REVIEW ARTICLE

The Therapeutic Potential of Nanobodies

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Abstract

Today, bio-medical efforts are entering the subcellular level, which is witnessed with the fast-developing fields of nano-medicine, nanodiagnostics and nanotherapy in conjunction with the implementation of nanoparticles for disease prevention, diagnosis, therapy and follow-up. Nanoparticles or nanocontainers offer advantages including high sensitivity, lower toxicity and improved safety—characteristics that are especially valued in the oncology field. Cancer cells develop and proliferate in complex microenvironments leading to heterogeneous diseases, often with a fatal outcome for the patient. Although antibody-based therapy is widely used in the clinical care of patients with solid tumours, its efficiency definitely needs improvement. Limitations of antibodies result mainly from their big size and poor penetration in solid tissues. Nanobodies are a novel and unique class of antigen-binding fragments, derived from naturally occurring heavy-chain-only antibodies present in the serum of camelids. Their superior properties such as small size, high stability, strong antigen-binding affinity, water solubility and natural origin make them suitable for development into next-generation biodrugs. Less than 30 years after the discovery of functional heavy-chain-only antibodies, the nanobody derivatives are already extensively used by the biotechnology research community. Moreover, a number of nanobodies are under clinical investigation for a wide spectrum of human diseases including inflammation, breast cancer, brain tumours, lung diseases and infectious diseases. Recently, caplacizumab, a bivalent nanobody, received approval from the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) for treatment of patients with thrombotic thrombocytopenic purpura.

Key Points

Antibodies, major macromolecules used for targeted therapy, led to significant improvement in clinical care and quality of life of cancer patients.

However, antibody limitations in terms of size, incomplete tumour penetration and possible immunogenicity led to the development of a new generation of petite drugs and medicines.

Biological (nano)drugs, including nanobodies, offer new possibilities for treatment of not only cancer, but also a variety of human diseases on a subcellular level that will revolutionize the (bio)medical fields, as confirmed by the EMA and FDA approval of caplacizumab.

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1 Introduction

1.1 Antibodies for Cancer Therapy

Cancer is considered a cluster of diseases with different molecular changes, including gene mutations and amplifications, copy number alterations, changes in tumour suppressor and DNA repair genes, and epigenetic modifications [1, 2]. Development of a successful tumour therapy is challenging due to low specificity of the drug and toxic effect on adjacent non-tumour cells. An active targeting therapy relies on the specific delivery of an active drug to the target using different possible affinity reagents such as those mediated by a lectin-carbohydrate, ligand-receptor or antibody-antigen recognition [3–5]. Obviously, for maximal effect, the specific receptor targeted by the affinity reagent should be overexpressed at the surface of the diseased cells. Thus, active targeting refers to site-specific ligand-mediated accumulation of drugs into the diseased site due to an increased expression of a specific biomarker for that malignancy [6].

Immunoglobulins (Ig) or antibodies are soluble glycoproteins playing an essential role as the natural therapeutic compound in vertebrates [7]. Five different classes of

antibodies (IgG, IgM, IgA, IgD and IgE) are elicited by the immune system as a response to non-self molecules (antigens), except in autoimmune disease conditions, with the purpose of their neutralization or elimination. The complex structure of antibodies is highly conserved among mammals. Antibodies consist of two identical heavy and two identical light chains connected by interchain disulphide bonds and non-covalent interactions as shown in Fig. 1a [8]. The antigen-binding site of antibodies comprises three loops of variable sequence and length within the variable heavy (V_H) domain and three variable loops within the variable light (V_L) domain [8]. Due to their specificity and affinity for their cognate antigen, antibodies play an important role in biomedical research and they are a preferred vehicle for the specific delivery of a therapeutic drug to its target.

Antibodies were the first macromolecules used for targeted delivery [9]. Monoclonal antibodies have been employed for several decades in clinical practice for treatment of malignancies [10]. These include anti-carcinoembryonic antigen (CEA) antibody arcitumomab, anti-prostate-specific membrane antigen (PSMA) antibody capromab, anti-cluster of differentiation 20 (CD20) antibody rituximab, anti-human epidermal growth factor receptor 2 (HER2) antibody trastuzumab, anti-vascular endothelial growth factor (VEGF) antibody bevacizumab,

anti-cluster of differentiation 52 (CD52) antibody alemtuzumab and three anti-epidermal growth factor receptor (EGFR) antibodies cetuximab, panitumumab and matuzumab [11–13], which are extensively reviewed elsewhere [14, 15]. These monoclonal antibodies are used for systemic tumour treatment and aim to block or neutralize growth factors or their receptors [16]. Antibodies are used for targeting cancer cells using one of the following mechanisms: killing by immune cells (opsonization); modification of biological processes like apoptosis; or delivery of cytotoxic agents like chemotherapeutics [17]. However, an issue with the use of murine antibodies in clinical care is that they elicit an immunogenic response in patients, that is, the generation of human anti-mouse antibodies (HAMA) targeting the murine idiotopes on the administered antibodies, which leads to their neutralization [18]. Different methods have been implemented to reduce immunogenicity, one of which is the construction of chimeric antibodies—monoclonal antibodies comprising human heavy and light chain constant regions and murine variable domains. The introduction of chimeric antibodies led to a decrease of immunogenicity from 50–75% in case of HAMA to 30% in case of human anti-chimeric antibodies (HACA) [18]. A further reduction of the immunogenicity could be obtained by grafting the antigen-binding loops of the V_H and V_L into a human
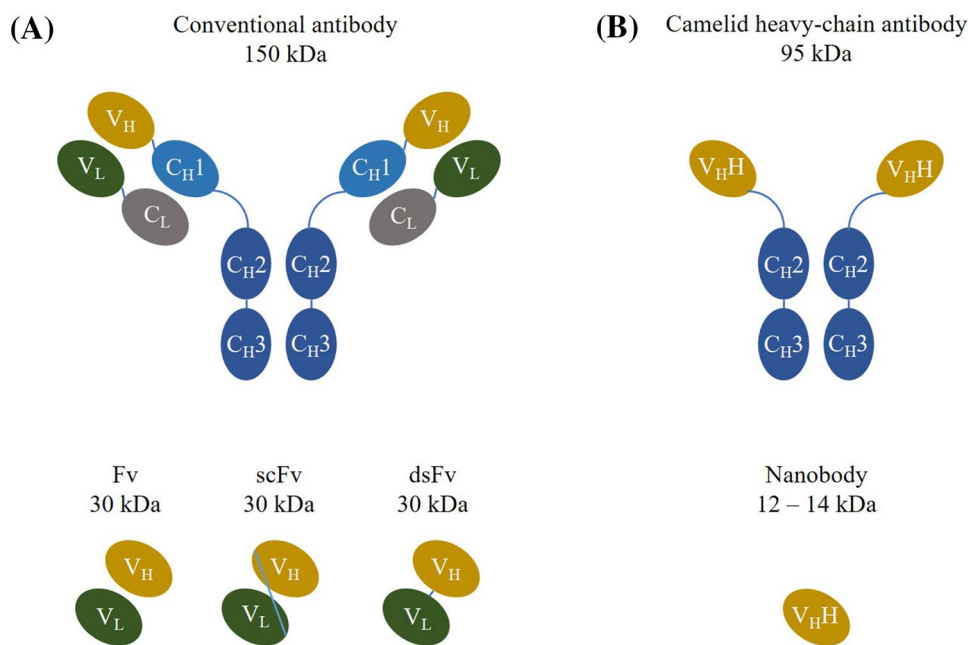

Fig. 1 Graphical representation of different antibody structures. **a** Classical antibodies consist of two identical heavy (variable—V_H and constant—C_H1/2/3 domains) and two identical light (variable—V_L and constant—C_L domain) chains connected with disulfide bonds. The antigen-binding region (variable fragment—Fv) consists of V_H and V_L connected with a linker peptide or stabilized with a disulfide bond in the cases of single-chain variable fragment (scFv) and disulfide-stabilized variable fragment (dsFv), respectively. **b** Camelid heavy-chain antibodies consist of two identical heavy chains only (variable—V_HH and constant—C_H2/3 domains). The antigen-binding region consists of a single variable domain V_HH or nanobody

IgG antibody. Another complication of using antibodies originates from their relatively large size, with dimensions of 14.2 nm × 8.2 nm × 3.8 nm, and corresponding large molecular mass of 150 kDa, which limits their penetration into tumour tissue. In vivo studies suggest that only 0.001–0.01% of the injected antibodies accumulate per gram of solid tumour [19, 20]. The poor penetration can also be a result of the 'binding site barrier' effect, first postulated by Weinstein [21, 22], in which antibodies with high affinity towards their antigens bind strongly to the antigen they first encounter. It was previously reasoned that antibodies with the highest affinity towards their antigens would lead to the best tumour targeting affect, as their slow k_{off} rates would ensure prolonged in vitro retention of the antibody on the tumour cells. However, Adams et al. showed that high affinity limits tumour penetration and intratumoural diffusion [23] (i.e., antibodies are trapped at the tumour periphery). Other factors that contribute to uneven and heterogeneous distribution of the antibodies are antigen density, vascularization, capillary permeability, tissue structure and composition, extracellular matrix components, interstitial pressure and degree of necrosis [17, 22]. In the case of cancer therapy, the 'binding site barrier' phenomenon leads to incomplete tumour penetration and therefore suboptimal therapeutic efficiency. Consequently, to improve the effect of antibody therapy, antibodies with moderate affinity towards their antigens are considered more advantageous than high affinity antibodies; the latter can be used as appropriate vehicles for delivery of different agents (e.g., toxins and chemotherapeutics) [23]. As a result of the suboptimal antibody concentrations, many patients also develop resistance to the antibody-based therapies, which leads to additional treatment failure [24].

Another weakness of antibodies is their fragility, which allows only intravenous or subcutaneous administration. Moreover, due to the complex hetero-tetrameric structure and posttranslational modifications, therapeutic monoclonal antibodies are mainly expressed in mammalian cells, which results in high expenses during their large-scale production [25]. As a result, so far in tumour management, benefit from the employment of monoclonal antibodies has been proven for only a small number of patients [26].

Passive targeting is usually preferred for the treatment of solid malignancies. Passive targeting employs the increased vascular permeability and retention effect, as well as the poor lymphatic drainage to achieve drug accumulation into the tumour microenvironment [3, 27, 28]. Increased vascular permeability allows for small molecules to pass freely into the tumour interstitium, while the lack of intact lymphatic drainage limits their removal, leading to an effective accumulation of the drugs into the tumour [9, 29, 30]. This results in up to 100 times higher drug concentration into the tumour, which leads to greater tumour cytotoxicity [31, 32]. The ability of a therapeutic drug to penetrate the tumour is not dependent solely on the physicochemical properties of the drug, but it is also influenced by the biology of the tumour, the microvasculature and the interstitial pressure [33]. Once the therapeutic antibody is administered, it can either enter the tumour from the vasculature (extravasation) or from the surrounding tissue (surface uptake) [34]. Besides this, antibody targeting involves diffusion and binding into the tumour interstitium, plasma clearance and internalization as well as catabolism into tumour cells [35]. As described by the Stokes–Einstein equation ($D = \frac{k_B T}{6\pi \eta r}$, where k_B is Boltzmann's constant, T is the absolute temperature, η is the dynamic viscosity and r is the radius of the spherical particle) for diffusion of spherical particles through a liquid, the diffusion rate is inversely proportional to the molecular radius [17]. In the case of antibodies, the diffusion coefficient varies between 5 and 50 μm^2/s [34]. Hence, passive targeting is a non-controllable process since not all drug molecules diffuse at an equal rate. Problems that arise from passive targeting mechanisms are unequal permeability of blood vessels throughout the tumour, which leads to uneven drug distribution and appearance of multidrug resistance (MDR). Transporter proteins that are overexpressed on the surface of cancer cells expel drugs from cells leading to development of MDR that ultimately results in drug resistance and treatment failure [30]. It is suggested that for effective therapy, nanoparticles ought to be in the size range between 5 and 200 nm. This will allow them to pass through the pores between endothelial cells, which vary in size from 50 to 200 nm [9, 36]. Nanoparticles < 5 nm are not recommended for therapeutic use as they might be quickly eliminated from circulation through renal clearance, while those > 200 nm will be captured by the liver and spleen reticuloendothelial system [36]. The best performing nanoparticles are those with a diameter of < 100 nm and with a hydrophilic surface [5, 10].

Although the introduction of antibodies into clinical care initially showed great success, their limitations in size, poor penetration, long serum half-life, strong background signal, heterogeneous distribution and immunogenicity strongly suggests that further progress could be achieved in this field. As the trend in targeted drug delivery moves towards the use of smaller and highly specific molecules, switching from classical antibodies to camelid nanobodies can help in circumventing some of the existing complications in patient care. In this review, we briefly outline the beneficial properties of camelid nanobodies in comparison with human immunoglobulins in treating cancer patients. We evaluate the potential role of nanobodies for in vivo imaging and therapy. We also illustrate the development of nanobodies

as biodrugs for treatment of breast cancer, lung diseases and brain tumours. Finally, we present the prospective use of nanobodies as next-generation therapeutic biodrugs by describing caplacizumab, the first EMA- and FDA-approved therapeutic nanobody construct acting against the rare blood disorder thrombotic thrombocytopenic purpura.

1.2 Nanobodies, the Smaller Variant of Antibodies

The occurrence of functional heavy-chain-only antibodies in the serum of camelids (dromedaries, camels, llamas, alpacas, guanacos and vicuñas) was discovered serendipitously nearly 30 years ago [37]. As the name suggests, heavy-chain-only antibodies lack light chains and also the first constant C_H1 domain within the heavy chain is absent (Fig. 1b), which leads to their size reduction. Heavy-chain-only antibodies have a molecular mass of 95 kDa, while their variable antigen-binding domains (V_HH) have a prolate shape with dimensions of 4 nm×2.5 nm×3 nm (Fig. 2) and are usually 12–14 kDa [38–41]. This variable domain of these heavy-chain antibodies comprises full antigen-binding potential, strong affinity to its cognate antigen and so it is considered to be the smallest naturally occurring, intact antigen binding fragment [42]. Their petite size in the low nanometre size range inspired Ablynx to introduce the name 'nanobodies'. Nanobodies are extensively used for research purposes in academia, but are also identified and produced by numerous commercial companies, including Ablynx (now Sanofi), VHsquared, Chromotek, Camel-IDS, Hybrigenics and many others [10].

Nanobodies have the same structural architecture as V_H domains of human immunoglobulins: four conserved sequence regions (framework regions—FR1/2/3/4) are surrounding three hypervariable antigen-binding loops (complementarity determining regions—CDR1/2/3) [42–44]. The paratope of nanobodies comprises three CDRs, and also, regularly, framework residues are involved in the antigen interaction. Nevertheless, CDR3 is the main contributor for antigen recognition and specificity, whereas CDR1 and CDR2 assist in the binding strength [45–47]. The CDR3 of nanobodies is on average 18 amino acids long, which is substantially longer than the average 12- or 14-amino acid-long CDR3 loop of V_H domains from mouse or human antibodies, respectively [20, 44]. A notable exception to this extended nanobody CDR3 loop are the llama nanobodies, where a fraction has a significantly shorter CDR3 consisting of only 4–8 amino acids [42]. The longer length of the majority of the CDR3 suggests a greater structural flexibility in the antigen-free form, which supposedly will be fixed in one single conformation upon antigen binding and thus creates an entropic penalty for binding. The presence of an additional interloop disulphide bond between either CDR1 (in camels) or CDR2 (in llamas) and CDR3 reduces this entropic penalty
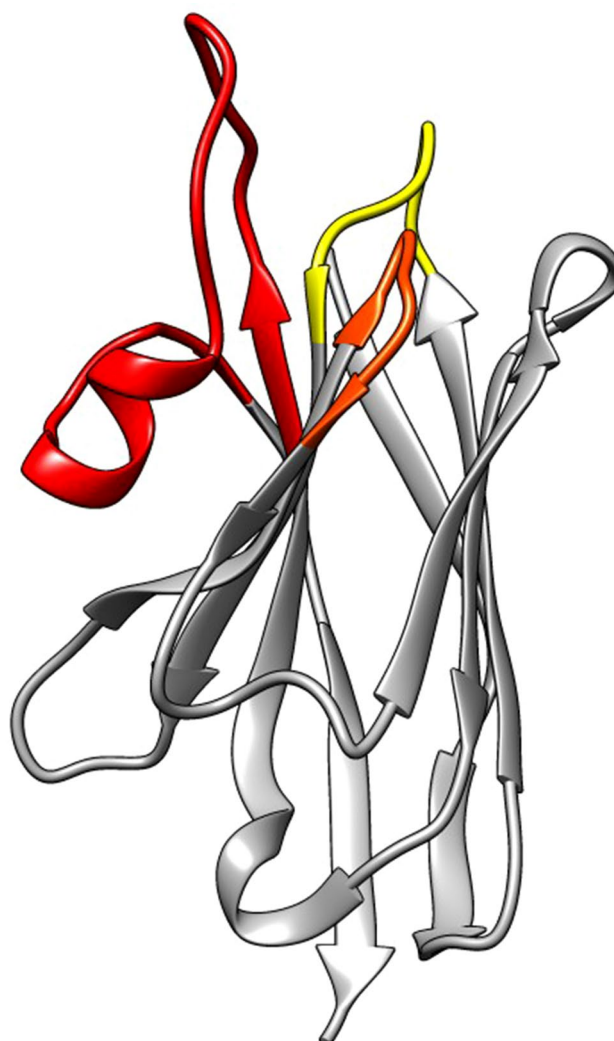

Fig. 2 Ribbon representation of a nanobody (pdb 1JTT). The framework regions are in grey, the hypervariable H1, H2 and H3 antigen binding loops are in yellow, orange and red, respectively

and at the same time increases the conformational stability of nanobodies [48]. Furthermore, a hallmark difference between a human V_H and a camelid V_HH is noted within the framework 2 region where four highly conserved and hydrophobic amino acids (V42, G49, L50 and W52 in V_H) are substituted in nanobodies by smaller and/or more hydrophilic amino acids (F42 or Y42, E49, R50 and G52) (amino acid numbering is according to the international ImMunoGeneTics (IMGT) information system). This increased frequency of occurrence of polar and charged amino acids at the solvent-exposed site of the autonomous V_HH explains their enhanced solubility in polar solvents (i.e. water) [42]. The extended CDR3 loop of nanobodies has the capacity to form a finger-like structure or a convex paratope (Fig. 2) that penetrates into the small cavities on the surface of the antigen or interacts with antigenic concave surfaces, respectively.

Therefore, the paratope of nanobodies are perfectly shaped to recognize specifically the catalytic site enzymes and so many nanobodies act as modulators of the enzymatic activity [20, 45, 49, 50]. In sharp contrast, the paratopes of classical immunoglobulins form cavities, grooves or flat surfaces that are associated with small chemical groups (haptens), peptides or epitopes on large proteins, respectively. Hence, since nanobodies and classical antibodies prefer differently shaped epitopes, they will normally not compete for binding on the same antigen. This is an extremely useful property if one wants to quantify antigens of infectious agents in the presence of host serum. Moreover, nanobodies can be selected or developed to distinguish between different protein isoforms, a very important property to develop a highly specific diagnostic test. In addition, the utility of nanobodies as probes in immunocytochemistry, immunohistochemistry and immunoblotting has been demonstrated [51, 52]. However, as nanobodies recognize mostly conformational epitopes, their application as probes in western blot is less successful [53].

Moreover, nanobodies in general have unexpected physical properties: prolonged shelf life at $+4\,^{\circ}\mathrm{C}$ and at $-20\,^{\circ}\mathrm{C}$, tolerance to increased temperature (60–80 °C, several weeks at 37 °C), resistance to proteolytic degradation, exposure to non-physiological pH (pH range 3.0–9.0), elevated pressure (500–750 MPa) and chemical denaturants (2–3 M guanidinium chloride, 6–8 M urea), all of which barely harm their antigen-binding capacity [54]. The nanobody robustness is mainly attributed to its efficient refolding capacity after chemical or thermal denaturation [55], although this reversible refolding upon thermal denaturation was recently questioned [56]. The monomeric structure of nanobodies and the lack of post-translational modifications allow for their expression in microbial systems including *Escherichia coli*, *Saccharomyces cerevisiae* and *Pichia pastoris*, which reduces the manufacturing costs [20, 57]. Nanobodies can be produced in milligram quantities per litre of culture in shake flasks, which means low-cost manufacturing and availability of sufficient amounts of purified nanobodies with consistent properties [58].

The sequence identity between nanobodies and the V_{H} of human immunoglobulins of family III is above 80% [59]. In their extensive analysis, Klarenbeek et al. assessed the homology between the camelid germline IgV gene repertoire and their human counterparts and found 95% sequence identity of the camelid IGHV family 3 with its human FR counterpart [60]. This means that nanobodies will have a low immunogenic profile and are thus suitable for human administration [61]. Still, the sequence of a therapeutic nanobody can always be 'humanized' if wanted [62]. However, additional engineering might be required after the humanization process to restore the original affinity of the nanobody for its target. A number of nanobodies have been identified

as lead compounds and reached advanced preclinical stages, while several of them are currently being tested in clinical trials as presented in Table 1 (data obtained from https://clinicaltrials.gov) [63–65]. Diagnosing cancer in early stages requires the use of imaging agents that are able to penetrate tumour tissues and bind to their targets with high specificity while the excess, unbound agent is rapidly removed from the body. The size of nanobodies makes them suitable agents for in vivo imaging [66–68]. Their short half-life in the bloodstream assures a high tumour to background ratio shortly after administration, which is a desired property of imaging agents [10, 38, 40, 69]. High antigen specificity is crucial for reducing side effects and minimizing the chance of obtaining false positives [54]. However, for molecular imaging, nanobodies have to be labelled with a diagnostic radioisotope, which can be gamma-emitting nuclides for single photon emission computed tomography (SPECT) or positron-emitting nuclides for positron emission tomography (PET) [70]. PET isotopes ^{68}Ga and ^{18}F are most appropriate for human use due to the short half-lives of 68 and 110 min, respectively. One such example is the use of an anti-HER2 nanobody for detection of HER2 expression in breast cancer using PET–computer tomography (PET/CT) and for clinical PET/CT and molecular imaging (iPET) [68, 71]. The authors examined safety, biodistribution, dosimetry and tumour targeting potential of ^{68}Ga-HER2 nanobody in breast cancer patients and observed a complete absence of adverse effects. Therefore, they concluded that the procedure is safe to use in human patients. They noticed only a low background signal, rapid renal clearance of the tracer (60–90 min post-injection) and highest uptake in the metastatic lesions, kidneys, liver and the intestines. Early and late half-lives of the ^{68}Ga-NOTA anti-HER2 nanobody were calculated to be 2.9 and 25.5 min, respectively, while at 1-h post-injection only 10% of the tracer was monitored. The ^{68}Ga-NOTA anti-HER2 nanobody is currently being prepared for a phase II clinical trial (ClinicalTrials.gov NCT03331601) evaluating its potential to detect brain metastasis in breast cancer patients; the trial is expected to be completed in 2021. Although the biodistribution of nanobodies is found to be antigen-specific, a potential problem in using nanobodies as in vivo imaging probes is their accumulation in the kidneys, which is a consequence of their renal elimination [66]. Accumulation in the kidneys might limit the use of nanobodies as detection probes to screen organs located in the vicinity of kidneys, such as the pancreas [54].

Although the small size of a monomeric nanobody is beneficial in many cases, it can be a disadvantage for therapy as nanobodies will be rapidly cleared through the kidneys since their molecular mass is well below the 50–60 kDa renal threshold for glomerular filtration. Therefore, if the antigen is not circulating in the patient's blood, only a marginal fraction of the administered nanobody will reach its

Table 1 Nanobodies that have entered in clinical trials

Nanobody drug	Disease	Target	Clinical trial	Phase of clinical development	References
Caplacizumab ALX-0681 ALX-0081	Thrombotic thrombocytopenic purpura	Ultra large von Willebrand factor	NCT03172208 NCT02878603 NCT01151423 NCT02189733 NCT02553317 NCT01020383	I III II I III II	[98, 139–143]
Ozoralizumab ATN-103	Rheumatoid arthritis	TNF	NCT01007175 NCT00959036 NCT01063803 NCT00916110	I/II I/II II I	[144]
Vobarilizumab ALX-0061	Rheumatoid arthritis	IL6	NCT02518620 NCT02309359 NCT02287922 NCT02101073 NCT01284569	II II II I I/II	[145]
Vobarilizumab ALX-0061	Systemic lupus erythematosus	IL6	NCT02437890	II	
ALX-0171	Lower respiratory tract infection	RSV	NCT02979431 NCT02309320 NCT01483911 NCT01909843 NCT01875926 NCT03468829 NCT03418571	II I/II I I I II II	[114, 146]
ALX-0761 M1095 MSB0010841	Psoriasis	IL17A, IL17F and IL17A/F	NCT03384745 NCT02156466	II I	[147]
Bispecific nanobody-derived CAR-T cells	Refractory/ relapsed B-cell lymphoma	CD19/CD20	NCT03881761 NCT03664661	I I	[105, 148]
α-ADAMTS-5 nanobody M6495	Osteoarthritis	ADAMTS-5	NCT03583346 NCT03224702	I I	[149, 150]
VHH batch 203027	Diarrhoea	Rotavirus	NCT01259765	II	[151]
[^{131}I]-SGMIB anti-HER2 VHH1	Breast cancer	HER2	NCT02683083	I	
68-GaNOTA-anti-HER2 VHH1	Brain metastasis of breast carcinoma	HER2	NCT03331601	II	
ALX-0651	Healthy volunteers	CXCR4	NCT01374503	I	
PF-05230905	Healthy volunteers		NCT01284036	I	

Phase I: dosing, toxicity and excretion in healthy subjects; Phase II: safety and efficacy in large patient cohorts; Phase III: multi-centred, randomized, placebo-controlled trials; Phase IV: post-marketing studies

ADAMTS-5 α-A disintegrin and metalloproteinase with thrombospondin motifs-5, *CAR* chimeric antigen receptor, *CD* cluster of differentiation, *CXCR4* chemokine receptor type 4, *HER2* human epidermal growth factor receptor 2, *IL* interleukin, *RSV* respiratory syncytial virus, *TNF* tumour necrosis factor

cognate target. Thus, in order to obtain a high target load in vivo, a monomeric nanobody should be frequently administered. Alternatively, multimeric nanobody constructs or nanobodies fused to serum albumin (either directly or via an albumin-binding nanobody) can be produced for increasing their half-life in blood [40, 72]. In one study, the authors fused a bivalent α-EGFR nanobody to a nanobody binding to human serum albumin and managed to increase the serum half-life of this α-EGFR-α-EGFR-α-Alb nanobody to 2–3 days [26].

1.3 Nanoparticles in Nanomedicine

Important developments in the clinical care of cancer patients including genomic profiling, immunotherapy and targeted therapy, emerged in the last decades [73]. One such

revolutionary approach is the use of nanoparticles (metallic, inorganic, organic and biological) for medical purposes.

Nanomedicine is a rising interdisciplinary field that is based on the use of nanoparticles in health and medicine with the objective to prevent, to diagnose and to treat disease at a molecular level [29, 74, 75]. In general, nanoparticles are small molecules with sizes ranging from 1 nm to 1 μm; for biological purposes, however, the size usually varies between 10 and 800 nm [76]. The aim of nanomedicine is development of novel imaging and therapeutic agents with enhanced efficacy, improved safety and lower toxicity [27, 77]. Besides nanomedicine, the fields of nanodiagnosis and nanotherapy are also rapidly developing. Nanodiagnosis stands for the planned use of devices with at least one dimension in the nanometre scale for detecting events that occur at a molecular level [78]. Nanodiagnosis will provide early disease monitoring with high sensitivity. For performing a successful diagnosis, nanoparticles need to meet several criteria: rapid extravasation from the blood vessels, fast penetration into the desired tissue, and eventually the unbound agent should quickly re-enter the blood vessels and be eliminated by the body, preferably via the kidneys, to generate a high signal-to-noise ratio in targeted tissues [29]. Until now, several nanoparticles have been developed for use in diagnostics with the most common being gold nanoparticles, dendrimers and quantum dots [10, 76]. In addition, nanoparticles can be combined with existing imaging technologies to obtain a more precise diagnosis.

The term nanotherapy describes the use of nanoparticles for drug delivery and therapy. The failure of current tumour therapy often results from a lack of effective drug delivery, toxicity issues, acquired drug resistance and poor drug solubility in aqueous solutions [28, 79]. Implementation of nanoparticles in tumour therapy can significantly improve the clinical outcome due to their beneficial properties such as small size, water solubility, improved stability, increased bioavailability and better pharmacogenetic features [80, 81].

The use of nanoparticles in oncology (i.e., nano-oncology) includes diagnostics as well as therapy [81, 82]. Thus, nano-oncology is a hybrid field between biochemistry, engineering technology and medicine with the aim of advancing tumour detection, diagnosis and therapy [83]. For successful implementation in clinical care, nanoparticles should be biocompatible, biodegradable, non-toxic, stable after administration and easily produced at large scale with controllable physical and chemical properties [84]. Different nanoparticles like liposomes, gold nanoparticles, iron-oxide nanocrystals and quantum dots are being manufactured for use in nano-oncology [3, 6, 10, 76]. However, our current knowledge of nanoparticle pharmacokinetics, biodistribution, metabolism and clearance is limited due to the small number of such particles being tested in clinical trials [83]. Additionally, potential toxicity issues for humans and the environment of the employment of inorganic nanoparticles are still to be determined [27, 85–87]. Once administered, nanoparticles will have the opportunity to interact with different biological environments (like cytoplasm and extracellular matrix), multiple cells, organs and tissues before they reach their specific target [27]. It is anticipated that nanoparticles that contain heavy metals can accumulate in vital organs such as the liver and brain, and can lead to tissue-specific toxicity [86]. Moreover, metallic (iron oxide and silver) nanoparticles can generate reactive oxygen species and lead to genotoxicity, while gold nanoparticles present risks for humans due to their affinity towards DNA and a low clearance rate. The properties of nanoparticles including their benefits and pitfalls have to be thoroughly examined and understood before they reach the patient. Nevertheless, the conjugation of nanobodies to nanoparticles could become an interesting combination, especially for nanomedicine and nano-oncology. Nanobodies are easily tailored for a directional conjugation to nanoparticles, which is expected to lead to an improved active targeting of the decorated nanoparticles to diseased tissues. However, the nanoparticle, or nanocontainer that can be loaded with drugs, will increase the size of the nanobodies, so that extravasation and tissue penetration will be less favourable.

2 Nanobodies as Therapeutics

Because nanobodies combine the beneficial properties of small molecules and monoclonal antibodies, they are an attractive agent for development of novel therapeutic strategies. Their small size makes them useful for targeting antigens residing in tissues that are weakly vascularized and poorly accessible. Nanobodies show better extravasation and tissue penetration than classical monoclonal antibodies, which is obviously crucial for therapeutic applications [39, 61]. The growth of solid tumours is dependent on the formation of new blood vessels (i.e. angiogenesis) and many nanobodies have been generated to interfere with this vascularization [88]. In one study, a nanobody against VEGFR2 was identified, which showed high antigen binding affinity ($K_D = 5.4$ nM) and inhibitory potential of capillary tube formation in vitro [89].

Blood and lymphatic vasculature differ significantly between tumours and healthy tissue. While normal blood vessels are compact and continuous, tumour blood vessels are leaky, containing pores that can reach over 100 nm in size. This leads to a phenomenon that is referred to as the "enhanced permeability and retention effect" (EPR) [80]. So far, potential targets for nanobody-based therapies are extracellular targets such as ligand receptors or transmembrane proteins with differential expression in the target cells of interest. To this end, nanobodies against transmembrane growth factor receptors EGFR1 (HER1), EGFR2 (HER2), VEGFR2, c-Met and chemokine receptor type 7 (CXCR7)

have been developed [61, 89–92]. These receptors have been linked to different malignancies. For example, VEGFR has been found expressed in different cancers including brain, lung, breast and colon cancer; c-Met is implicated in colon, breast and ovarian cancers and haematological malignancies; and the overexpression of CXCR7 is correlated with breast and lung cancers [61, 90]. Nanobodies against extracellular targets like HGF and chemokines have also been developed [26].

In addition, nanobodies can be used as drug carriers (nanocarriers) and other delivery systems for targeted therapy. This way, systemic toxicity will be avoided, hydrophilic drugs can be solubilized into lipid bilayers or micelles, and large amounts can be administered at once [53]. For such purposes, nanocarriers should be between 5 and 200 nm in size with hydrophilic surface [5, 36]. Examples of such cargoes with enhanced specificity for tumour cells expressing EGFR are nanobody-liposomes [93], nanobody-micelles [94] and nanobody-albumin nanoparticles [95].

For cancer therapy, nanobodies can be used either as antagonists, to prevent ligand binding and causing conformational changes that lead to activation of signalling cascades, or as allosteric inhibitors to modulate the enzymatic activity of their target proteins [61]. It is notable that anti-ligand nanobodies can be effective only if one single ligand is involved in the induction of the signalling cascade of the receptor [53].

Nanobodies show thermal resistance and stability against harsh conditions, chaotropic agents and pH extremes, so the route of administration can be intravenous, oral, intraperitoneal or intratumor. In each case, the nanobodies should be formatted in line with the stringent conditions that they will meet, like extreme pH and proteases after oral administration, and serum stability after an intravenous injection. Proper formulation is essential to ensure stability and drug release at the desired site, and to avoid unwanted side effects. In general, an extreme isoelectric point (pI) of the nanobody makes it less suitable for in vivo use. Monomeric nanobodies are rapidly cleared from the bloodstream via the kidneys and if such nanobodies are conjugated with toxic substances, their accumulation in the kidneys might lead to unwanted renal toxicity [66].

Despite the weaknesses of nanobodies for therapeutic applications (short serum half-life and rapid renal clearance), their superior properties (natural origin, small size, low immunogenicity, strong antigen-binding affinity, stability and water solubility) allowed for a number of them to enter clinical trials [64, 68, 96–98]. Three major points have to be addressed for drug candidates to enter clinical trials:

(a) does the drug reach the target organ in sufficient amounts to give the desired effect?

(b) is the specific binding of the drug and the target enough for the biological activity?

(c) does the compound exert the functional modulation of the target? [99].

The clinical trial database https://clinicaltrials.gov was searched for the keywords 'nanobody', 'nanobodies', 'VHH' and 'Ablynx' (access date 19 April 2019) and yielded the results presented in Table 1. Nanobodies used in clinical trials are of different formats such as bivalent monospecific, bivalent bispecific, bivalent bispecific albumin-conjugated, trivalent bispecific and bispecific chimeric antigen receptor (CAR) T cell.

For activating T cells in vitro, anti-'cluster of differentiation 3' (CD3) antibodies are used. Still, therapeutic anti-CD3 antibodies induce systemic inflammation and adverse effects. This led to the development of bispecific T-cell engagers (BiTE) that are smaller than antibodies (11 nm in length and 55 kDa in size) and are composed of two single-chain variable fragments (scFv) with unique antigen specificity connected by a short amino acid linker [100]. What distinguishes BiTE from other scFv is that one receptor usually targets the CD3 subunit of the T-cell receptor, which allows for targeting T cells, while the other receptor targets a specific tumour antigen. In this regard, smaller sized BiTEs can be generated by substituting one or both the scFv with nanobodies. An example is the Her2 × CD3 bispecific antibody using anti-CD3 clone UTCH1 scFv and anti-Her2 nanobody, which showed strong anti-tumour effect in vitro and inhibits tumour growth in vivo [101, 102]. A similar approach was used by Mølgaard et al. with the generation of the bispecific light T-cell engager (LiTE) consisting of anti-CD3 UTCH1 scFv and anti-EGFR nanobody [103]. The model shows high tissue penetration, but requires continuous injection for therapeutic purposes. As an alternative, chimeric antigen receptor (CAR) T cells expressing non major histocompatibility complex (MHC) antigen receptors can be used. CAR T cells with nanobodies as the antigen-recognizing agent against MUC1 and EGFR have already been described [104, 105].

A different approach is the use of nanobodies in photodynamic therapy (PDT)—a minimally invasive and non-toxic treatment that induces cell death by activating a photosensitizer with light exposure [106]. The use of antibodies (both classical and heavy-chain) in PDT is termed 'photoimmunotherapy'. Van de Broek et al. developed a method for conjugation of gold nanoparticles with anti-HER2 nanobodies to target HER2-expressing carcinoma cells [6]. The authors proved specific interaction of the carcinoma cells with the anti-HER2 conjugated gold nanoparticles, while binding was not observed for the controls.

In addition to these, nanobodies conjugated to various nanoparticles (liposomes, micelles and albumin nanoparticles) have also been explored [53]. A multivalent nanobody-liposome system targeting EGFR-overexpressing tumour cells was described by Oliveira et al. [93]. In this study, the authors show that liposomes conjugated to anti-EGFR ectodomain (EGa1) associate to a greater extent with human tumour cells than liposomes without the conjugated nanobody. Later, an improved liposome-nanobody system loaded with an anti-IGF-1R kinase inhibitor (AG538) was introduced [107]. This AG538-loaded liposome-nanobody system showed good growth inhibition of head and neck cancer cells in vitro. Similar systems were developed by conjugating polymeric micelles to anti-EGFR (EGa1) nanobodies [94, 108]. The results show that anti-EGFR nanobody-conjugated polymeric micelles bind more effectively to EGFR-expressing cells compared with the unconjugated micelles. In addition, the authors stated that unspecific binding of the micelle-nanobody conjugate to EGFR-negative cells was not observed. Lastly, development of anti-EGFR nanobody-albumin nanoparticles (NANAPs) has also been reported [95]. The study by Altintas et al. showed successful internalization of the NANAPs and inhibition of 14C squamous head and neck cancer cell proliferation, while no such effect was observed for non-targeted formulations [95]. Overall, these studies present the potential that nanobodies hold for the development of targeted therapies either alone or in combination with different molecular structures. However, further studies are needed to determine the efficacy and safety of these combined therapeutic systems.

2.1 Nanobodies Against Breast Cancer

Breast cancer is a heterogeneous disease with different morphologies and molecular profiles. The human epidermal growth factor receptor (HER) consists of four receptor tyrosine kinases (HER1/2/3/4), which play important roles in extracellular transmission of signals that lead to cancer cell growth, survival and differentiation [109]. Activation of these receptors leads to signalling cascades that promote cell proliferation, angiogenesis, invasion and metastasis. HER2 is a transmembrane protein that is found overexpressed in breast cancers, non-small-cell lung cancers, gastric cancer, colon cancers and ovarian cancers [110]. Although the humanized IgG1 monoclonal antibody trastuzumab is used to treat breast cancer patients, the therapy is most effective for patients with HER2-positive malignancies. The patients with low or heterogeneous HER2 expression benefit only marginally from the therapy [110]. A nanobody directed against HER2 that can be internalized by breast carcinoma cells expressing the receptor was developed [110]. The authors observed different tumour targeting properties of

their nanobody, depending on the strategy that was used for labelling ($[^{131}I]IB$-Mal-$_D$-GEEEK vs Iodogen). A higher ratio of tumour to healthy tissue signal was obtained when labelling with $[^{131}I]IB$-Mal-$_D$-GEEEK [110]. However, the issue with kidney toxicity still remains to be solved.

Another receptor molecule that is frequently overexpressed and dysregulated in breast cancer is VEGFR2 [53]. Behdani et al. selected an anti-VEGFR2 nanobody that binds to cell surface VEGFR2, and proved that it efficiently inhibits capillary tube formation in vitro [89]. Further characterization of the nanobody and evaluation of its potential in vivo is still to be published.

2.2 Nanobodies Against Lung Diseases

For the treatment of lung diseases, drugs can be either administered by systemic injection (intravenously or subcutaneously) or by inhalation [111]. Inhaled drug delivery offers a number of advantages like a direct drug delivery at high concentrations, a fast onset of the drug action, a smaller dose needed to achieve a therapeutic effect and a lower systemic exposure that decreases the appearance of side effects. Administration by inhalation of anti-IL13 Fab fragment on murine models for asthma and a chimeric anti-EGFR monoclonal antibody (cetuximab) for lung cancer is being tested [112, 113]. If delivered through the lungs either as an aerosol or dry powder, it is important for the drugs to be able to maintain their structural integrity and bioactivity [111]. In this regard, the high stability of nanobodies, their resistance to harsh conditions, robustness and solubility are favourable properties for development of alternative administration routes like pulmonary delivery. A 42-kDa trivalent nanobody (ALX-0171) targeting the surface glycoprotein fusion (*F*) protein of human respiratory syncytial virus (RSV) is currently in clinical development for pulmonary administration in the form of an aerosol [114]. ALX-0171 drug inhalation allows for rapid drug delivery to the site of infection, which will shorten disease time. A phase I clinical trial confirmed that ALX-0171 was well received in healthy adult volunteers and in subjects with hyper-reactive airways [111]. However, Sanofi, which acquired Ablynx in mid-2018, has decided to discontinue the clinical tests of the inhaler-administered ALX-0171 for treatment of RSV-infected children.

Another example for the use of nanobodies in the development of lung-targeting drugs is the targeting of pulmonary surfactant protein A (SPA) [115]. Due to the exclusive expression of SPA on alveolar epithelial type II cells, it can serve as a molecule for specific targeting of lung diseases. In one study, the authors selected two nanobodies, Nb6 and Nb17, that showed high specificity for SPA [115]. To evaluate the lung-targeting ability of Nb17, the authors performed in vivo imaging using murine models and showed

fast accumulation of Nb17 in the lungs, and complete clearance from the blood circulation within 6 h after injection. In addition, at least in mice studies, a minimal retention of Nb17 in liver and spleen was detected.

2.3 Nanobody Applications for Central Nervous System Pathologies

2.3.1 Nanobodies Passing the Blood–Brain Barrier

The human brain is an immunologically unique and privileged organ as it is protected by the semi-permeable physical blood–brain barrier (BBB) [116]. Although the main role of the BBB is regulation of homeostasis and protection of the brain from damaging agents, for therapeutic applications it becomes an obstacle as it prevents the passage of high molecular weight drugs [117]. Different mechanisms, including physical methods (disruption with osmotic shock, magnetic gradient and ultrasound) [118], use of cell-penetrating peptides [119] as well as receptor-mediated transcytosis (e.g., transferrin receptor, insulin receptor and low-density lipoprotein receptor-related protein) [120, 121] have been explored for increasing BBB permeability. Among these strategies are the so-called 'Trojan horses' that are ligands or antibodies targeting receptors of the BBB resulting in the transfer of various molecules into the brain either by non-specific charge-mediated adsorptive endocytosis or by energy-dependant receptor-specific endocytosis/transcytosis [122]. One of the most interesting technologies is the transport pathway through the transferrin receptors on the surface of microvascular endothelial cells [123]. The transferrin receptor is highly expressed at the BBB and mediates iron delivery to the brain through binding and trafficking of transferrin protein [124]. Hence, different antibodies against the transferrin receptor have been developed [125, 126]; antibody release into the in vitro model of the brain parenchyma can be improved by binding to the transferrin receptor at acidic pH and decreasing antibody affinity to the receptor [127]. In addition to antibodies, nanobodies against the transferrin receptor have also been raised [128]. Furthermore, two nanobodies able to cross the BBB in vitro, FC5 and FC44, were described previously [129, 130]. Abulrob et al. examined the mechanism by which FC5 transmigrates through the BBB in vitro and in vivo [131]. The results of their study show that transcytosis of FC5 through the BBB is independent of charge, but undergoes actin- and PI3 kinase-dependent transcytosis through clathrin-coated endocytic vesicles and depends on the recognition of specific oligosaccharide epitopes on the surface of human brain cerebromicrovascular endothelial cells. The authors also show that FC5 migration through the BBB does not involve interactions with the transferrin receptor [131]. In a different study, Haqqani et al. compared single domain FC5, monovalent and bivalent FC5 fused to the N-terminus of human Fc (Mono-FC5Fc and Bi-FC5Fc, respectively) and demonstrated that both Mono-FC5Fc and Bi-FC5Fc exhibit better BBB transcytosis than the single domain FC5 [132]. Although FC5 shows binding potential towards brain endothelium when compared with endothelium from lung, umbilical cord and even astrocytes, its specificity is not restricted to humans, but also binds to mouse and rat cerebral endothelial cells [131]. The above findings predict a good potential for the use of FC5 as a transport mechanism through the BBB, albeit further optimization will be necessary.

As administered nanobodies distribute widely and rapidly through the human body, they give new opportunities for molecular diagnosis of pathologies of the central nervous system. However, accessing the brain for therapeutic purposes is a challenge due to the existence of the BBB [67]. Without active transport, this biological barrier is only permeable for lipophilic molecules with a size below 400 Da [91]. One of the reasons for the limited ability of nanobodies to cross the BBB is thought to be their short half-life in the bloodstream [133]. However, monomeric nanobodies have been reported to be able to cross the BBB freely, even if their molecular mass (15 kDa) is well above the natural size barrier [129, 134, 135]. Nevertheless, this presumed passive BBB passage of nanobodies failed to deliver therapeutic amounts into the brain [136]. It is well established that proteins can cross the BBB by adsorptive-mediated endocytosis (AME). The AME process is triggered by electrostatic interactions between cationic proteins with basic pI and anionic charges present on the target cell surface [134]. The authors of one study reported that nanobodies with basic pI targeted against glial fibrillary acidic protein (GFAP) will spontaneously cross the BBB, and/or will be able to penetrate cells to bind its intracellular target in vivo [134]. This suggests that nanobodies with high pI could also be used as intrabodies. A limitation of this approach was the relatively large amount of nanobody (2 mg) that was injected into the murine model to obtain the desired effect [134].

We thus conclude that nanobodies are superior to currently used antibodies for transport through the BBB because they show better penetration and more homogeneous distribution. However, in the case of systemic administration, their small size is considered a limitation due to rapid clearance from the body.

2.3.2 Nanobodies Against Brain Tumours

The application of nanobodies for targeting tumours of the central nervous system is also explored. Grade IV gliomas, glioblastomas, are brain tumours that are among the deadliest human malignancies. Amplification of the transmembrane glycoprotein EGFR is common in glioblastomas. *EGFR* gene amplifications and activating mutations

are observed in up to 70% of glioblastomas and play an important role in gliomagenesis [72]. α-EGFR nanobodies that inhibit EGF-induced signalling and cell proliferation in vitro have already been developed [137]. A combination of a nanobody targeted against EGFR and a pro-apoptotic EGFR-specific nanobody and their immune-conjugates against tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) was used as a targeted treatment for glioblastoma [72]. Nanobody conjugates were secreted from neural stem cells on site with sustained release and showed sufficient tumour uptake level in glioblastoma. The combination caused decreased glioblastoma growth and invasiveness both in vitro and in vivo.

2.4 Caplacizumab for Thrombotic Thrombocytopenic Purpura

The first EMA- and FDA-approved 28-kDa nanobody is the bivalent nanobody caplacizumab (ALX-0681) for treatment of thrombotic thrombocytopenic purpura (TTP) [98]. TTP is a blood disorder where the activity of von Willebrand factor (vWf)-cleaving protease ADAMTS-13 is inhibited (enzyme activity < 10%), which leads to platelet consumption in vWf-platelet aggregates and microvascular thrombosis [98]. In approximately 20% of the cases diagnosed with TTP, the disease has a fatal outcome. The current treatment consists of daily plasma exchange to restore ADAMTS-13 and remove vWf autoantibodies, as well as immunosuppressive therapy to supress anti-vWf antibodies [98]. Relapses are commonly encountered after treatment and are a permanent risk as they can occur within 30 days after the last plasma exchange or even 10–20 years later. Almost 15 years ago, a nanobody that recognizes the active state of vWf was developed [138]. The nanobody, named caplacizumab, allows the detection of vWf in plasma of patients with ADAMTS-13 deficiency [64]. There are two types of caplacizumab, ALX-0681 and ALX-0081, for subcutaneous and intravenous administration, respectively. The bivalent nanobody shows an inhibitory effect on platelet aggregation in blood, a good safety profile and efficiency. The phase III HERCULES double-blinded, randomized, placebo-controlled trial proved that, in combination with current therapy, caplacizumab treatment with subcutaneous administration aids in faster resolution of the disease, fewer TTP-related deaths and less disease recurrence [98]. The trial was multi-centred and took place in 92 laboratories worldwide. During the trial, patients that received caplacizumab showed faster platelet count normalization than the ones receiving placebo. In addition, lower incidence of TTP recurrence and TTP-related deaths were observed in the caplacizumab-treated patients compared with those receiving placebo [98].

3 Expert Opinion

Since the discovery of naturally occurring heavy-chain antibodies in sera of camelids and the development of technologies to clone and identify their antigen-binding fragments, known as nanobodies, the number and range of possible applications with nanobodies seem to have exploded. Supported by intrinsic beneficial biochemical and biophysical properties, nanobodies are a robust targeting entity that is easily assembled or incorporated into more complex, pluripotent constructs. Even in the absence of an intrinsic therapeutic effect, their conjugation with chemotherapeutic agents generates highly promising targeted drug delivery compounds. Although small monomeric nanobodies are often superior over classical antibodies for therapeutic applications, they still have their own drawbacks such as a fast renal clearance preventing a high load at the diseased tissue and inducing kidney toxicity. However, tools and strategies are available to engineer the nanobodies into next-generation constructs of higher efficacy and with fewer side effects.

It can be tricky to compare the performance of classical antibodies with nanobodies for therapeutic applications. Although their size, complexity, glycosylation, presence or absence of Fc, epitope-specificity and manufacturing are widely different, they both have an IgG origin and share a high specificity for their cognate target, which they bind with high affinity as well. Consequently, the bivalent antibodies might perform better due to longer blood circulation time, avidity effects, the capacity to cross-link or dimerize their antigens and in triggering an Fc-mediated immune response. In contrast, monomeric nanobodies have a much faster extravasation and an improved tumour penetration and better targeting of cryptic epitopes but result in lower loads at lesions due to their very fast blood clearance via the kidneys. Although nanobodies are easily dimerized or multimerized and could be equipped with a serum albumin binding nanobody to obtain avidity effects and to improve their blood retention, such engineering will inevitably reduce their diffusion advantages. Finally, if wanted, nanobodies can also easily be equipped with the Fc regions (hinge, C_H2 and C_H3 domain, most likely of human IgG1) to reconstitute a humanized heavy-chain-only antibody format to include the Fc-mediated effector functions, and increase the blood retention time as well.

Compliance with Ethical Standards

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