI 1034020	Aβ	AD	Phase I	Interventional study in male healthy volunteers i.v. or s.c. injected with Nb	Clinical trial stopped prematurely because of drug-related severe adverse effects	NCT01958060
B10	Amyloid fibrils	AD	*In vitro*	Dot blot, ThT fluorescence assay, X-ray diffraction	Distinguished Aβ amyloid fibrils from disaggregated Aβ peptide	[270]
V31-1	Intraneural Aβ oligomers	AD	*In vitro*	Cytotoxicity assay in SK-N-SH cells, ThT fluorescence assay, ELISA	Prevention of Aβ fibril formation	[271]
ni3A and pa2H	Aβ deposits	AD	*In vivo*	APP/PS1 mice	Nb coupled to ^{99m}Tc allowed detection of vascular or parenchymal Aβ deposits	[149]
NbSync2 and NbSyn87	α-Synuclein	PD	*In vitro*	NMR spectroscopy	Provided structural information about fibrillar maturation	[275,276]
iVHH	Htt	HD	*In vitro, ex vivo*	ELISA, immunoprecipitation on human brain tissue	*In vivo* detection of human htt	[279]
Nb 3F5	α-Helical domain of mutant PABN1	OPMD	*In vitro*, *ex vivo*, *in vivo*	Cell proliferation assay in Hela and Cos-1 cells, *ex vivo* staining of human muscle tissue, OPMD model in *Drosophila*	Intrabodies with high therapeutic value	[280,281]
FAF Nb1-3	C68 fragment of mutated gelsolin	Gelsolin amyloidosis	*In vitro*; *in vivo*	Heterozygous D187N gelsolin transgenic Nb-expressing mice	Expressed as intrabody	[283,284]
PrioV3	Cytosolic PrP^{Sc}	Prion disease	*In vitro*; *in vivo*	Susceptible N2a cells; neurotoxicity studies, brain biodistribution and residence time	Nb crossed BBB via clathrin-mediated transport; potential valuable therapeutic tool for treatment of protein-misfolding disease	[298,299]
Nb 24	Fragment of truncated β2-microglobulin (ΔN6β2m)	Dialysis-related amyloidosis	*In vitro*	X-ray diffraction	Can be used as crystallization chaperone	[301]
Nb 484	MoPrP(23–230)	Transmissible spongiform encephalopathy	*In vitro*	SPR, amyloid seeding assay in ScGT1 cells	Inhibits prion propagation; can be used as crystallization chaperone	[303]
	Bax	Oxidative stress	*In vitro*	Oxidative stress model in SHSY-5Y cells	Expressed as intrabody	[287]
Bone disorders						
ALX-0141	RANKL	Postmenopausal osteoporosis, RA, cancer-related bone metastasis	Phase II	In patients with breast cancer-derived bone metastasis	Trivalent Nb	[328]

[a] *Abbreviations*: ART2, ADP-ribosyltransferase 2; Aβ, Amyloid beta; hMSC, human mesenchymal stem cells; HUVEC, human umbilical vein endothelial cells; ICV, intracerebroventricular; MARSA, monoclonal affinity reagent sandwich assay; MLD, minimum lethal dose; Nsp3,4A and 9, nonstructural protein 3, 4A, and 9; PMN cells, polymorphonuclear cells; $TCID_{50}$, tissue culture infectious dose 50; ThT, thioflavin T; VP1, viral protein 1.

TABLE 2

Overview of companies that use or develop Nb-based technologies.

Company name	Services and/or applications	Company (headquarters)
121 Bio	Develops approaches for enzymatic conjugation and Nb development. Their aim is to advance immunotherapy, patient selection, and management, thereby meeting important unmet needs	Biopharmaceutical start-up company (USA)
AbbVie	Has a global license agreement with Ablynx for development of ALX-0061 for RA and SLE	Pharmaceutical company (USA)
Ablynx	Has proprietary and partnered programs with Nbs used as therapeutics for cancer, inflammation, and immune diseases	Biopharmaceutical company (Belgium)
Agrosavfe	Develops Agrobodies as biopesticides to control pests and diseases on crops and harvested products (crop protection)	Spin-off of VIB (Flanders Institute for Biotechnology) (Belgium)
Boehringer Ingelheim (BI)	Has a global strategic alliance with Ablynx for the discovery, development, and commercialization of up to ten different Nb therapeutics	Pharmaceutical company (Germany)
Camel-IDS	Involved in the development, preclinical validation, and clinical translation of Nbs as molecular-imaging probes for cancer in the form of targeted radionuclides	Spin-off company of VUB (Free University Brussel) (Belgium)
ChromoTek	Develops and commercializes better tools for cell biology and proteomics; offers Nano-Traps (Nbs for immunoprecipitation), Nano-Boosters (Nbs directed against GFP or RFP coupled to superior fluorescent dyes), or Chromobodies (intracellular Nbs fused to a fluorescent tag to detect endogenous proteins)	Spin-off of the Ludwig Maximilians University Munich (Germany)
ConfoTherapeutics	Offers Confobodies for drug discovery programs on GPCRs, addressing unmet medical needs	Drug discovery company: Spin-off of VUB (Vrije Universiteit Brussel) and VIB (Belgium)
Creative Biolabs	Constructs immunized and non-immune libraries, thereby generating Nbs against any target; additionally offers single-domain maturation services	Biotechnology company (USA)
EddingPharm	Has an exclusive royalty-bearing license with Ablynx for the development and commercialization of ALX-0141 and ATN-103 for all indications, including RA, in mainland of the People's Republic of China, the Hong Kong and Macao Special Administrative Regions, and Taiwan	Pharmaceutical company (China)
GenScript	Offers a comprehensive Nb service for research and therapeutic applications via selection and optimization of Nbs from immunized llamas of synthetic libraries	Biotechnology company (USA)
Genzyme (Sanofi)	Has an exclusive research collaboration with Ablynx for the investigation and development of Nbs to treat MS	Pharmaceutical company (USA)
Hydribody by Hybrigenics Services	Selects and validates antibodies derived from a fully synthetic humanized VHH antibody library; provides Nbs fused to the tag of choice	Biopharmaceutical company (France)
Merck & Co (Merck Sharp & Dohme)	Has a research and license agreement with Ablynx; licensed for the preclinical and clinical development and commercialization of Nbs for immuno-oncological applications	Pharmaceutical company (USA)
Merck KGaA	Has entered into four collaborations with Ablynx to co-discover and co-develop Nbs against targets in immunology (rheumatology) and oncology	Pharmaceutical company (Germany)
Novartis	Has a license agreement to discover and develop Nb-based therapeutics against several disease targets that are difficult to address with conventional antibodies	Healthcare company (Switzerland)
Novo Nordisk	Has a global exclusive collaboration and licensing agreement with Ablynx for the discovery and development of novel multi-specific Nb drug candidates	Pharmaceutical company (Denmark)
ProSci Inc	Offers custom single-domain antibody services from immunization to production	Biotechnology company (USA)
QVQuality	Develops custom-made Nb-based imaging agents for imaging and research purposes; provides monoclonal selection of VHH, custom immunization of llamas, and large-scale production for clinical testing; additionally, provides C-direct labeling of Nbs with imaging agent of interest	Biotechnology company (The Netherlands)
Taisho Pharmaceutical Co.	Has an exclusive license agreement with Ablynx for the development and commercialization of ozoralizumab in Japan	Pharmaceutical company (Japan)

currently on the market [38]. Although they can evoke antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) via the immunoglobulin G (IgG) Fc-effector domain, the large size of mAbs limits tumor penetration and binding to targets that are difficult to reach [39,40]. Additionally, their high costs limit their use on a wide scale in oncology. Nbs can be produced more economically during discovery and have been introduced into cancer treatment strategies. In contrast to mAbs,

they are distributed homogeneously in tumor tissue [41]. As anticancer biological agents, Nbs can be used as antagonistic drugs, but due to the absence of an Fc-effector domain, their efficacy as a pure immunotherapeutic is inferior to that of mAbs [43]. Nevertheless, the absence of the Fc-domain in Nbs can reduce the number of unwanted immune-mediated adverse effects that are elicited by this domain. However, more promising approaches were recently introduced, such as their use as targeting moieties linked to effector domains and radionuclides. Additionally, they can be decorated on nanoparticles (NPs) that can be filled with other (small-molecule) anticancer drugs for active targeting to the specified tumor cells [42]. Nonetheless, two aspects need to be considered when such Nb – effector domain complexes are generated. First, the stability of the Nbs, which are reportedly very stable, might be attenuated [38] and, second, a change in Nb-binding affinity has been reported [43].

Direct antagonistic effects

Nbs were first developed as antagonists that bind extracellular proteins that have a unique expression pattern or are overexpressed on tumor cells, such as human epidermal growth factor receptor 2 (HER2), which is overexpressed in approximately 15–30% of breast cancers and 10–30% of gastric/gastroesophageal cancers [44]. Antagonistic Nbs can control tumor cell growth and proliferation and are able to induce apoptosis by suppression of signal transduction. Consequently, monovalent and bivalent epidermal growth factor receptor (EGFR, also known as HER1)-specific Nbs and biparatopic Nbs that target different epitopes of EGFR have been introduced. These Nbs successfully inhibit solid tumor growth both *in vitro* and *in vivo* [45–47]. Nbs targeting hepatocyte growth factor (HGF) or its receptor (c-Met) have also proven their efficacy in eradicating solid tumors and multiple myeloma by preventing the signaling cascade [48,49] (extensively reviewed in [38,42]). Targeting two different tumor-related proteins simultaneously, as has been suggested for EGFR and HGF, is assumed to have stronger antitumor effects because simultaneous blockage achieved by linking two Nbs, would, for example, overcome acquired resistance to inhibitors of the signaling pathways [48]. In this regard, a bifunctional multivalent antibody comprising Nb and affibody moieties (MaAbNA; Fig. 1a) in which an anti-EGFR Nb is coupled to two anti-HER2 affibodies, has been developed [50]. Affibodies are Ab mimetics comprising three α-helices derived from protein A of *Staphylococcus aureus*. Additional coupling of the MaAbNA to the chemotherapy drug doxorubicin enhanced the tumoricidal properties even more. Recently, an anti-EGFR Nb fused to the tumor-penetrating and cell-internalizing peptide internalizing (i) RGD improved *in vivo* tumoricidal actions compared with non-fused Nbs as a result of better tumor penetration [51]. Additionally, the fusion protein also improved the efficacy of a chemotherapeutic drug, paclitaxel, when it was co-administered [52]. In addition, CapG is a constituent of the actin cytoskeleton and its overexpression is involved in tumor dissemination and metastasis. To prevent cancer metastasis, anti-CapG Nbs designed as transbodies (Fig. 1a) have been developed. Here, the Nbs were equipped with the T3S signal sequence of the *E. coli*-secreted protein F (EspF) and were delivered via injection into (cancer) cells by bacteria that harbor a functional type III protein secretion system [53,54]. Coupling to a variant of substance P (SP) has also been proposed to deliver cargo intracellularly via receptor-mediated delivery when SP interacts with its receptor, neurokinin-1, which is overexpressed in many cancers [55,56]. Nbs targeting the trypsin-like serine protein urokinase-type plasminogen activator (uPA) have also been generated because this protein has major roles in adhesion, migration, invasion, and metastasis [57]. Metastasis can also be prevented by targeting angiogenesis in tumors. In this context, drugs directed against chemokine receptors have gained attention because these receptors are involved in proliferation, metastasis, and angiogenesis. Nbs against the G-protein-coupled chemokine receptor CXCR7 effectively reduced head and neck cancer by inhibiting angiogenesis [58]. Additionally, Nbs directed against vascular endothelial growth factor (VEGF)-A and VEGF receptor 2 (VEGFR2) were developed because these receptor are overexpressed in tumor vasculature and suppression of signal transduction may inhibit neovascularization and tumor metastasis [59–61]. Inhibition of the angiogenesis pathway with a humanized trispecific Nb that blocks both VEGF and angiopoietin-2 (Ang2), another importer player in angiogenesis, was found to be superior in patient-derived xenograft studies and *in vivo* in tumor-bearing mice compared with inhibition of the individual pathways [62]. Nbs against the metastatic factor carbonic anhydrase IX (CAIX) have also been reported. This enzyme is expressed under hypoxic conditions and its inhibition could reduce malignancy and tumor cell survival [63].

Another elegant way to prevent cancer progression is by intervening at the level of the brain–adipocyte axis, of which leptin is an important mediator [64]. Leptin is involved in multiple peripheral pathways (e.g., as a mitogen) and its levels are positively correlated with the cancer risk, indicating that Nbs neutralizing the leptin receptor (LepR) might be useful [65]. The anticancer effect was demonstrated in a melanoma mouse model in which local administration of an anti-LepR Nb inhibited tumor regression. However, since systemic administration only leads to blockade of the important central actions of leptin without having antitumor properties, development of peripherally acting Nbs is recommended [66].

As mentioned above, the lack of an Fc-effector domain and subsequent ADCC limits the use of Nbs as anticancer agents. In 2008, two Nbs (C21 and C28) isolated from a llama immune library were found to specifically bind Fc-γ-RIIIa, an Fc-receptor class that binds to the Fc portion of Abs with important antitumor effects [67]. This receptor is found on natural killer (NK) cells, mononuclear phagocytes, and neutrophils, and the advantage of this approach is that these Nbs do not engage or compete with serum IgG for receptor binding, unlike conventional Abs. Moreover, their cytotoxic activity is independent of Fc glycosylation and Fc-γ-RIIIa polymorphism and, unlike anti-CD3 bispecific antitumor Abs, they do not engage regulatory T cells because these cells do not express Fc-γ-RIII. Thus, upon Nb binding to the Fc-γ-RIII of NK cells, an agonistic reaction is elicited that boosts an interferon (IFN)-γ response. Fusion of this Nb with an anticarcinoembryonic antigen (CEA) Nb led to *in vitro* lysis of CEA^+ tumor cells because of NK cell activity, as well as to *in vivo* reduction of tumor growth in CEA^+ tumor xenografted mice [68]. This new concept boosts ADCC and is a promising approach to optimize not only Nb-based antagonistic anticancer therapies, but also other Nb-based therapies, such as for acute brain injury [69].

Nbs linked to effector moieties

Given that Nbs lack Fc-effector activity, they can be used as vehicles to actively target cargo, such as drugs or macromolecules, specifically to the tumor or other target cells. This heightens the antitumor effects of these drugs and reduces systemic toxicity because of the local enrichment. The procedure to generate such immunotoxins (Fig. 1a) is reviewed in [42]. When an antagonistic Nb is linked to an effector moiety, the advantages of the two molecules are combined, namely the high specificity, intrinsic therapeutic effect, and high tissue penetration of the Nb with the tumor-destructive properties of the toxin. This is illustrated by the efficient tumor inhibition obtained by VEGFR2 Nbs conjugated to *Pseudomonas* exotoxin A [70] and with the therapeutically active major histocompatibility complex (MHC)-II Nbs conjugated to DM1, a potent inhibitor of microtubule polymerization [71]. Promising results were also obtained with neural stem cells (NSC) transfected with a lentiviral plasmid encoding secreted bivalent anti-EGFR Nbs and the cytotoxic TRAIL recombinant protein. It was shown that NSC specifically homed to tumors and, subsequently, the engineered NSC released the Nb-TRAIL construct at the tumor site. Thereby, the construct targets both the cell death and cell proliferation pathways and inhibits invasiveness of glioblastoma tumor cells [72]. Another excellent example is a Nb conjugate providing selective activation of a co-administered anticancer prodrug, whereby a Nb against tumor-associated CEA was coupled to β-lactamase. The combined molecule was carried specifically to the surface of CEA-expressing tumor cells, where β-lactamase activated the co-administered anticancer cephalosporin nitrogen mustard prodrug [73]. Additionally, in the MaAbNA-PEG2000-adriamycin construct, several beneficial antitumorigenic approaches are combined: two different biomarkers are targeted (HER2 and EGFR1) and the construct is coupled to the anticancer drug doxorubicin to further increase the antitumor efficacy [50]. An immunotherapeutic can also be designed that destroys tumor cells via antibody-dependent cellular cytotoxicity, as seen with a HER2-specific Nb equipped with a molecule that recruits anti-dinitrophenyl Abs, which subsequently provoked tumor cell destruction [74].

The newest advances in circumventing tumor evasion were achieved by engineering T cells with novel chimeric antigen receptors (CARs) using Nbs as binding and/or targeting moieties (Fig. 1a). T cell therapy involves the adoptive transfer of T lymphocytes specific for a tumor antigen into patients with cancer. Although this approach has problems such as the immunogenicity of the antigen-binding domain, this can be overcome with Nbs [75,76]. Thus, this approach combines the advantages of T cell-based immunotherapy (i.e., efficient tumor penetration, cytokine release, and cytotoxicity) and Nb-based immunotherapy (i.e., small size, low immunogenicity, and high specificity for tumor-associated antigens) [77]. A hinge spacer links the Nb to the intracellular signal transduction domains of T cell receptors, comprising conventional binding domains and co-stimulatory endodomains, such as CD3ζ, OX40, and CD28. Mucine 1 (MUC-1) [78], tumor-associated glycoprotein 72 (TAG-72) [76], and HER2-specific [79] T cells have already been introduced that highly express stable CARs *in vitro*. Additionally, CARs equipped with a Nb specific for the melanocyte differentiation antigen glycoprotein 100 (Gp100) were expressed by transgenic human primary T cells and could suppress an established melanoma *in vivo* [80].

Nbs incorporated into drug delivery systems

Nbs can also be chemically attached to the surface of other drug delivery systems, such as nanosized drug carriers or NPs, which can then be encapsulated with nonspecific drugs for active delivery to the site of interest. This is an attractive approach because it protects the body against systemic toxicity and allows solubilization of hydrophobic drug in hydrophilic structures, such as liposomes or micelles (Fig. 1b). Additionally, it permits administration of larger drug doses simultaneously, which could reduce the administration frequency and immunogenicity [42]. When antagonistic Nbs are used, the Nb itself also inhibits tumor growth and in the absence of other drugs; thus, Nbs can also have a dual function in such cases. An additional advantage of this larger Nb–NP is its increased circulation time, although this is accompanied by poorer but sufficient tumor diffusion. Nevertheless, tumor accumulation will still be possible based on the enhanced permeability and retention (EPR) effect and via lymphatic drainage [38,81].

Multiple NPs and drug carrier systems have been described, such as liposomes functionalized with Nbs via maleimide-PEG-DSPE linkers and micelles conjugated to Nbs via polymer chains at the end of hydrophilic PEG-blocks. Furthermore, cross-linked albumin NPs decorated by Nbs (NANAPs) have also been reported (Fig. 1b) [42].

Important advances have been made with a Nb coupled to a drug delivery system that targets EGFR (Ega1). There are reports of constructs in which the Nb is linked to PEG-liposomes (immunoliposomes; Fig. 1b). Loading the liposomes with an insulin growth factor 1R (IGF-1R) kinase inhibitor provided a dual-active nanomedicine that simultaneously blocked both EGFR and IGF-1R [82,83]. Additionally, conjugates to micelles containing the chemotherapeutic doxorubicin [84], or NANAPs filled with a multikinase inhibitor [85] have been created, of which led to EGFR downregulation and subsequent tumor cell proliferation inhibition. Another EGFR-targeting Nb was biotinylated and linked via streptavidin to a bionanocapsule (Fig. 1b). Under normal conditions, this hollow NP only allows targeted drug or gene delivery to hepatocytes, but following biotinylation, it is redirected to target EGFR-expressing tumor cells as a result of the Nbs [86]. Also interesting are albumin NPs targeting the HGF receptor (HGFR or Met) (anti-Met NANAPs), because Met expression in cancer is correlated with poor prognosis [87]. This approach results in Met downregulation because of receptor-mediated uptake and subsequent lysosomal degradation of the NP by the Met-expressing cells [88].

A new interesting class of carrier systems is the polymersome (Fig. 1b), which architecturally resembles liposomes but is highly stable and can encapsulate larger amounts of hydrophilic drugs compared with micelles. This makes them particularly interesting for the delivery of cargo intracellularly or for the controlled release of drugs. As an example, tumor vessel-targeting polymersomes decorated with Nbs that target PlexinD1 (a transmembrane protein overexpressed in tumor vasculature) or polymersomes coupled to HER2 Nbs have been developed [89,90].

Finally, extracellular vesicles (EVs) (Fig. 1b) are increasingly recognized as the newest system to transfer biological cargo inside cells because they are rapidly taken up by the cells; however, they do not target specific cell types and are rapidly cleared [91].

Incubation of EVs with PEGylated micelles conjugated with an EGFR-specific Nb led to incorporation of the Nb-PEG-micelles into the EV membranes. Thus, the cells can be effectively targeted (e.g., tumor cells) and the circulation time of the EVs is increased, all without altering the morphological and biophysical characteristics of the vesicles [92].

Nbs in targeted radionuclide therapy and photodynamic therapy

Radioimmunotherapy (RIT) is the combination of radiation therapy with Ab immunotherapy and has become an attractive strategy in cancer treatment because it allows the selective destruction of cancer cells and constitutes less invasive radiotherapy: the Ab recognizes and binds the surface of the primary tumor site and disseminated disease tissue and thereby delivers high doses of radiation directly to the tumor without any damage to healthy tissue. Currently, there is only one FDA-approved radiolabeled anti-CD20 mAbs on the market (^{90}Y-ibritumomab tiuxetan), and 44 radiolabeled mAbs are in clinical trials for the treatment of solid tumors and hematological malignancies [93]. Unfortunately, the use of Abs again has important drawbacks, such as poor tumor penetration and undesirable pharmacokinetics of the targeting vehicles, such as an overly long half-life. By contrast, radiolabeled Nbs accumulate specifically at the target site, which limits the toxicity to healthy tissue, and are cleared rapidly from the blood [94]. However, this rapid renal clearance results in kidney retention, where it might lead to unwanted radiation damage. Approaches to reduce such kidney retention are reviewed in [94]. Radiotracers in current clinical use are predominantly β-emitting radioisotopes, mainly yttrium-90 (^{90}Y), iodine-131 (^{131}I), and lutetium-177 (^{177}Lu), but α-emitters are also beginning to emerge [95].

Efforts have been made to treat multiple myeloma with ^{177}Lu-labeled anti-idiotypic Nbs against M-proteins. These proteins are mAbs produced by malignant plasma cells and targeting them limits disease progression [96]. D'Huyvetter *et al.* generated a HER2-specific Nb (2Rs15d) linked to ^{177}Lu using the bifunctional 1B4M-DTPA chelator with a metal-binding moiety that interacts with the radionuclide and a chemically reactive functional group interacting with the Nb. To overcome kidney nephropathy because of unwanted irradiation caused by kidney retention, the Nb was co-infused with the plasma expander Gelofusin in mice bearing small, established HER2$^+$ tumors. This led to almost complete blockade of tumor growth and a significant difference in event-free survival between the treated and untreated mice [94]. Another anti-HER2 Nb (5F7GCC) was radio-iodinated with the residualizing agent *N*-succinimidyl 4-guanidinomethyl 3-$^{125/131}$I-iodobenzoate (*I-SGMIB) [97] or with *N*(ε)-(3-[^{131}I]iodobenzoyl)-Lys^5-*N*(α)-maleimido-Gly^1-GEEEK ([^{131}I]IB-Mal-D-GEEEK) [98]. Labeling with ^{131}I, as in the ^{131}I-SGMID-Nb-conjugates, yielded a new promising conjugate for targeting β-particle radiotherapy, because this labeling exhibited favorable radiation dosimetry [97].

In contrast to RIT, in which radioisotopes are used to kill the cancer cells, photodynamic therapy (PDT) induces cell death by activating a photosensitizer (PS; Fig. 1a) by light exposure. This treatment therapy is considered to be minimally invasive and nontoxic. To actively target PS, mAbs or Nbs can be used, an approach known as 'photoimmunotherapy' (PIT). Anti-EGFR monovalent or biparatopic Nbs linked to a traceable PS (IRDye700DX) combine molecular imaging with cancer therapy and are specific and potent partially because of internalization. This is an excellent example of how this technology is paving its way to clinical application [99]. A more recent innovative approach to destroy tumor cells is with branched gold NPs (Fig. 1b). Given their large absorption cross-section, gold NPs are photothermal and can destroy tumor cells upon laser irradiation because of consequent heat production. It has been shown that anti-HER2 Nbs conjugated to branched gold NPs accumulate on the surface of HER2-expressing tumors and can effectively be used as antigen-targeted photothermal anticancer agents [100].

Nbs used for *in vivo* medical imaging

The use of radiopharmaceuticals in medical imaging has become widespread [101]. This technique enables not only easy, non-invasive investigation of biological processes, but also early detection of disease and monitoring of disease progression and response to therapy [102,103]. Several imaging techniques have already been incorporated into daily clinical use: nuclear imaging [e.g., single photon emission computed tomography (SPECT) and positron emission tomography (PET) using radionuclides, such as ^{99m}Tc, ^{89}Zr, or ^{68}Ga], optical imaging [near-infrared (NIR) using NIR fluorescent dyes], and ultrasound imaging using microbubbles (Fig. 1b) [104,105]. PET scanners have higher image resolution than SPECT, and NIR imaging requires a shorter time than PET or SPECT. NIR is also more sensitive and safer because it uses non-ionizing radiation [42], however, although NIR already has better tissue penetration, it is still limited to a few millimeters and, thus, is mainly applicable for surface-located therapies [106].

Targeted NIR dyes and functionalized microbubbles as *in vivo* molecular imaging tracers are not yet on the market and targeted SPECT or PET radionuclides could be optimized. Again, the Nb platform is well suited for this purpose for the same reasons as those mentioned in section *Nbs in targeted radionuclide therapy and photodynamic therapy* [93,107]. Nbs rapidly and specifically bind to tumor antigens, and unbound Nbs are quickly cleared from the blood. In this regard, the use of short-lived PET-radioisotopes, such as gallium-68 (^{68}Ga, $t_{1/2}$: 68 min) or fluorine-18 (^{18}F, $t_{1/2}$: 110 min) [108], is favorable because this significantly lowers the radiation burden in patients. Additionally, as early as 1 h post-injection, the target:nontarget (healthy tissue) ratio of the tracer is higher than can be obtained with Abs [109,110]. Such high ratios are recommended because nonspecific binding and high background can make interpretation of the images difficult or faulty. Thus, Abs are not always suitable for this purpose because they have a long half-life and slow diffusion rate; therefore, the optimal contrast between the target and surrounding tissue is obtained only after 2–4 days, necessitating the use of long-lived and toxic radioisotopes, such as ^{124}I and ^{89}Zr [107]. Finally, Bannas *et al.* compared Nbs with mAbs labeled with AlexaFluor 680 for *in vivo* T cell imaging with NIR fluorescence and concluded that Nbs are best suited for non-invasive short-term use [111]. However, again, the disadvantage of the quickly cleared Nbs is the intense kidney retention, which makes it difficult to observe lesions in nearby organs [112], although efforts have been made to reduce the retention by co-injecting the positively charged plasma-expander GeloFusin [113]. Renal uptake can also be diminished by removal of the his$_6$-tag at

the C-terminal end of Nbs, although this tag facilitates purification and is highly suited for easy site-specific labeling with radioisotopes such as ^{99m}Tc [114]. In absence of the his_6-tag, site-specific labeling of the Nbs can be achieved with Sortagging, without comprising their antigen-binding sites. Sortagging is a versatile chemo-enzymatic sortase-catalyzed transacylation reaction that leads to robust and reproducible labeling of proteins that are provided with a C-terminal sortase-recognition site, such as LPXTG, at the place where the tag is desired [115–118]. Finally and importantly, Nbs should not exert any agonistic activity, because this could lead to adverse pharmacological effects during imaging [119].

In oncology, Nbs targeting HGF [48], HER2 [98,120–123], prostate-specific membrane antigen (PSMA) [124,125], CEA [126], or CIAX [127] have been developed for non-invasive preclinical screening of, for example, breast cancer or prostate cancer, and for monitoring cancer therapy by SPECT/CT or PET/CT [102,128]. As already mentioned, overexpression of EGFR is found in many types of human epithelial cancer and different visualization techniques offer good signals and distribution. Conjugation of EGFR-specific Nbs to ^{99m}Tc [114,129] or ^{68}Ga [130] for nuclear imaging, or to the fluorophore IRDye800CW for NIR [109] have been described. A bimodal platform using small unilamellar vesicles (sULVs) (30-nm diameter) provided more sensitivity because it combines two imaging techniques, magnetic resonance imaging (MRI) and optical imaging. The vesicles were loaded with Gd, a contrast agent for MRI, linked to the NIR dye Cy5.5 (to enable optical imaging), and finally functionalized with a Nb against insulin-like growth factor-binding protein 7 (IGFBP7), which is overexpressed in brain tumor vessels. This approach combines the anatomical information provided by MRI with the molecular characteristics of the disease provided by the Nbs and NIR. *In vivo* proof-of-principle confirmed that the contrast between brain and tumor tissue 2 h post-injection was higher with the targeted approach than with the untargeted approach [131]. One can also combine two independent and validated tumor markers, for example via co-injection of CAIX and HER2 Nbs conjugated to the same or different fluorophores (IRDyes) to assess the simultaneous expression status of both proteins in breast cancer. This dual-spectral imaging setting resulted in an increased tumor:background ratio and, thus, can improve early detection of heterogeneous tumors [132]. Next, an emerging contrast-specific ultrasound-imaging mode utilizes microbubbles targeted by coupling Nbs to their surface. Hernot *et al.* described vascular cell adhesion molecule-1 (VCAM-1)-specific Nbs coupled to microbubbles via streptavidin-biotin bridging and injection of those microbubbles enabled measurement of the echo intensity in the tumor 10 min post-injection [133]. Recently, nanobubbles were coupled to PSMA-specific Nbs and facilitated prostate cancer imaging. Given that nanobubbles are smaller than microbubbles, they can pass more easily through small (neo)vasculature [134]. Multiphoton microscopy using fluorescent nanocrystals, quantum dots (QD), is another high-quality imaging technique. Hereby, QDs can be functionalized with Nbs in a highly oriented manner, resulting in nanoprobes that can be used to immunolabel tumor-associated biomarkers on tumor cells [135]. An anti-CEA Nb linked to CdSe/ZnS QD clearly discriminated CEA-overexpressing tumor areas from normal tissue [135,136] and HER2-specific QDs were also shown to have superior performance in low-expressing lung cancer cells compared with mAbs conjugated to conventional dyes [137]. CEA-specific Nbs conjugated to a NIR QD (Qd800) are also available and it was shown that Qd800-conjugated Nbs against wild-type and mutant EGFR (EGFRvIII), which is detected in a high percentage of brain cancers, could accurately determine the level of tumor aggressiveness and resistance [138,139].

Molecular imaging can also be helpful for purposes other than diagnosis or disease monitoring in oncology. For example, NIR imaging has been successfully introduced during image-guided surgery. The anti-EGFR Nb 7D12 conjugated to IRDye800CW provided the surgeon with real-time visualization of tumors during resection of orthotopic tongue tumors and cervical lymph node metastases [140]. In immunology, Nb-based molecular imaging may be valuable for diagnosis and for drug efficacy assessment, for example by visualization of inflamed areas. In this context, De Groeve *et al.* developed two Nbs with different cellular specificities against bone marrow-derived cells. Imaging of the *in vivo* distribution of myeloid cells can be useful as an *in vivo* sensor of the status of ongoing immune and inflammatory responses in cancer or autoimmune diseases [108,116]. Furthermore, their potential can be extended to other diseases in which activated inflammatory cells have crucial roles in pathogeneses, such as type I diabetes mellitus and atherosclerosis [141]. For example, Nbs directed against the macrophage-mannose receptor (MMR) or against a complement receptor of the Ig superfamily (CRIg), which is expressed on rheumatoid arthritis (RA) macrophages, have been developed to track macrophages in the synovial fluid of arthritic joints [142,143]. This enables the prediction of which patient will develop clinical symptoms before onset of macroscopic clinical symptoms. Approaches to track macrophages can also be applied in cancer research. One example is the use of ^{99m}Tc or ^{18}F-labeled anti-CD206/MMR Nbs, which can be used to visualize the strongly proangiogenic tumor-associated macrophages residing in tumor stroma [112,144]. In another example, anti-CD11b and anti-class II MHC Nbs were site-specifically labeled with ^{18}F or the NIR dye Alexa Fluor 647 (AF647) in a sortase-catalyzed reaction. The targeted CD11b$^+$ and MHCII$^+$ cells can serve as sentinels to detect the tumor tissue they surround or invade and, consequently, the labeled Nbs can track disease evolution [71,116]. Additionally, with the generation of Nbs against VCAM-1 conjugated to ^{99m}Tc or ^{18}F, accurate and non-invasive tracers were developed to target vulnerable atherosclerotic plaques during atherosclerosis [113,145,146]. Atherosclerotic plaques can also be visualized using ^{99m}Tc-conjugated Nbs that target the vulnerable Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) [147]. In addition to the monitoring of disease evolution, the long-term efficacy of cell-based therapies, which are potentially useful after myocardial infarction, can also be non-invasively evaluated with Nbs. In this approach, the sensitivity of bioluminescent imaging and SPECT/CT imaging is combined when ^{99m}Tc-labeled anti-YFP Nbs are used that track transplanted YFP/GLuc-transfected cells. Ultimately, this could be used as a reporter system for such cell-based therapies [148]. Finally, Nbs are helpful in the diagnosis of neurological diseases, such as Alzheimer's disease (AD). Nb pa2H, directed against amyloid-β deposits and linked to ^{99m}Tc, was detectable in brain 24 h post-injection [149]. Other applications of Nbs in this field have been reviewed elsewhere [102,150].

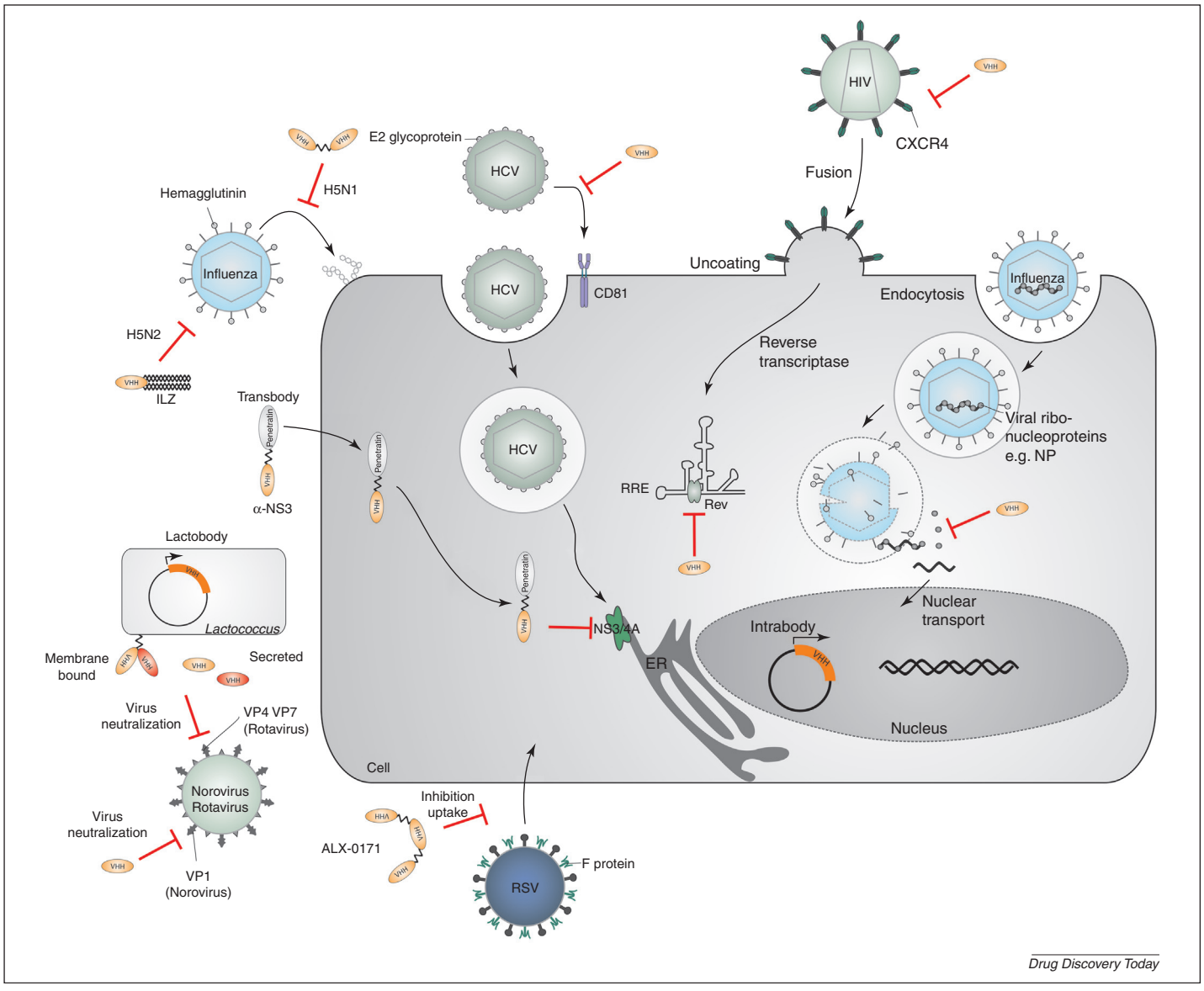

FIGURE 2

Overview of the possible applications of nanobodies (Nb) to target viruses. Nbs can interfere at different levels of the viral replication cycle; for example, by preventing virus–cell attachment, such as Nbs against influenza virus and hepatitis C virus (HCV) that prevent viral uncoating and viral uptake (ALX-0171). Intracellularly expressed Nbs (intrabodies) or cell-penetrating Nbs (transbodies) are useful to interfere with viral nuclear translocation, such as intrabodies targeting the nucleoprotein of influenza virus or the multimerization protein Rev of HIV, and the transbody against NS3/4A of HCV. Prophylactic bivalent Nbs, expressed as lactobodies by modified *Lactobacilli*, protect against viral escape mutants of norovirus. Additionally, Nbs against rotavirus have also been developed.

Nbs to combat infections

Nbs targeting viruses

To fight viruses and prevent their spread, Nbs can interfere at different levels of the viral replication cycle, such as by preventing virus–cell attachment, viral entry, and viral uncoating [151,152] (Fig. 2). Nbs can also be used to broaden our understanding of viral cell–cell transmission. For example, intranasally administered neutralizing Nbs can protect against different influenza virus types (e.g., H5N1 or H5N2) by preventing viral attachment to the host and subsequent viral replication [153,154]. A Nb directed against hepatitis C virus (HCV) specifically prevents viral cell entry and cell–cell transmission [155]. The possibility of expressing Nbs as intrabodies might be an advantage in the treatment of viral infections because they would be able to interfere with intracellular viral actions and disrupt virus replication at an early stage. Indeed, an intracellularly expressed Nb that interferes with Rev multimerization of HIV-1, a protein that is involved in nuclear trafficking of viral mRNAs, efficiently inhibited this crucial step in virus replication [156,157]. Moreover, this Nb prevented replication of different HIV subtypes, pointing towards broad-spectrum anti-HIV activity. Additionally, Nbs are useful in the study of the mechanisms of oligomer assembly of HIV [158]. In other studies, a Nb expressed in the cytosol that targets the nucleoprotein of influenza virus potently inhibited nuclear translocation [159] and another Nb expressed in the cytoplasm blocked the replication of porcine reproductive and respiratory syndrome virus (PRRSV) [160]. However, the feasibility of the intrabody approach in patients as treatment has been questioned. Recently, transbodies were

described with serine protease-specific Nbs that were molecularly linked to a cell-penetrating peptide, penetratin (Fig. 1). These humanized anti-HCV Nbs effectively penetrated the cell and markedly reduced HCV RNA replication [161].

Nbs can be formatted by the multimerization of different VHH fragments that target different epitopes, or for example, by the addition of an isoleucine zipper domain to monovalent Nbs, which leads to post-translational trimerization of the Nb. These are attractive approaches because they not only lead to broader neutralization capacities because of the increased avidity and affinity, but might also prevent virus escape [154,162–164]. Other viruses that have been tackled by Nbs are extensively discussed in [165].

Prophylactic Nbs can be generated too, for example by generating modified lactobacilli that produce VHH antibody fragments, called 'lactobodies' (Fig. 1a). Oral administration of lactobodies expressing surface-anchored anti-rotavirus (RV) Nbs might be prophylactic against RV-induced diarrhea [29,166,167]. *Lactobacillus paracasei* expression of bivalent Nbs or co-expression of two individual Nbs even led to protection against escape mutants and can also be used therapeutically [168,169]. To further extend treatment options, a rice-based expression system was developed so that anti-RV Nbs are expressed in, and administered via, rice (MucoRice-ARP1). This approach was shown to be protective in mice. MucoRice-ARP1 rice powder or rice water offer novel approaches to prevent and treat RV-induced diarrhea, thereby reducing the medical and economic burden in developed and developing countries. This alternative strategy could also complement current vaccine-based prophylaxis in situations where live attenuated vaccines are contraindicated [170]. Finally, two groups recently also described Nbs against the diarrheagenic human norovirus: these Nbs might be further developed as diagnostic tools, but more importantly as immunotherapy for treatment and immunoprophylaxis [171,172].

Nbs targeting bacteria

Currently, bacterial infections are mainly treated with antibiotics. However, the emergence of antibiotic resistance and the high cost of currently available treatments highlight the urgent need for new alternative therapeutic approaches [173]. Nbs to combat bacteria can be raised against bacterial surface proteins to block bacterial attachment to host cells (Fig. 3). Based on this principle, Nbs against the lectin domain of F18 fimbrial adhesin of the enterotoxigenic *E. coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC) prevented attachment *in vitro* [174]. Nbs might be prone to proteolytical cleavage, although to a lesser extent than Abs; however, the challenge in tackling enteric pathogens in particular is to design orally administered Nbs that survive the gastrointestinal tract. Nevertheless, Harmsen *et al.* selected Nbs against ETEC F4 fimbriae that were resistant to proteolytical degradation in the upper gastrointestinal tract. Oral administration of these protease-resistant Nbs to piglets reduced ETEC-induced diarrhea by inhibiting fimbrial cell adhesion [175–177]. Nevertheless, to prevent enteric infections, mucosal immunity is needed and the success of oral immunization requires the generation of protective secretory IgA at the intestinal surface. Therefore, the anti-F4 Nbs were grafted onto porcine IgA Fc and expressed in *Arabidopsis thaliana* seeds (Fig. 3) [178]. Proof-of-efficacy was provided because a diet comprising Nb-IgA-expressing seeds reduced bacterial attachment and reduced overall infection pressure in weaned sensitive piglets ($F4R^+$). This strategy could form the basis for oral feed-based passive immunization [178,179]. A different anti-adhesion Nb approach against the oral bacterium *Streptococcus mutans* also enables more selective killing of bacteria. In this research, bacteria-specific Nbs were fused to glucose oxidase, a nonspecific antimicrobial agent. Even at low concentrations, the fusion protein was able to selectively kill *S. mutans*, and so might be beneficial as prophylaxis against dental caries [180,181]. The success of passive immunization with lactobacilli producing scFv Abs against *S. mutans* is an example of how this approach can be further extended using Nbs [182].

Bacterial motility can also be targeted with Nbs (Fig. 3). Pentameric Nbs enhance antigen agglutination, and pentavalency of Nbs can be conferred by exploiting the homopentamerization properties of nontoxic verotoxin B via linking the Nbs to that toxin [183]. Subsequently, the high-avidity pentabodies (Fig. 1a) that bound the flagella of *Campylobacter jejuni* and other specific protease-resistant antiflagella Nbs demonstrated remarkable stability and potently inhibited the motility of *C. jejuni* [184]. The pentabody was also potent *in vivo*, reducing *C. jejuni* colonization in the ceca of infected chickens [31]. By targeting the flagella, both bacterial motility and biofilm formation can be inhibited, as in the use of antiflagellin Nbs against *Pseudomonas aeruginosa* [185]. Another protein that is important during biofilm formation is the biofilm-associated protein (Bap) and, consequently, anti-bap Nbs were developed as a strategy to combat *Acinetobacter baumannii* [186]. Nbs that prevent bacterial secretion of toxins can also be designed, such as Nbs against the type VI secretion system of Gram-negative bacteria [179].

Another elegant approach is the development of Nbs that are not directed against the bacteria as such but against their virulence factors, such as the secreted enzymes that are pathogenic or confer resistance against antibiotics (Fig. 5d). Antibiotic resistance is a major problem, because the bacteria continuously respond to new antibiotics with modified systems exhibiting broadened specificity [187]. One such system is β-lactamase, which confers resistance against β-lactam antibiotics. Highly inhibitory Nbs against two types of β-lactamase (TEM-1 and BcII) cn be used to reverse the antibiotic resistance, and Nbs against other types of β-lactamase could also be produced [188]. However, resistance to this therapy is still possible because some bacteria will escape again by the generation of enzyme variants that are not recognized by those Nbs. In addition, Nbs against UreC, a subunit of urease produced by *Helicobacter pylori*, inhibit urease activity, which can alter the survival of *H. pylori* in the acidic stomach. This explains why such Nbs are thought to a novel class of treatment for *H. pylori* infection [189,190]. Finally, because many bacteria achieve virulence via the secretion of toxins, potent neutralizing Nbs directed against such toxins have been generated, as described below.

Nbs against parasites and fungi

The use of Nbs against parasites is new but is gaining increased attention (Fig. 4). This is illustrated by frequently described Nbs used against African trypanosome parasites, such as the potent trypanolytic Nb An46 (Fig. 4a). Via antigenic variation of the variant-specific surface glycoprotein (VSG) on the densely packed

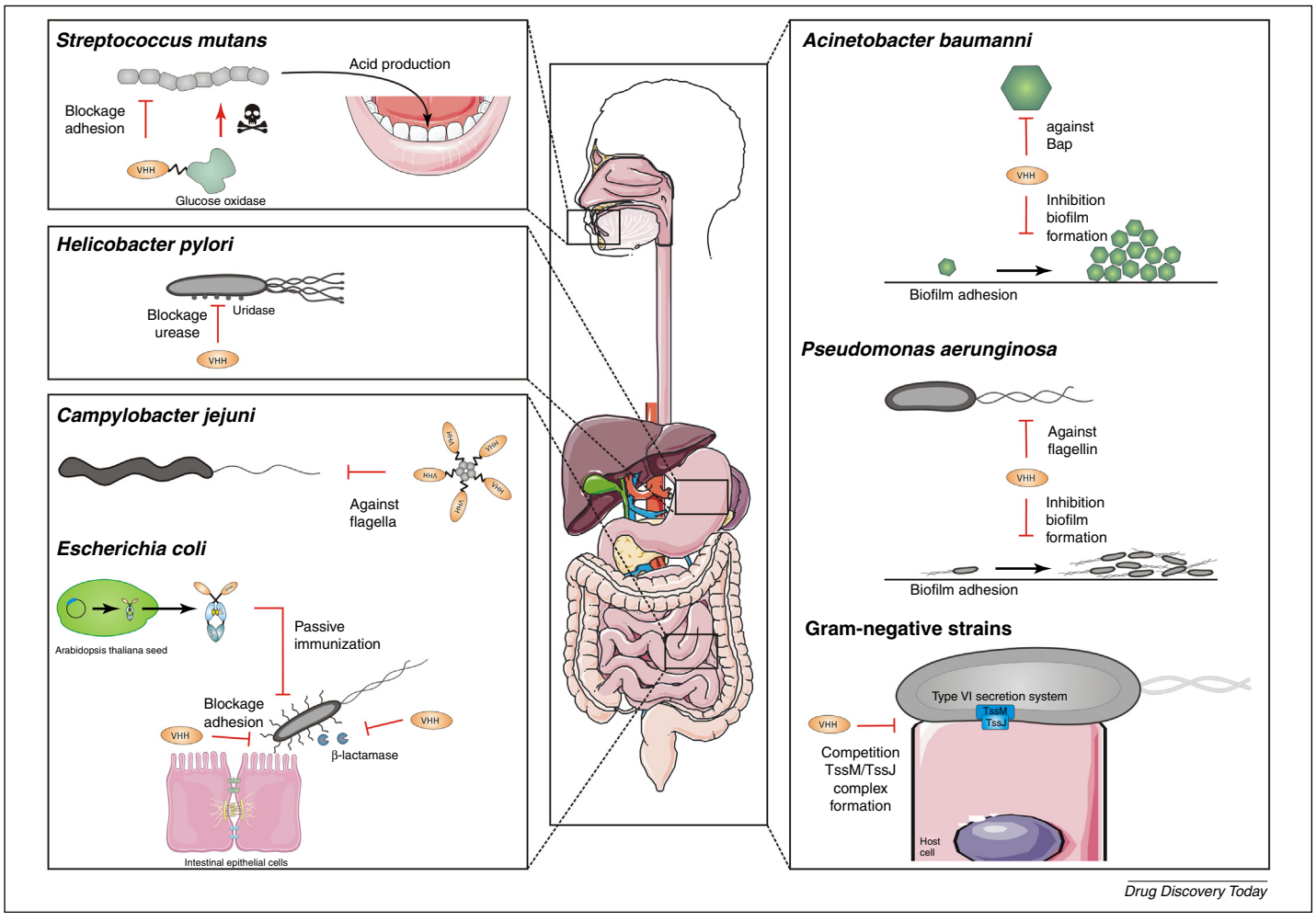

FIGURE 3

Overview of possible applications of nanobodies (Nbs) against different bacterial species. Nbs against bacterial surface proteins can interfere with bacterial attachment to host cells, such as the prophylactic anti-F4 Nbs generated against enterotoxigenic or shigatoxin-producing *Escherichia coli*. Grafted onto porcine IgA Fc and expressed in *Arabidopsis thaliana* seeds, the Nbs can be used for the oral feed-based passive immunization of piglets. Antiadhesion Nbs against buccal *Streptococcus mutans* linked to the nonspecific antimicrobial agent glucose oxidase prevent dental caries. Bacterial motility can be targeted *via* Nbs against the flagella of bacteria, such as that of *Campylobacter jejuni*, and biofilm formation of *Pseudomonas aeruginosa* or *Acinetobacter baumannii* can be altered using Nbs against important biofilm-formation mediators. Nbs against the type VI secretion system prevent bacterial secretion of toxins by Gram-negative bacteria. The secreted effector molecules, such as enzymes that are indispensable for bacterial environmental adaption, can also be neutralized by Nbs, such as those against β-lactamase or UreC, a subunit of urease produced by *Helicobacter pylori*.

surface of the Afican trypanosome parasite, these hemoparasites have evolved a potent immune evasion system to avoid Ab-mediated elimination; this makes Nbs an attractive alternative to bind the difficult-to-reach conserved VSG epitopes. Such Nbs target the distinct VSG epitopes of bloodstream trypanosomes, and some have been shown to exert direct *in vitro* and *in vivo* trypanolytic activity by disturbing the complex endocytotic machinery in the flagellar pocket of the parasite [191]. It was also demonstrated that the effects of the Nbs depend on their low molecular weight, monovalency, and high affinity [192]. Non-trypanolytic VSG-specific Nbs were converted to an immunotoxin via linking to β-lactamase. The enzyme then activated the nontoxic prodrug cephalosporin mustard to the highly toxic PDM specifically at the surface of the parasite and killed it [193]. In another approach, immunotoxins were generated by linking VSG Nbs to truncated apolipoprotein L-I (Apo-L-I), a component of high-density lipoprotein (HDL) that confers innate immunity and lyses almost all African trypanosomes. Given the structural alteration, truncated Apo-L-I also lysed the otherwise resistant strain *Trypanosoma brucei rhodesiense*, which causes human African trypanosomiasis. Infected mice treated with this immunotoxin promptly cleared the parasite from their circulation [194,195]. A major fear relating to human African trypanosomiasis is its progression from a hemolymphatic to an encephalitic stage. This can be countered with Nbs that cross the blood–brain barrier (BBB). Caljon *et al*. reported that Nb An33 crossed the BBB *in vivo* under healthy conditions and even more so under pathological conditions, in which BBB permeability becomes an issue [196]. However, in healthy conditions, only 0.0005% of the injected dose was found in the brain parenchyma, and even though the amount increased 20-fold during the late encephalitic stages, the proportion of dose recovered in brain remained

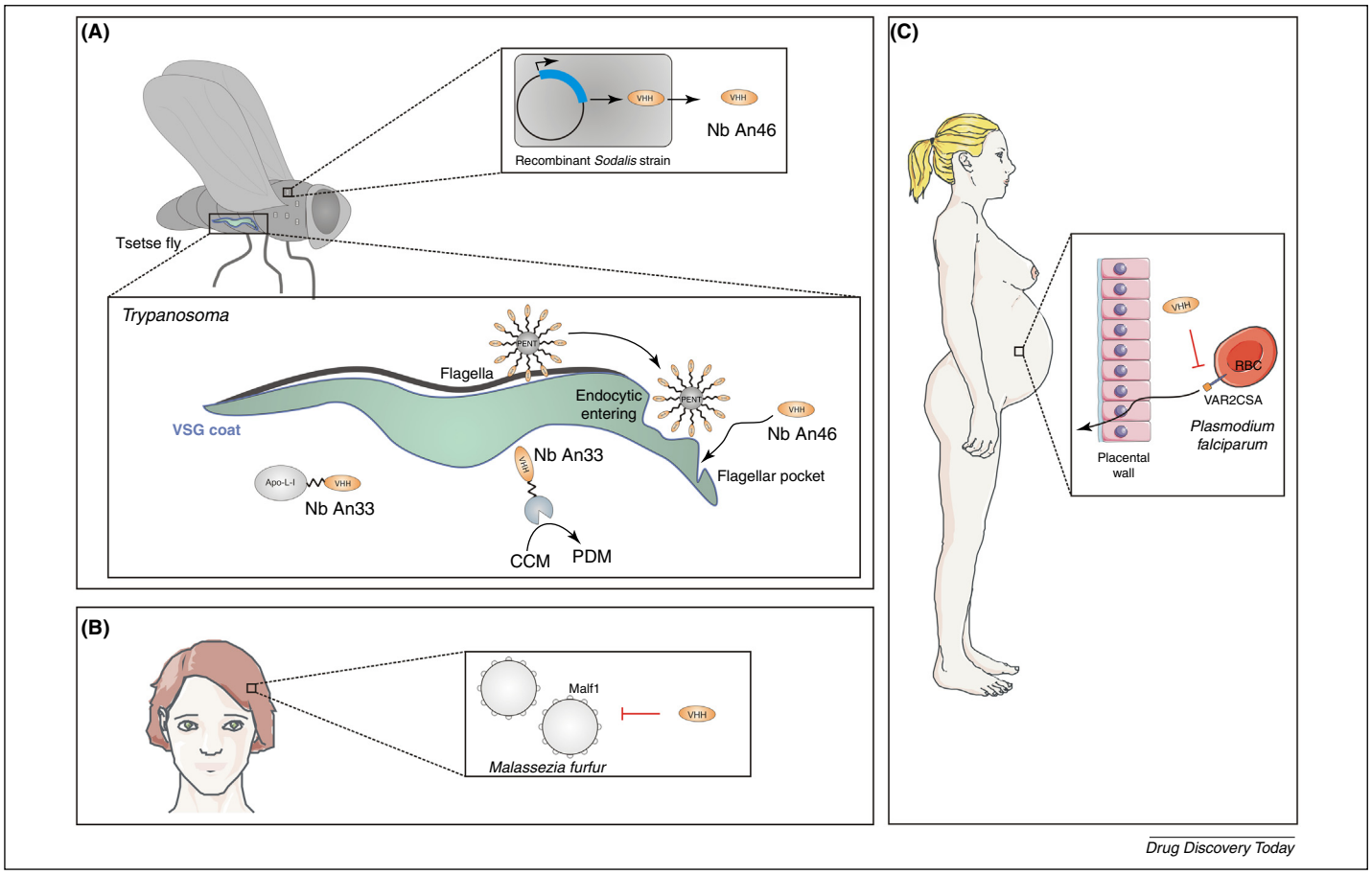

FIGURE 4

Overview of the antiparasitic actions of nanobodies (Nbs). **(a)** African trypanosomiasis, caused by the parasite *Trypanosoma brucei*, is a serious medical problem because of drug resistance. Multiple Nbs have already been generated against the parasite. Trypanolytic Nb An46 disturbs the endocytotic machinery of the parasite in the flagellar pocket of the parasite. Nontrypanolytic Nb An33 has been made more potent, such as by linking the Nb to enzymes that convert the nontoxic prodrug cephalosporin mustard (CCM) into the highly toxic PDM at the parasite surface. Linking this Nb to apolipoprotein L-I (Apo-L-I) resulted in an immunotoxin that lyses almost all African trypanosomes. More effective treatments are obtained by nanoparticles (NPs) that are loaded with the first-line antitrypanosomiasis drug pentamidine and coupled to Nb An33 to effectively target the drug to the parasite. Parasite development in the tsetse fly and spread of the parasite *via* this fly can be controlled *via* expression of trypanolytic Nbs in genetically modified symbionts of the tsetse fly. **(b)** The versatile possibilities of Nbs are illustrated with Nbs used in medical shampoos against *Malassezia furfur* that prevent dandruff formation. **(c)** Nb-based vaccines against pregnancy-associated malaria (PAM) have been developed. *Plasmodium falciparum*-infected red blood cells express VAR2CSA, enabling adhesion to the placenta *via* binding to placental CSA. Nbs against VAR2CSA prevent placental adhesion and are useful against PAM.

minimal. To control parasite development in the fly and thereby limit the spread of this vector-borne disease, genetically modified disease vectors that interfere with pathogen transmission could be a solution. Thus, a recombinant *Sodalis* strain, a vertically transmitted microbial symbiont of the tsetse fly (*Glossina* spp.) was engineered to express tryptanolytic Nbs, and proof-of-concept was demonstrated because the flies released the Nbs extracellularly [197,198] (Fig. 4a). Two promising systems were then engineered to actively target the trypanocidal drugs to the parasite surface. These comprised PEGylated poly lactic-co-glycolic acid (PLGA) or chitosan NPs conjugated with anti-VSG Nbs, which were loaded with the first-line drug against trypanosomiasis acute infection, pentamidine (Fig. 1b) [105,199]. *In vivo*, a ten-times lower dose than the minimal full curative dose of free pentamidine incorporated in these PLGA NPs cured all the infected mice, whereas a 100-times lower dose cured 60% of them [199]. Moreover, the chitosan NPs were 100 times more effective than free pentamidine and could circumvent the resistance acquired because of impaired uptake of the drug [105]. In addition, Nbs that target the paraflagellar rod protein of different trypanosomes have been described, but they are mainly useful as diagnostic markers of trypanosomiasis [200].

Finally, an original idea that further illustrates the versatile possibilities of Nbs is their use in medicinal shampoo. The fungus *Malassezia furfur* (Fig. 4b) is implicated in the formation of dandruff and Nbs against the cell wall protein Malf1 remain stable in shampoos that prevent dandruff formation. Nbs are highly suitable for this approach because they remain stable under harsh conditions, such as high urea concentrations, and the presence of both nonionic and anionic surfactants can easily be selected during panning [18].

Nbs as neutralizing and/or detoxifying agents

By using Abs as neutralizers of potent toxins, spectacular results have been obtained in the development of antivenom therapies. However, the use of Nbs could further enhance toxin neutralization (Fig. 5). Given their good tissue distribution, Nbs can more easily reach and neutralize toxins. However, some toxins are small,

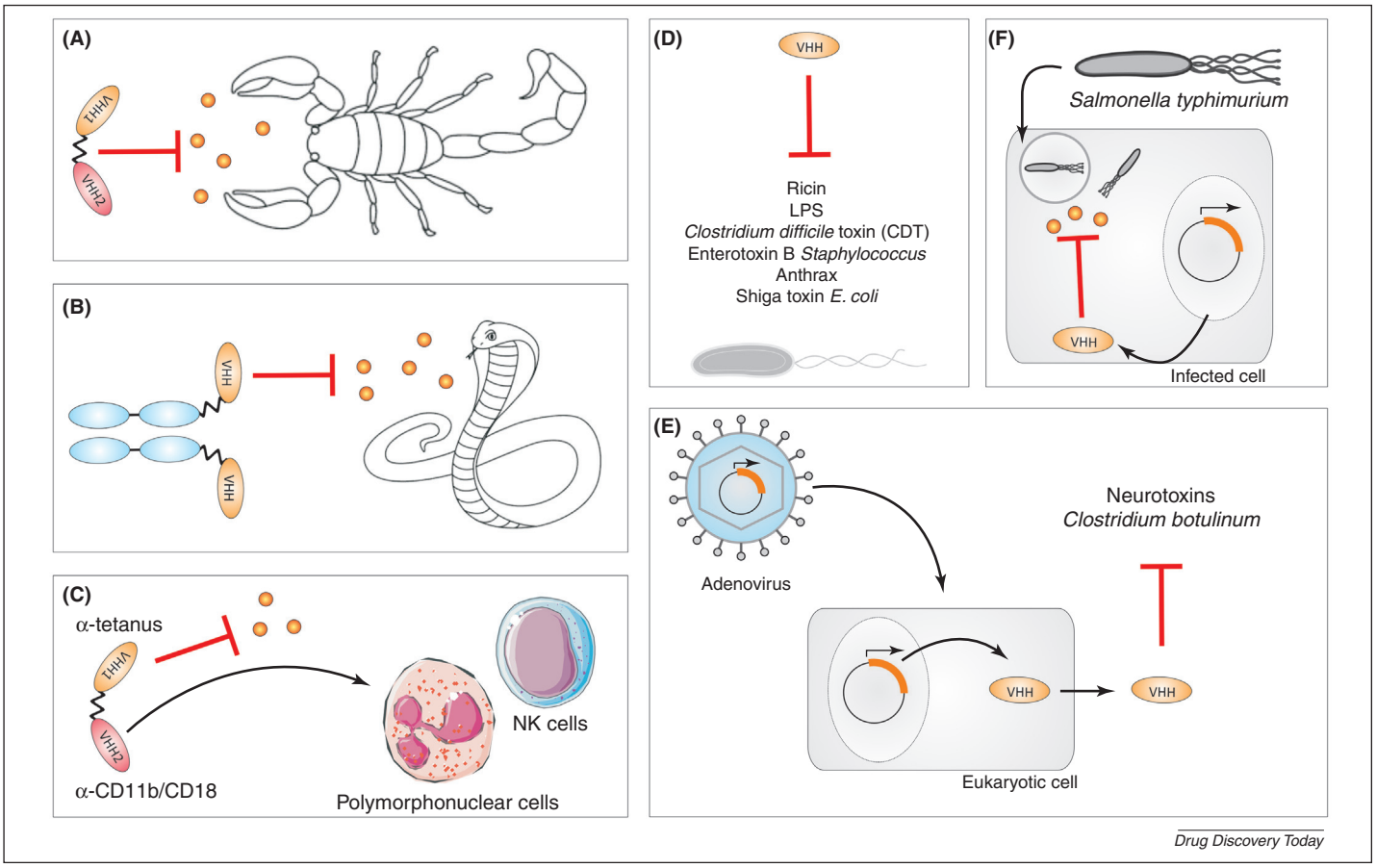

FIGURE 5

Nanobody (Nb)-based strategies against toxins and venom. Neutralizing Nbs have been generated against the toxic venom fractions of the *Androctonus australis hector* and *Hemiscorpius lepturus* scorpions **(a)** and the cobra **(b)**. Multivalency or fusion to human Fc fragments increases the potency of those Nb-constructs. **(c)** To increase the potency of neutralizing Nbs, a Nb specific for the CD11b/CD18 receptor was generated that could be linked to an original antitetanus toxin Nb. This approach resulted in a construct with effector functions that facilitates the elimination of the toxin–immunocomplexes *via* natural killer (NK) cells or polymorphonuclear cells. **(d)** Nbs against small toxins, such as lectin-ricin lipopolysaccharide (LPS) and bacterial proteins, such as shiga toxins, clostridium difficile toxin A and B (CDT), staphylococcal enterotoxin B and anthrax, originating from *Vibrio cholera* and *Bacillus anthracis* respectively, have been shown to be effective. **(e)** Nbs against the botulinum neurotoxins produced by *Clostridium botulinum* have been designed and, when are expressed in an adenoviral vector by adenoviral infected cells, they work as a prophylactic agent against the toxins. **(f)** Intracellular Nbs provide protection against the intracellular *Salmonella typhimurium* toxins when the Nbs are expressed as intrabodies.

nonimmunogenic polypeptides, so obtaining a proper immune response can be problematic. Nevertheless, Nbs have been raised against different toxic venom fractions of the *Androctonus australis hector* scorpion venom (AahG50, AahII, and AahI') [201–204]. Multivalency induced by polymerization of two Nbs against the two most toxic venom fractions improved their affinity. Additionally, proof-of-principle was obtained from experiments on rats, in which the bispecific Nbs had good pharmacodynamic properties and effectively protected against envenoming [205,206]. *Hemiscorpius lepturus* is another scorpion and the most dangerous in Iraq; heminecrolysin (HNc) is the major hemolytic and dermonecrotic venom fraction known from this species. Anti-HNc Nbs were raised and completely protected against HNc-induced envenoming [207]. Neutralizing antivenom Nbs against α-cobratoxin were also generated and fused to a human Fc fragment, thus retaining their high binding affinity to the toxin via the Nb but exerting the immunological properties of conventional Abs [208] (Fig. 5a,b).

Another example is the enzyme ricin, which is classified as a potential biothreat agent; therefore, there is a need for neutralizing agents against it. Although some Abs are available, bispecific heterodimeric Nbs directed against the two subunits of ricin offered significant protection against ricin challenge in mice [209–211]. More detailed information about the crystal structure of the RTA subunit of ricin can be obtained by using ricin-specific Nbs as crystallization chaperones. This could highlight toxin-neutralizing approaches that can be used in the design of subunit vaccines [212] (Fig. 5d).

Other examples are Nbs directed against bacterial compounds or toxins, for example the Nb against *Neisseria meningitidis* lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. *Neisseria meningitidis* is a major health threat that causes meningitis in young children. The anti-LPS Nb blocked binding of LPS to target cells of the immune system, which abolished LPS signaling in whole blood [213]. Antitoxin Nbs were also successfully generated to neutralize the cholera toxin

(staphylococcal enterotoxin B) [214], the *Clostridium difficile* toxin (CDT) originating from *C. difficile* [215–217], the Shiga toxins [218], and the toxins from *Bacillus anthracis* (anthrax) [219]. Additionally, multiple Nbs against the dangerous botulinum neurotoxins (A and E) have been described [220,221] and some provided prophylactic protection when given as a genetic therapy using an Nb-expressing adenovirus vector [222] (Fig. 5e). Finally, even Nbs expressed as intrabodies (Fig. 5f), for example against the *Salmonella typhimurium* toxins, proved to be efficacious *in vitro*. This approach is necessary because this pathogen is intracellular and secretes its toxin directly into the cytosol of the host cell [223]. However, in such cases, it is not clear how these Nbs can be used in humans.

Recently, a Nb was generated to increase the potency of neutralizing Nbs. It was based on the generation of bispecific constructs in which the antitoxin Nb was linked to a Nb specific for the CD11b/CD18 receptor (Mac-1) (Fig. 5c). Using this approach, the construct was found to have effector functions, facilitating the elimination of the toxin–immunocomplexes [224]. This antiendocytotic receptor Nb was linked to an anti-tetanus Nb and, *in vivo*, led to spectacular results in that mice survived a tenfold higher lethal dose compared with mice treated with monomeric Nbs. Another way to promote toxin clearance is by co-administration of an effector Ab (efAb) that binds to epitopic tags that are engineered onto each anti-toxin Nb (Fig. 1a). The Nb binds first to the toxin and subsequently the efAb binds those complexes via the tags on the Nbs. Thus, the toxin becomes decorated with Nb–Ab complexes and liver clearance is promoted [218,225].

Nbs used as diagnostics

Nbs could be exploited not only as therapeutic agents, but also for diagnostic purposes or used in biothreat assays that allow fast detection of biothreat organisms or agents, such as biological weapons. As therapeutic agents, Nbs are directed against a highly conserved epitope of the virus and can be integrated into routine practice for the early diagnosis of viruses. Early diagnosis is favorable, especially for highly pathogenic and potentially lethal viruses, such as the Ebola virus [226], or for viruses for which no specific cure exists, such as Hantavirus, which causes Hantavirus Pulmonary Syndrome. For example, a Nb against recombinant N protein ($prN\Delta_{85}$) of a Hantavirus strain was used in enzyme-linked immunosorbent assays (ELISA) and could rapidly detect the native viral antigen in serum samples [227]. Also, species-specific Nbs against *Taenia* and *Brucella* were developed that enable the easy distinction between different bacterial species [228,229]. Such Nbs can also be included in other systems; they can act as capture agents in enzyme immunoassays (EIA) or surface plasmon resonance (SPR) assays for the early and highly accurate diagnosis of viral, bacterial, or fungal infections. Additionally, rapid and sensitive detection assays for influenza H3N2 and H5N1 were developed using a double-sandwich ELISA model in which biotinylated Nbs were immobilized onto the surface of streptavidin-coated plates. The autologous red blood cell (RBC) agglutination assay is another diagnostic method for certain diseases. This system uses Nbs that indiscriminately bind all human RBC by binding to human glycophorin A. Fusion between those Nbs and the HIV-1 antigen p24 induced RBC agglutination when added to whole blood taken from a patient with HIV because of the presence of Abs against p24 in the patient's plasma. Although the authors stressed that an HIV diagnosis assay will not be established in daily routine practice because of the existence of quicker and less labor-intensive methods, this principle could still be useful for the diagnosis of other diseases, such as sleeping sickness [230]. Also, the evolution of HIV infection can be monitored using anti-HIV-1 Nbs as a piezoimmunosensor. These are non-labeled biosensors displaying Nbs that, in this case, are specific for the HIV-1 virion on the surface of gold-coated crystal piezoelectric sensors. The sensor resonance frequency is monitored and is a measure of the amount of antigen. The possibility of displaying the Nbs at high density will augment their diagnostic potential for the rapid and sensitive detection of antigens in complex biological samples [231]. Furthermore, an innovative competitive diagnostic assay was designed based on Nb technology to identify exposure to trypanosome-transmitting vectors, such as the tsetse fly. Nbs against Tsal, a protein in fly saliva, were generated and used in an assay to detect exposure to the vector. This assay had improved accuracy because Nb binding to Tsal protein was inhibited by the anti-Tsal Abs in plasma produced by the exposed host [232].

Nbs in infection: prospects

Finally, it should be mentioned that the unique characteristics of Nbs could be further exploited for the rational design of subunit vaccines against bacteria or parasites or to design more specific drugs. For these purposes, Nbs can be used as crystallization chaperones for which their properties fit perfectly: they are stable, yield an 'induced-fit' antigen–Nb complex, and improve crystal packing and X-ray phasing [233]. Used a chaperones, Nbs can open new avenues by identifying critical, conserved epitopes of antigens. Indeed, they have already been used to provide structural information relating to viral or parasitic components, such as HIV-1 Nef or the D3 domain of *Plasmodium falciparum* [234,235] (Fig. 4c). In this regard, vaccines against pregnancy-associated malaria (PAM) could be developed. In RBC infected with *P. falciparum*, the most pathogenic malaria-causing species, parasitic adhesion proteins enable the parasite to evade system clearance. Multiple *P. falciparum* infections lead to the generation of neutralizing Abs against those proteins, but women who are pregnant for the first time become susceptible again as a result of placental tropism, because of VAR2CSA expression by the parasite: via this protein, the parasite can adhere to the placenta, where the parasites then accumulate. Thus, VAR2CSA is the main candidate to use in the design of vaccines against PAM. Nbs could be used to identify the VAR2CSA epitopes that are essential for placental adhesion, eventually leading to rational vaccine development [236].

Nbs against inflammation

Inflammation is an important and tightly controlled defense mechanism against infection and injury. Acute inflammation is usually beneficial and meant to restore homeostasis, but when the inflammation persists, it progresses to a chronic state, resulting in a variety of inflammatory diseases, including autoimmune diseases, inflammatory bowel disease (IBD), or atherosclerosis [237]. In addition, systemic inflammatory response syndrome (SIRS) is a condition resulting from an uncontrolled inflammatory response in which the fragile balance between pro- and anti-inflammatory

signals is lost [238]. When infection is present, SIRS is called 'sepsis'. Anti-inflammatory drugs and drugs that interfere with the inflammatory cascade are of potential value [239]. Strategies to alleviate the inflammation are mainly anti-inflammatory drugs and drugs that interfere at the cytokine level. Additionally, G-protein-coupled receptors (GPCRs) are universally acknowledged to be interesting molecular drug targets because they are expressed on many different cells and tissues and are involved in many acute and chronic inflammatory diseases. Unfortunately, even though many marketed small-molecule drugs successfully target GPCRs and new research has revealed detailed structural information about GPCRs, making rational drug design possible, the number of new molecular entities that were approved has stagnated over the past decade [240,241]. Currently, only a few GPCRs are targeted, which provides many opportunities to investigate their interaction with Nbs [242].

Autoimmunity and inflammatory diseases

Given that tumor necrosis factor (TNF) is a major key player in inflammation, it is not surprising that anti-TNF Nbs have been developed, as discussed below. Nbs were raised against both murine and human TNF and bivalency increased their neutralizing potential [242]. The Nbs were effective inflammation suppressors in the mouse collagen-induced arthritis model, and might be a promising and cost-effective alternative treatment for RA because current anti-TNF therapeutics impose a heavy economic burden on health services [243]. Another study also showed impressive results in mouse models of chronic colitis when mice were orally gavaged with *Lactococcus lactis* expressing bivalent anti-TNF Nbs as lactobodies [244].

Although anti-TNF Nbs might replace the more immunogenic anti-TNF Abs in current clinical use, many of the adverse effects related to TNF inhibition will remain [245]. Thus, a more selective trivalent Nb was recently developed that specifically targeted human TNF receptor 1 (TNFR1) [30]. TNF signals through two receptors (TNFR1 and TNFR2), whereby TNFR1 is involved in proinflammatory signaling, whereas TNFR2 has important immunomodulatory functions [246]. Approaches that interfere with all TNFs prevent signaling through both receptors, leading to adverse effects because signaling through TNFR2 is also abolished [245]. TNFR one silencer (TROS) is a trivalent biparatopic Nb linked to an albumin-binding Nb that selectively competes for binding with TNF on TNFR1. *In vitro*, *ex vivo*, and *in vivo* evidence demonstrated its efficacy in inhibiting TNFR1 [30]. Further *in vivo* characterization is needed to show whether it has beneficial effects in diseases in which anti-TNF therapy is either not useful or even exacerbates the disease, as in multiple sclerosis (MS) [247]. Additionally, TROS is a candidate treatment for autoimmune diseases that are currently approached with anti-TNF blockers: the specific TNF/TNFR1 inhibiting approach could reduce the adverse effects related to total TNF inhibition [248].

Chemokine receptors are also attractive drug targets, and blocking the most promiscuous of all, GPCR CXCR2, has the potential to treat a variety of inflammatory diseases, including asthma, MS, RA, IBD, and more. However, it remains challenging to find specific inhibitors with high avidity without off-targets effects. Consequently, CXCR2-directed Nbs have been generated and characterized, and a biparatopic construct has been developed that acts as an inverse agonist. It binds two distinct epitopes of CXCR2 that are unreachable for small drug molecules and further development of this biologic might eventually lead to a promising new drug [249].

Furthermore, autoimmune diseases can also be tackled with anti-IgG Nbs to deplete auto-IgG by plasmapheresis, a blood purification method. Patients with systemic lupus erythematosus (SLE) or Goodpasture syndrome were treated with hemodialysis and the auto-Abs were effectively removed from the blood on an affinity column that used the anti-IgG Nbs as ligand [250]. Eventually, this approach could be extended to other autoimmune diseases that involve many auto-IgGs (e.g., myasthenia gravis). Development of Nbs against one specific IgG subtype or isotype might also be possible.

SIRS and sepsis

For SIRS and sepsis, the most important lethal conditions in the intensive care unit (ICU), there are no treatments besides supportive care [239]. One reason for this is the highly heterogeneous population with different pathology features. Good biomarkers might be helpful to stratify patients into more homogeneous subpopulations [251]. Procalcitonin (PCT) is a valuable diagnostic biomarker of bacterial infections and is already frequently used for risk assessment of seriously sick patients progressing to severe sepsis and septic shock. Nbs against PCT, which were able to detect PCT in serum with high sensitivity, could be an additional tool for patient stratification [252,253]. Development of targeted treatments might also be useful. Recently, Nb 14 was developed, which specifically targets MMP8. A detrimental proinflammatory role has been attributed to MMP8 and several research groups have proposed MMP8 as a potential drug target for the treatment of sepsis. Additionally, MMP8 was found to be upregulated in patients with sepsis and was correlated with high mortality [254–256]. Demeestere *et al.* provided proof-of-principle of MMP8 inhibition by Nb 14 *in vitro* and demonstrated for the first time the possibility of expressing a Nb systemically by *in vivo* electroporation of muscles, thereby protecting against LPS-induced endotoxemia [257]. As mentioned above (*Nbs as neutralizing agents*), a Nb against LPS has also been developed. LPS, derived from the outer membrane of Gram-negative bacteria, stimulates immune cells to produce large amounts of inflammatory mediators. Although the Nb was raised against *Neisseria meningitidis*, it also bound to LPS from other bacteria. Accordingly, the anti-LPS Nb is a leading candidate in the development of therapies against LPS-mediated sepsis [213]. Although anti-LPS Abs did not prove their efficacy in clinical trials [258], anti-LPS Nbs can be used during selective plasmapheresis in patients with sepsis to increase LPS clearance from the blood during plasma exchange.

Immunotherapy to intervene in the intrinsic immune system

Previous approaches aimed to reduce the proinflammatory functioning of cytokines, but several cytokines also have anti-inflammatory, anticancer, or antiviral properties [259]. Unfortunately, the intrinsic toxicity of these cytokines has hampered their use in medicine. However, this dilemma can be circumvented by linking the cytokines (mostly engineered in a less toxic and/or less active form) to Nbs that guide the so-called 'immunocytokines' (Fig. 1a) to the desired target cells. The activity of the mutant cytokine is

restored as a result of its accumulation at the desired location. This 'activity-by-targeting' approach was validated for mutant type I IFNs fused to a Nb targeting the murine leptin receptor and led to antiviral activity on targeted cells [260]. Chimeric proteins comprising mutant leptin or IFN fused to an anti-TNFR1 Nb or to a Nb specific for programmed cell death 1 ligand 2 (PD-L2), respectively, were also described. This approach can be extended easily by changing the cytokine or the Nb that can envision virally infected cells, tumor cells, or particular immune effector or regulatory cells.

Another completely different way to deal with inflammation is by interfering with T cell functioning. First, a multivalent Nb has been described that selectively binds the first extracellular loop of the voltage-gated Kv1.3 ion channel, which is crucial for T cell activation. In general, ion channels are difficult to target with either small-molecule drugs or Abs; thus, a Nb that targets this ion channel would be the first-in-class treatment for a range of autoimmune and inflammatory diseases. *In vitro*, the Nb potently blocked the gating-dependent channel, stimulating the T cell response, and *in vivo* proof-of-concept was provided in a rat delayed-type hypersensitivity model [261]. In addition, the ARCT2/P2X7 pathway of T cells could be targeted with Nbs. ADP-ribosyltransferase C2 (ARTC2.2) is an ectoenzyme expressed at the surface of T lymphocytes and, in the presence of NAD^+, it ADP ribosylates several cell surface target proteins and thereby regulates their functions. The ATP-gated ion channel P2X7 is the most prominent ARTC2.2 target on T cells. P2X7 is widely expressed on T cells, microglia, and macrophages, and is involved in inflammatory and neuronal diseases. A low concentration of NAD^+ induces long-lasting P2X7 activation and ultimately triggers cell death, to which regulatory T cells (Tregs) and NK T cells (NKT) are sensitive [262]. ARTC2.2 was the first ectoenzyme targeted with Nbs, and the Nb s+16a-Fc fusion protein prevented ADP ribosylation. Proof-of-concept was obtained by finding that the Nb, in presence of α-galactosylceramide, restored the NKT population of diabetogenic NOD-CD38$^{-/-}$ mice, which are naturally depleted, and this eventually led to inhibition of the development of type 1 diabetes mellitus [263]. In addition, long-term treatment with the Nb expanded the suppressive Treg activity. Furthermore, this Nb is a valuable tool for studying how the ARTC2/P2X7 pathway regulates immune cells and its possible *in vivo* pathophysiological implications [264]. Intervening at the level of this pathway may provide therapeutic options to manipulate immune responses in several inflammatory diseases [263], explaining why both agonistic and antagonistic Nbs against the target of ARTC2.2, P2X7, have been developed with best-in-class opportunities. These Nbs could either block or potentiate P2X7 on T cells and macrophages. Agonistic Nbs boost immunity and are useful in oncology; by contrast, antagonistic Nbs will block the inflammatory response and could be used as anti-inflammatory agents in inflammation or neurological disorders [265]. *In vivo* proof-of-concept has been given in inflammatory mouse models of glomerulonephritis and atopic dermatitis [261,265].

Lentiviral vectors provide unique properties to boost the immune response in vaccination and immunotherapeutic strategies against viral, cancer, or autoimmune antigens. These are intrinsically immunogenic and capable of providing long-term antigen expression and delivering foreign genes to a variety of dividing and nondividing cells. This leads to the activation of cellular immunity and humoral responses [266]. Using Nbs, lentiviral vectors can be specifically addressed towards antigen-presenting cells (APCs) or human myeloid dendritic cells (DCs), where they are transduced by vectors. This is imperative for the activation of antigen-specific T cells, which is required for a proper immune response. To incorporate APC and/or DC-specific Nbs on the envelope of the lentivirus, the budding mechanism of lentiviral vectors is exploited. Additionally, a binding-defective but fusion-competent vesicular stomatitis virus glycoprotein was encoated on the viral surface. A mouse bone marrow-derived DC-specific Nb expressed by lentiviral vectors selectively transduced human lymph node-derived DCs and macrophages *in vitro*. *In vivo*, it enhanced the immune response mediated by conventional DCs after intranodal administration. This technology can further enhance the potential of lentiviral vectors as general gene delivery vehicles because it can be used to target different cell types and, thus, induce specific immune responses [267].

Other ways to generate effector functions on Nbs aiming to increase the potency of Nbs are also discussed in the section 'Nbs as neutralizing and/or detoxifying agents'. They include generation of an anti-CD11b/CD18 receptor Nb [224] and co-administration of effector Ab (efAb) that are specific to tags engineered onto anti-toxin Nbs [218,225]. Both approaches favor toxin–immunocomplex clearance.

Nbs in neurodegenerative and other amyloid disorders

There are currently only symptomatic treatments for neurodegenerative disorders; no disease-modifying or neuroprotective therapies that alter the natural disease course are available. However, such disorders are responsible for a major portion of the economic burden on the healthcare system, and as the population continues to age, the economic impact will grow proportionally [268]. Consequently, new and affordable therapies are needed.

Alzheimer's disease

AD is the most common neurodegenerative disease. It is characterized by the cerebral deposit of aggregated amyloid-β (Aβ) peptide plaques and formation of neurofibrillary tangles [269], resulting in dementia and loss of cognitive functions. Aβ plaques are formed via proteolytic cleavage of a large precursor protein, amyloid precursor protein (APP), by enzymes such as Beta-site APP-cleavage enzyme (BACE-1). Nbs that are selective for different amyloid (precursor) peptides have been produced, and Nbs that can prevent the formation of mature Aβ fibrils by stabilizing Aβ protofibrils have been identified [270,271]. Nevertheless, it is still debatable whether stabilization of the oligomeric intermediate, and thereby prevention of Aβ assembly, is desirable [272]. This might explain the lack of follow-up papers, given that the last paper to describe Aβ-targeting Nbs was published in 2009. Recently, a biparatopic Nb BI 1034020 was designed that targets two different epitopes of Aβ with high affinity. The aim was to reduce the free Aβ peptide levels in plasma to prevent the formation of new Aβ plaques and to clear the existing plaques, but the initiated clinical trials were prematurely terminated (see 'Further clinical trials'). The drug-related adverse effects seen in this trial might be explained by the stimulation of, rather than reduction in, Aβ aggregation, because the Nb-construct targets two different epitopes of Aβ. Again, this failure raises the question of whether

targeting Aβ is the proper strategy to approach AD. Thus, another attractive strategy is the inhibition of BACE-1, with BACE-1 Nbs having been generated and patented [273]. For diagnostic purposes, Aβ-specific Nbs coupled to ^{99tm}Tc enabled the *in vivo* detection of vascular and parenchymal Aβ deposits because they could cross the disrupted BBB, making them promising tools for such applications [149].

Parkinson's disease

Some attempts have been made to use Nbs to tackle the second most common neurodegenerative disease, Parkinson's disease (PD). PD is characterized by the loss of dopaminergic neurons in the substantia nigra, and misfolding of α-synuclein (α-syn) into fibrillar aggregates seems to have a prominent role in the pathogenesis of this disease [269]. Consequently, reduction of the intracellular levels of α-syn is a logical therapeutic approach, the aim being the prevention of misfolding, aggregation, and toxicity [274]. NbSyn2 is a Nb directed against the C-terminal part of monomeric α-syn, but the Nb could not prevent aggregation because it bound both monomeric and aggregated protein [275,276]. The recently generated NbSyn87 binds epitopes other than NbSyn2, but again it bound both monomeric and fibrillar forms. Nevertheless, those Nbs provide information about possible conformational rearrangements during fibrillar maturation and, therefore, are valuable for gaining knowledge about the structure of α-syn [277].

Huntington's disease and oculopharyngeal muscular dystrophy

Huntington's disease (HD) is a progressive autosomal dominant genetic neuropsychiatric disorder that affects muscle coordination and can be accompanied by behavioral disturbances. Given that HD has a well-described target, namely the misfolded mutant Huntingtin protein (mHTT), therapies directed against it seem promising [278]. Patients who are predisposed to developing HD can be selected by genomic screening, making it possible to start therapy before the onset of disease signs and symptoms. However, while numerous reports have described the use of Abs or scFvs, only one group has reported the generation of a Nb directed against HTT [279]. Further studies on Nbs in this context are needed.

Oculopharyngeal muscular dystrophy (OPMD) has some of the characteristics of HD. It is caused by expansion of a GCG repeat in the *PABPN1* gene encoding a polyalanine repeat at the N terminus of the polyA binding nuclear 1-protein (PABN1). The hallmark of OPMD is progressive weakening of specific muscles. Nbs binding an α-helical domain of mutant PABN1 were generated, and Nb 3F5 was successfully expressed as an intrabody in the nucleus. In a cellular model of OPMD, it not only prevented aggregation, but also *led* to clearance of existing aggregates [280]. These results were confirmed in *Drosophila*: intrabody 3F5 was expressed in myocyte nuclei and prevented and restored muscle degradation [281].

Other applications of Nbs in neurodegenerative disorders

Hereditary gelsolin amyloidosis is a rare autosomal dominantly inherited amyloid systemic disorder caused by a point mutation in the *GSN* gene encoding the actin-binding gelsolin protein. The disease is characterized by peripheral neuropathy and ophthalmological and dermatological aberrations [282]. This genetic defect causes expression of mutated gelsolin, which leads to the deposition of gelsolin amyloid (AGel) peptides because of misfolding and aberrant pathological furin proteolysis [283]. Three Nbs have been developed that shield the mutant gelsolin from pathological furin proteolysis. *In vitro*, this approach was effective in protecting against gelsolin degradation, and *in vivo* it reduced gelsolin build-up in the endomysium and improved muscle contractility [284]. Following this success, another gelsolin Nb was generated and expressed as an intrabody in the endoplasmic reticulum (ER) to protect gelsolin from proteolysis in the *trans*-Golgi network. Transgenic mice that expressed this Nb in the ER were able to counter amyloidogenesis at an early disease stage [283]. Nbs generated against other amyloidosis disorders, such as those induced by oxidative stress is another possibility for the treatment of neurodegenerative diseases. Although the mechanism of oxidative stress-induced cell death is not yet clear, it is involved in the development of both AD and PD [286]. This was the rationale for developing inhibitory intrabodies against Bax, a proapoptotic factor involved in the early phase of apoptosis. Such intrabodies were found to protect mitochondria against oxidative stress [287].

Nbs in neurodegenerative diseases: prospects

In contrast to the mounting number of publications on the potential uses of intrabodies (expressed mostly as scFv) in neurological disorders [274,288], only a few intracellularly expressed therapeutic Nbs have been generated for such diseases. Successful therapeutics are still missing, particularly for PD and HD. The expression of Nbs as intrabodies for these purposes should be considered because intrabodies can interfere with am early step in pathogenesis, even before the misfolding of α-syn or HTT occurs, and evidence for the validity of this effective approach was provided in OPMD [274]. Delivery of the gene encoding the intrabody can be provided by lentiviral vectors that encode Nbs on their surface to specifically target the neuronal cell of interest [289]; however, the feasibility of the use of lentiviral vectors in humans still has to be validated.

Gaining access to the central nervous system (CNS) is another challenge to the successful treatment of neurodegenerative disease because this is generally a prerequisite for a proper pharmacological effect. Many promising therapeutics fail to cross the BBB and reach the cytosol of affected cells after systemic administration, because the BBB is only permeable to lipophilic molecules up to 400 Da. The finding that BBB transport is possible via the Nb platform is of paramount importance because, unlike conventional Abs, several Nbs were reported to cross that barrier, partially because of the absent Fc-receptor-mediated efflux to the blood. Other mechanisms by which Nbs are transported vary from one Nb to another. First, spontaneous transport might be feasible because of the small size or high isoelectric point (p*I*) of the Nb, and pathological inflammatory conditions that compromise the BBB integrity increase their transport [149,196,290]. However, the doses recovered in brain after administration are low and probably not of therapeutic significance; thus, efforts are required to modify Nbs in such a way that their brain-crossing properties are improved without losing their size-based advantages. Second, receptor-mediated transcytosis (RMT) is feasible by linking Nbs to physiological shuttle molecules, such as transferrin, insulin-like growth factor, or low-density lipoprotein [291–293]. Recently, new proteins that are highly expressed by brain

endothelial cells were identified. Thus, Nbs directed against those new targets could be developed to enhance brain uptake [294]. Furthermore, Nb FC5 engages active RMT by binding to a BBB-enriched transporter, possibly the luminal α (2,3)-sialoglycoprotein receptor, which triggers clathrin-mediated endocytosis. This enabled transcellular migration across human cerebromicrovascular endothelial cells (HCEC) to be established [295–297]. A second Nb, Nb PrioV3, generated against the disease-associated prion protein PrP^{Sc} to inhibit prion proliferation, was also reported to cross the BBB via clathrin-mediated RMT, both *in vitro* and *in vivo* in mouse brain parenchyma [298,299]. Finally, Nbs have already been tested as a modular BBB delivery platform to facilitate the passage of cargo such as macromolecules or small-molecule drugs into the CNS, and so could offer many opportunities for the treatment of neurodegenerative disease or CNS infections [196]. Fusion of the BBB-crossing Nb FC5 to a human IgG Fc domain that prolongs circulation time and to a neuropeptide enhanced brain exposure both *in vitro* and *in vivo* [300]. An additional way to deliver therapeutic Nbs into the brain would be to incorporate the Nb into a BBB-targeting NP, as already suggested [301]. In conclusion, the BBB remains a major hurdle to overcome and, despite years of extensive research, limited progression has been made. Thus, there is room for improvement in the search for therapeutic Nbs against neurodegenerative diseases.

Finally, Nbs can offer unique properties to study the intermediates of aggregation-prone polypeptides that lead to protein misfolding and aggregation, the hallmarks of many neurodegenerative diseases. Used as probes, Nbs make it possible to unravel the molecular mechanisms that form the basis of the pathology. Additionally, studies have shown that Nbs could be used for the diagnosis of amyloidosis at an early stage of disease by recognition of aggregation-prone oligomers in blood, as recently reviewed elsewhere [287,302–304].

Clinical studies with Nbs

Since the discovery of HcAbs in camelids in 1993, their antigen-binding domains (known as Nbs) have gathered considerable attention because of their characteristics that are more beneficial in some applications compared with (monoclonal) Abs and ScFv Abs. Publications on Nbs have increased exponentially over the past decade, and some Nbs are now in clinical trials. In 2007, the first clinical studies with a Nb (ALX-0081) against the von Willebrand factor (vWF) was performed by Ablynx. Later, other clinical trials followed (see Fig. 6 for an overview of all clinical trials). At first, Ablynx was the market leader, although the company now collaborates with other companies, such as Merck & Co., Eddingpharm and AbbVie (Table 2). In addition to the Nbs in clinical trials described below, there are other Nbs in the pipeline in the fight against inflammation, cancer, hematological, and respiratory diseases. More patient-friendly administration routes, such as inhalation, oral delivery, or topical administration, are likely to promote successive Nb therapies.

Nanobodies against inflammation and autoimmune diseases

ALX-0061

Given that interleukin (IL)-6 is a key player in several inflammatory diseases and anti-IL6 mAbs are in therapeutic use, a Nb that binds and blocks the IL6 receptor (IL6R), with best-in-class potential, was designed. ALX-0061 comprises a monovalent anti-IL6R Nb linked to an anti-HSA Nb to extend its half-life, creating a bivalent, bispecific Nb. The good preclinical results in a human IL6-induced inflammation model in *Macaca fascicularis* (cynomolgus monkeys) and in a collagen-induced arthritis model in rhesus monkeys supported a Phase I/II placebo controlled trial [305]. This trial evaluated the safety, pharmacokinetics, and pharmacodynamics of single and multiple intravenous (i.v.) injections in healthy individuals and patients with RA. Proof-of-concept for use of ALX-0061 in RA was obtained in a Phase IIa study on patients with moderately to severely active RA. There were no reports of antidrug Abs and, at all administered doses, ALX-0061 showed good 24-week safety and efficacy results [306,307]. ALX-0061 was further developed for RA and SLE and a Phase IIb RA monotherapy and combination study with methotrexate is ongoing, alongside a Phase II RA study to evaluate long-term safety and efficacy (Clinical trial identifier: NCT02287922 and NCT02518620). In addition, a Phase II clinical study with patients with moderate to severe seropositive SLE is ongoing, aiming to evaluate the safety and efficacy of ALX-0061 in SLE and to identify the optimal dosage (Clinical trial identifier: NCT02437890) [308,309].

ATN-103 (ozoralizumab) and ATN-192

Given that current anti-TNF therapies have revolutionized the treatment of autoimmune diseases, such as RA and psoriasis, Ablynx developed a humanized, trivalent, bispecific Nb, ATN-103 (ozoralizumab) that binds with high affinity to TNF. It comprises two anti-TNF Nbs and one Nb that binds HSA to extend serum half-life. Results of studies in a transgenic mouse model of polyarthritis indicated that ozoralizumab is superior to infliximab and can decrease clinical scores and reverse the pathology. The first clinical trial with ozoralizumab (Clinical trial identifier: NCT00916110) was successfully performed on healthy male Japanese volunteers to evaluate the safety of a single subcutaneous (s.c.) or i.v. injection of the Nb. A Phase I/II was initiated to evaluate the safety of ozoralizumab administered to patients with stable RA taking methotrexate (Clinical trial identifier: NCT00959036) and proof-of-concept was demonstrated in patients with active RA in a Phase I/II interventional, multiple ascending dose (MAD) study (Clinical trial identifier: NCT01007175). Consequently, ozoralizumab met both the primary and secondary endpoints (ACR20 and ACR50) without any reports of severe adverse events (SAE). Patients from this study were eligible to enroll in an open-label extension (OLE) study (Clinical trial identifier: NCT01063803) that provided data on long-term safety and tolerability. The study showed DAS28 remission in 38% and EULAR response in 97% of patients. Although the OLE study reported the presence of neutralizing antidrug Abs against ozoralizumab, all of the volunteers completed the trial [310].

A second anti-TNF Nb was developed, ATN-192, formerly known as PF-05230905, which is the PEGylated form of ATN-103 and a Phase I clinical trial on healthy volunteers was successfully completed (Clinical trial identifier: NCT01284036).

ALX-0761

In addition to the Nbs directed against the most prominent proinflammatory players in autoimmune diseases, a trivalent Nb (ALX-0761) has also been developed that not only neutralizes two other important proinflammatory cytokines, IL17A and IL17F, but

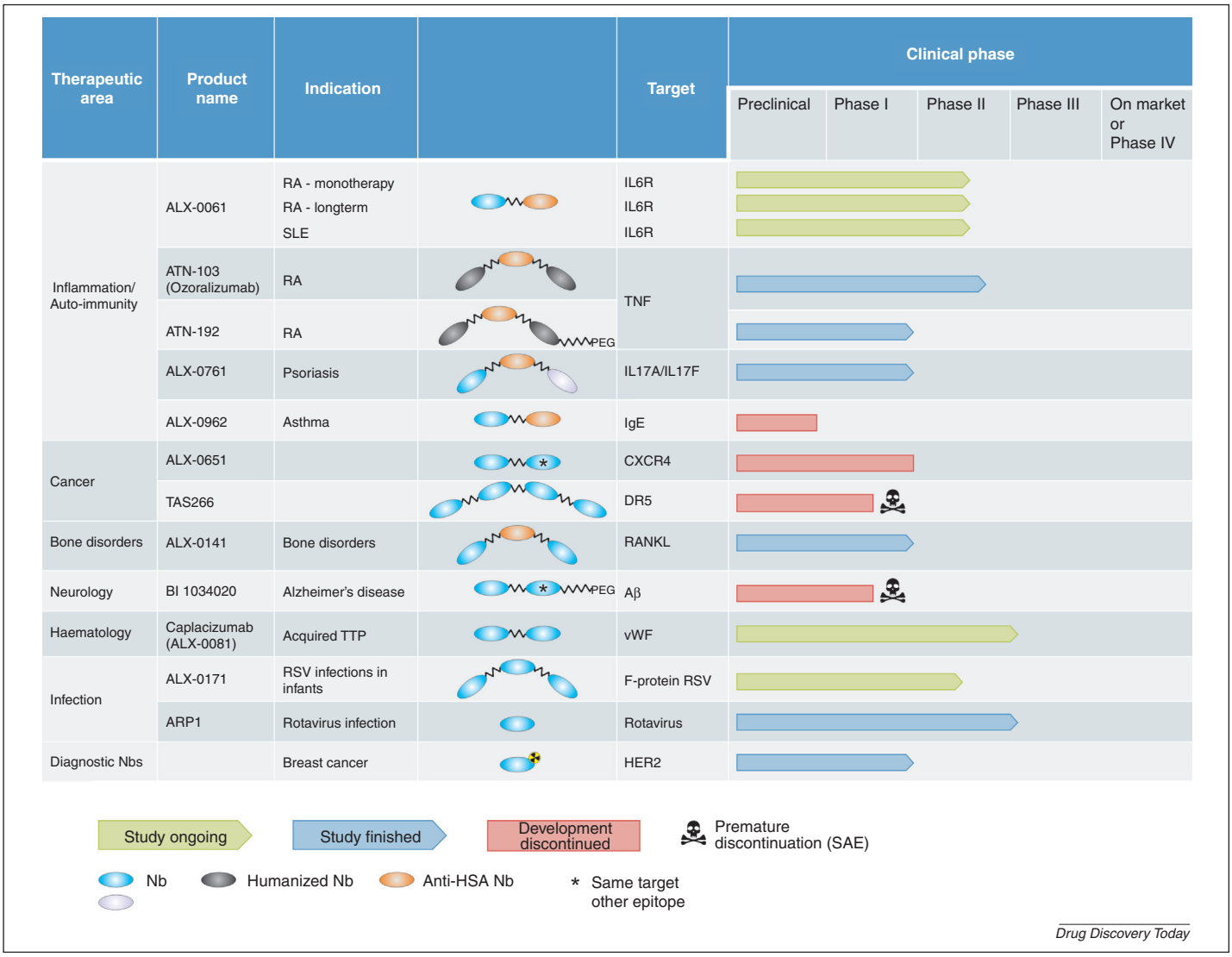

FIGURE 6

Overview of clinical trials with nanobodies (Nbs). All candidate Nbs that are or were in clinical development for the treatment of a range of diseases. The figure shows either the current phase of development for each Nb or when development was discontinued.

also binds HSA. In an RA model in cynomolgus monkeys, ALX-7061 met the primary endpoint, which was a significant improvement of the symptoms and, thus, achieved proof-of-concept. A Phase Ia study on healthy volunteers was initiated and successfully completed [311] and a Phase Ib multiple ascending dose study is ongoing with patients with moderate to severe psoriasis (Clinical trial identifier: NCT02156466). Primary outcome measures of this multicenter, randomized trial include the presence of adverse events, local injection site reactions, and pharmacokinetic and pharmacodynamic profiling [312].

ALX-0962

IgE Abs initiate an allergic reaction upon allergen exposure, leading to allergic asthma. Therefore, IgE neutralization is an attractive way to treat moderate to severe allergic asthma. Inhaled corticosteroids and long-acting β-agonists are the main therapies to treat asthma, and only one anti-IgE Ab, omalizumab, has been approved and made available. A potent monovalent anti-IgE Nb, coupled to a HSA-binding Nb, ALX-0962, has also been developed [313]. This bispecific Nb is five times more effective in the neutralization of human IgE than omalizumab and, unlike omalizumab, also abolishes allergen sensitivity. In 2013, preclinical studies were initiated on ALX-0962, but following review of the results, it was decided not to progress into Phase I studies because of insufficient differentiation from competitors [314,315].

Nbs against cancer

ALX-0651

ALX-0651 was the first clinically used biologic against GPCRs, directed against two different epitopes of CXCR4, and was a bivalent, biparatopic Nb. ALX-0651 might become a first-in-class drug, because there are currently no approved biologics against the difficult druggable GPCRs. Inhibition of CXCR4 leads to stem cell mobilization from the bone marrow and enables stem cell collection for transplant to patients with multiple myeloma and non-Hodgkin's lymphoma [316]. In cancer treatment, CXCR4 is a

validated target for small-molecule drugs, but there are currently no biologics that target it. A Phase I study was initiated on ALX-0651, with a biomarker ($CD34^+$ count) as dose-escalation endpoint (Clinical trial identifier: NCT01374503) and this study showed proof-of-concept in targeting GPCRs with biologics [317]. Despite these results, preliminary data indicated that ALX-0651 was unlikely to achieve better effects than standard care, so further development of ALX-0651 was not pursued beyond Phase I.

TAS266

TAS266, a Nb against death receptor 5 (DR5) has also been tested in clinical trials. DR5 is a key receptor on cancer cells that promotes controlled cell death in tumors via the activation of downstream caspases. Consequently, multiple therapeutic agonists that target DR5 have been developed; however, these failed in clinical trials possibly because of insufficient clustering and activation of DR5. Generation of constructs with higher valency increases the ability to kill tumor cells. TAS266 is a tetrameric agonistic Nb that can effectively cluster DR5 in its ternary active state, which is not achievable by conventional Abs because they need an additional crosslink via the Fc-γ receptors on the immune cells in the tumor microenvironment. It was shown that, *in vivo*, TAS266 had stronger antitumor effects than soluble TRAIL or agonistic Abs in multiple models of tumor xenografts, and that tumor regression was sustained after treatment cessation [318]. A Phase I clinical trial was initiated (Clinical trial identifier: NCT01529307), in which TAS266 was administrated via i.v. infusion to patients with advanced solid tumors over a time frame of 2 years. The study was terminated early because of unexpected hepatotoxicity reported in three out of four patients at the 3 mg/kg dose levels, possibly caused by immunogenicity to TAS266 [28,319].

Nbs against hematological disorders

ALX-0081 is a bivalent, monospecific Nb directed against the A1 domain of the blood glycoprotein vWF, which is involved in hemostasis. ALX-0081 inhibits platelet adhesion to the vessel walls and thereby controls platelet aggregation and clot formation. A Phase I study was initiated with healthy volunteers and patients with stable angina undergoing percutaneous coronary intervention. The study showed that i.v. injection of the Nb was well tolerated and that it inhibited vWF-mediated clotting, measured via the platelet aggregation biomarker, Ristocetin cofactor (RICO) [320]. In a comparative Phase II randomized trial, ALX-0081 was tested in patients at high-risk for acute coronary syndrome undergoing percutaneous coronary intervention, with the aim of comparing the bleeding risk after ALX-0081 to that of an approved GPIIb/IIIa inhibitor, abciximab, as well as evaluating the clinical effectiveness (Clinical trial identifier: NCT01020383) [321]. Although ALX-0081 did not increase bleeding risk, it did not significantly reduce the number of bleeding events compared with abciximab. Consequently, clinical development of ALX-0081 as an antithrombotic was discontinued, but the potential use of ALX-0681 (caplacizumab) for treatment of thrombotic thrombocytopenic purpura (TTP) was further investigated [322]. ALX-0681 is a variant of ALX-0081 that is administered s.c. instead of i.v., making it more applicable to a broader target group.

There is currently no approved therapy to treat the rare hematological disease TTP, making caplacizumab a first-in-class drug that holds promise as an adjunctive therapy to plasma exchange. Given the rareness of the disease, caplacizumab received orphan drug status in 2009. That year, positive Phase I results with caplacizumab were obtained and the favorable pharmacological behavior supported progression towards Phase II testing. Preclinical results in a baboon model of acquired TTP also justified the use of caplacizumab, because it was safe and completely prevented the rapid onset of severe thrombocytopenia [323].

The TITAN study, a Phase II single-blinded, placebo-controlled study, was initiated to examine the efficacy and safety of caplacizumab in patients with acquired TTP (Clinical trial identifier: NCT01151423) and proof-of-concept was achieved, because treatment with caplacizumab plus standard care resulted in a significant reduction in the time required to normalize the platelet count [324]. A Phase I trial demonstrated bioequivalence between a reconstituted lyophilized versus liquid formulation. This justifies the use of the lyophilized formulation in future Phase III studies and for commercialization (Clinical trial identifier: NCT02189733). A Phase III randomized clinical trial (HERCULES) has been started in 92 patients with acquired TTP and with that, caplacizumab is the first Nb to enter this phase of clinical development (Clinical trial identifier: NCT02553317). Potentially, caplacizumab could be launched on the market in 2018.

Nbs against viruses

ALX-0171

ALX-0171 is a first-in-class trivalent, monospecific Nb that targets the replication of the respiratory syncytial virus (RSV) by binding the F-protein on its surface, thereby blocking viral uptake into the cells. Currently, no specific treatment options are available. ALX-0171 was developed to treat infants and hospitalized patients, and its design and physical robustness allow optimal delivery to the site of infection (the lung) via inhalation. This approach offers fast onset of pharmacological response and leads to high local concentrations. *In vitro*, ALX-0171 administration resulted in a 10,000-times reduction of the viral titer, and *in vivo* intranasal administration in rats showed that the Nb reduced viral replication and leukocyte infiltration [325]. The first-in-man Phase I study on healthy male volunteers showed that different single or multiple ascending doses of ALX-0171 were well tolerated and did not affect lung functioning (Clinical trial identifier: NCT01483911). Two subsequent Phase I trials followed; a safety study on patients with hyper-responsive airways and a pharmacokinetic study on healthy subjects (Clinical trial identifier: NCT01909843 and NCT01875926). Positive results were obtained when ALX-0171 was administered by a nebulizer and, in a neonatal lamb model of infant RSV infection, ALX-0171 demonstrated preclinical proof-of-concept. The first-in-infant Phase I/IIa safety study is ongoing (Clinical trial identifier: NCT02309320), which will be followed by a Phase IIb study.

Anti-RV protein VHH (ARP1)

RV-mediated diarrhea in children is a serious problem in developing countries, with more than 125 million episodes of diarrhea yearly and high morbidity and mortality rates. Additionally, treatment options are limited and the cost of treatment and prevention is a critical limiting factor [166]. As described above, Nb-based efforts have already been made with the generation of anti-RV

lactobodies, lactobacilli that express anti-RV Nbs that have prophylactic as well as therapeutic properties. However, these lactobodies have yet to reach the clinical trial stage.

Another anti-RV VHH (anti-rotavirus protein 1 or ARP1) was identified after immunization of llamas with rhesus monkey RV. *In vitro*, ARP1 bound and neutralized a range of simian and rhesus (human-related) RV genotypes and, in an *in vivo* mouse pup model, ARP1 reduced the severity of RV-induced diarrhea [166,326]. Safety studies were conducted on healthy adults and children and, subsequently, a double-blind Phase II clinical study was set up in Bangladesh with male children with RV diarrhea. The aim was to evaluate the efficacy of orally administered ARP1 in reducing RV-induced diarrhea severity and duration, and duration of fecal clearance of the RV (Clinical trial identifier: NCT01259765). In this trial, ARP1 reduced stool output [327]. In India, a Phase II/III trial was initiated, in which investigators evaluated the efficacy of prophylactic treatment of ARP1 given as a food supplement to children aged between 6 and 12 months. Healthy children received ARP1 daily in orange juice, and the incidence of RV gastroenteritis was verified (Clinical trial identifier: NCT01265355). However, no results were available at the time of writing.

Nbs against bone loss-related disorders

ALX-0141

As a key mediator of bone resorption, receptor activator of nuclear factor-κB ligand (RANKL) is an important regulator of osteoclasts. A Nb targeting RANKL was developed for the treatment of bone loss-related disorders, such as (postmenopausal) osteoporosis and cancer-related bone metastasis. ALX-0141 is a trivalent, bispecific Nb comprising two RANKL-binding units and a HSA-binding Nb. S.c. injection of ALX-1041 in cynomolgus monkeys every 2 weeks increased bone mineral content and density [328]. A Phase I clinical trial with postmenopausal women followed up for 1 year indicated that ALX-0141 is well tolerated and effective because even the lowest dose strongly inhibited the bone resorption biomarker CTX-1 [329]. These data support further Phase II trials with ALX-0141, now known as EDP 406, for breast cancer-derived bone metastasis (www.eddingpharm.com).

Diagnostic Nbs

Anti-HER2 Nbs

Nbs offer great opportunities for imaging purposes because they have favorable properties, including high specificity, short residence in blood, and the ease with which they can be linked to radioisotopes. HER2 is an oncogene overexpressed in 20–30% of patients with breast cancer. Screening of the HER2 status in such patients is essential to select patients who are eligible for anti-HER2 therapy. Non-invasive screening of those patients with monovalent anti-HER2 Nb 2Rs15d is now possible [120]. 2Rs15d was linked to ^{68}Ga as a radiotracer via a NOTA-derivative for PET imaging. Preclinically, these Nbs showed highly specific contrast images of HER2$^+$ tumors in xenograft tumor-bearing mice without the appearance of toxicity [330]. The first-in-man clinical trial with this radiopharmaceutical in breast cancer recently finished (EudraCT 2012-001135-31). This trial aimed to assess the safety, human biodistribution, and dosimetry of these Nbs. Fifteen female patients with HER2$^+$ breast tumors first identified by immunohistochemistry were enrolled in a dose-ascending approach. This Phase I study indicated that ^{68}Ga-HER2 PET/CT is a safe procedure with potential for *in vivo* assessment of the HER2 status of tumors because tracer accumulation is high in HER2$^+$ tumors with low background [331].

Nbs against neurodegenerative diseases

BI 1034020

AD is characterized by the formation of amyloid plaques comprising mainly aggregated Aβ peptides in the brain. A half-life extended biparatopic Nb BI 1034020 was designed that targets two different epitopes of Aβ with high affinity. The aim was to reduce the free Aβ peptide levels in plasma to prevent the formation of new Aβ plaques and to clear existing plaques. In a preclinical mouse model, BI 1034020 significantly reduced the free Aβ in plasma. A Phase I study on healthy male volunteers was initiated to evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of i.v. or s.c. injections of BI 1034020 (Clinical trial identifier: NCT01958060). However, the study was discontinued prematurely because of a drug-related SAE in the first volunteer.

Concluding remarks and future perspectives

In 1993, functional HcAbs were discovered in camelids, in contrast to the nonfunctional HcAbs found in patients with heavy-chain antibody disease, which are short and truncated [332]. Given the unique molecular characteristics of their antigen-binding domain, the Nb, these Abs are amenable to many different applications and many *in vitro* proof-of-principle studies have been described. Additionally, *in vivo* studies have also shown that Nbs might offer great therapeutic and/or diagnostic opportunities because of their deep tissue penetration and rapid renal clearance. Their ability to tightly bind hidden and cryptic (immune-evasive) antigens broadens their applicability as diagnostic or therapeutic tools. These properties have been exploited in the fields of oncology, inflammation, infectious diseases, molecular imaging, and others, and could eventually make a substantial difference to therapy and diagnostic screenings. For example, as immunoprobes for directed non-invasive *in vivo* imaging, the success of Nbs is illustrated by anti-HER2 Nbs, which are currently in clinical trials. The use of Nbs in some, if not all, of the fields mentioned above will be accompanied by the development of smart innovative Nb-based systems to overcome some of the shortcomings of Nbs compared with Abs. This is illustrated by the antagonistic Nbs used in oncology. Not much was expected from them initially because they lacked the Fc-effector domain needed for cytotoxicity, although they have many advantages. However, clever rational design could overcome this limitation. Nbs have been designed to specifically increase the lethality of NK cells against cancer cells so that they kill them rather than only stopping their proliferation [68]. By innovative design, Nbs that promote toxin–Nb clearance were generated, and have the neutralizing potency of conventional Abs but with all the advantages of Nbs [224]. We expect that the more compact and stable Nbs will replace mAbs and their fragments, such as Fab and scFv Abs, in many areas because they are easier to produce. During the discovery and developmental phases, Nb expression in bacterial or yeast systems provides cheap in-house purification of the protein, especially compared with conventional Abs. Upstream process development (USP), which is the optimization of the fermentation process, leads to a reproducible and scalable process

that yields pure high-quality end products. However, once Nbs are in clinical trials, the manufacture process needs to be scaled up and has to be compliant with strict good manufacturing practices (GMP) guidelines [333]. Consequently, this scale-up process is accompanied by higher costs, making the cost difference between Nbs and Abs less relevant.

Notwithstanding all their therapeutic possibilities and positive aspects, there are still some obstacles to overcome to the use of Nbs. The potential of their application to neurodegenerative diseases requires considerable improvement. More specifically, the use of intrabodies makes it possible to intervene at early stages of disease, and properties of Nbs make them suited for engineering as stable well-folded intrabodies. This is in contrast to most Abs, which do not fold properly because they cannot form disulfide bonds in the reducing environment of the cytoplasm [285,334]. Undoubtedly, the major obstacle is the delivery of Nb-expressing vectors, but gene therapy is feasible because Nbs are simple molecules expressed from a single gene [335]. Strategies for continuous intracellular delivery have already been devised and could also be transferred to Nb applications [335]. Additionally, the expression of the Nb of interest as a transbody can be considered because the cell-penetrating protein of this fusion construct will guide the Nb inside the cell [161]. Penetratin of the HIV Tat protein can be used as protein transduction domains to facilitate internalization. Nevertheless, the applicability of this approach should be taken with caution because the Nbs end up in the endosomes, from which they have to escape to reach the cytosol. One can also exploit the bacterial type III protein secretion system (T3SS), which injects Nbs directly into cells [53]. However, until now, in neurology, most described Nbs were used as crystallization chaperones to elucidate the structure of protein aggregates and misfolded proteins, or as probes to understand molecular mechanisms. The information generated is useful for identifying new drug targets that might form the basis for new neuroprotective and prophylactic therapeutics, and novel studies are eagerly awaited. In addition, as anti-infectious agents, Nbs could also be the key to new therapies. To the best of our knowledge, no therapeutic Nb has yet been developed for the highly prevalent disease, malaria. Also, because of the emergence and spread of antibiotic resistance, there is an urgent need for new therapeutics against infectious agents. The modularity of Nbs is a favorable characteristic for designing new therapies, because coupling Nbs against different antigens or bacterial and/or viral structures could provide protection against escape mutants. The Nb platform could also be useful for developing lung-targeted drugs. Such drugs are mainly of interest for therapies of respiratory infections or refractory lung diseases. Local delivery maximizes local drug concentration and greatly limits systemic distribution. Although Nbs administered by inhalation with a nebulizer have been described and are in clinical trials (Clinicaltrial.gov: ALX-0171), Nbs directed against lung-specific ligands might also provide a way of specific targeting. One ligand of interest is pulmonary surfactant protein A (SPA) and, *in vivo*, anti-SPA Nbs specifically accumulated in the lung 15 min after i.v. administration without any acute or chronic toxicity observed [336].

Next, although the display technology of phages carrying an immune library is a powerful way to isolate and select the desired Nbs, some of the isolated Nbs have low affinity, partially because they are monovalent. Higher affinities are preferable, especially when clinical application is a goal. Although the construction of multivalent constructs can solve part of the problem, the Nb maturation can also be a solution. Given that the affinity maturation processes of Abs can improve their diagnostic and therapeutic efficacy, it is worthwhile to also apply this to Nbs [337]. Starting from a VHH library from immunized llamas, the error-prone polymerase chain reaction (PCR) technique, which is a widely used technique for Ab maturation because of its simplicity, versatility and cheapness, can be used. The desired Nb is then used as a starting material to construct a highly diversified phage VHH library and, subsequently, the constructed library of mutated Nbs is subjected to several rounds of panning. This can ultimately result in Nbs with 1.5-higher binding affinity than the parental Nb, as described by Hoseinpoor *et al.* [190]. The creation of family-specific libraries that contain homologous VHHs can also lead to the isolation of phages with similar nucleotide sequences as the parental VHH but with improved affinity or neutralization capacity [338]. Additionally, DNA shuffling techniques have been described for engineering more potent, stable, and protease-resistant Nbs [175]. This method relies on the random recombination of different orthologous parent gene segments by DNA fragmentation and PCR reassembly to create a diverse library of progeny genes. Unfortunately, the creation of such immune libraries and subsequent mutant libraries takes time and money. Indeed, even though the expression and production of Nbs are inexpensive, the immunization of llamas, alpacas, or camels is not, especially when compared with the immunization of smaller animals for the generation of Abs. Also, in some cases, immunization is not feasible when the antigens are toxic, lethal, or transmittable, or have a low immunogenicity; thus, this resulted in the need to look for alternatives. For example, one can start from non-immune, naïve, or synthetic VHH libraries [252,339], and high-affinity antigen-specific Nbs could be selected starting from semisynthetic libraries using the *in vitro* ribosome display technique, in which no cloning and transformation are required when the mutant library is constructed [340]. Furthermore, Koide *et al.* enhanced the affinity by more than 100-fold by combining molecular mechanisms and rational design [341]. However, non-immune libraries could lead to successful Nbs, although they did not outperform the phage display immune libraries in terms of selecting potent Nbs [342].

The cumbersome immunization of llamas or camels needed to generate Nbs can be counteracted not only with the above-mentioned non-immune, naive libraries, but also by immunization of smaller animals. Some research groups have generated transgenic mice expressing correctly folded Nbs in B cells because they carry a mini-Ig construct that encodes dromedary VHH or a hybrid llama/human antibody locus [343–345]. In mature B cells of healthy mammals, except camelids, cellular release of single H chains without L chains is normally prevented in the ER. However, conventional B cell development is blocked at an immature B cell stage in $L^{-/-}(\kappa^{-/-}\lambda^{-/-})$-deficient mice and this leads to spontaneous production of HcAbs [346].

Another important point is the impact that Nbs might have when they are released in the environment. Some Nbs are selected to be more stable than Abs, and so will not biodegrade immediately when they are exposed to the external environment. This has already been

demonstrated by stable Nbs present in shampoos used to treat dandruff [18]. The significance of excreted antibacterial or antiviral Nbs on emerging multiresistant bacteria needs to be studied further. Given that antibiotics present in groundwater can affect natural bacteria and agriculture, the risks of Nbs need to be evaluated and measures to inactivate or restrict the amount of released Nbs should be taken [347].

Finally, there are other promising small engineered antibody mimetic protein scaffolds, apart from Nbs, such as DARpins, which are derived from ankyrin proteins [348]; anticalins, which are based on the lipocalin scaffold [349]; affibodies [50,350]; and other non-immunoglobulin scaffolds, reviewed in [351]. These have similar advantages to Nbs, such as high specificity and affinity, robust biophysical properties, and low immunogenicity, and could also be expressed in microbial expression systems. Additionally, some of these scaffold proteins are already involved in clinical trials.

As a general conclusion, Nbs are versatile molecules with favorable properties and have been evaluated for both therapeutic and diagnostic applications, as well as research tools. The way is now open to further exploit the inexhaustible potential of Nbs and to further position them as pharmaceuticals for use in daily clinical practice.

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