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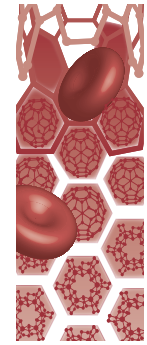
Nanomedicine



Nanobody-based cancer therapy of solid tumors

The development of tumor-targeted therapies using monoclonal antibodies has been successful during the last 30 years. Nevertheless, the efficacy of antibody-based therapy is still limited and further improvements are eagerly awaited. One of the promising novel developments that may overcome the drawbacks of monoclonal antibody-based therapies is the employment of nanobodies. Current nanobody-based therapeutics can be divided into three different platforms with nanobodies functioning as: receptor antagonists; targeting moieties of effector domains; or targeting molecules on the surface of nanoparticles. In this article, we describe factors that affect their performance at three different stages: their systemic circulation upon intravenous injection; their extravasation and tumor penetration; and, finally, their interaction with target molecules.

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Cancer therapy using monoclonal antibodies (mAbs) is a rapidly developing field. It has been more than 30 years since the first patient was subjected to a mAb therapy [1]. The introduction of mAbs for cancer treatment has been without doubt a remarkable success, bringing us closer towards personalized medicine. Until now, approximately 25 mAbs have been approved by the US FDA and are available on the market [2]. Most of them act by binding to transmembrane receptors or soluble ligands, thereby interfering with their signal transduction pathways, resulting in inhibition of tumor cell proliferation or angiogenesis. Due to the presence of an intact Fc domain, mAbs can evoke antibody-dependent cell-mediated cytotoxicity (ADCC) by attracting complement or effector cells of the human immune system to the cancer site. mAbs have also been used as targeting moieties for the delivery of nanomedicines or nanoparticles containing a cytotoxic payload. In another approach, mAbs have been directly conjugated to cytotoxic drugs (e.g., auristatin, maytansine, calicheamicin or doxorubicin). Several of these mAb–drug conjugates (ADCs) have already reached the clinical trial phase [3]. Nevertheless, the large size of mAbs alone (150 kDa; dimensions: 14.2 × 8.5 × 3.8 nm [4]), which is further increased by conjugation to a nanoparticle, is a considerable drawback, as it leads to limited tumor penetration and slow distribution [5–7]. To overcome the limitations of full-length mAbs, smaller formats have been generated, such as the naturally derived or synthetic antigen-binding fragment (Fab; ~50 kDa), variable fragment (Fv; ~15 kDa) or single-chain variable fragment (scFv; ~30 kDa). The advantage of smaller size is, in most cases, counterbalanced by decreased stability, resulting in aggregation (especially in the case of scFv), lower affinity and/or difficulties in large-scale production [8].

In the early 1990s, a new type of antibodies, the heavy-chain antibodies (HcAbs; ~95 kDa), were discovered serendipitously by Hamers-Casterman and coworkers [9]. In contrast to the well-conserved structure of IgG in mammals, which consists of two identical heavy chains and two identical light chains [10], members of the Camelidae family have additional IgG isotypes composed of a homodimer

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of heavy chains only [9]. These fully functional antibody structures retain high binding capacities, similar to those obtained with conventional mAbs, even though they lack the light chain. Due to the ease of immunization, HcAbs are mostly obtained from Camelidae (bactrian camels, dromedaries, alpacas and llamas), even though HcAbs have also been found in cartilaginous fish (e.g., sharks, rays and skates). Interestingly, the variable domain of the heavy chain from HcAbs (i.e., VHH, also referred to as nanobodies or single_domain antibodies), which is the domain that is responsible for antigen binding, is fully functional and currently the smallest naturally derived antigen-binding fragment (Figure 1).

In this article, we will describe the unique features of nanobodies that are particularly relevant in the context of cancer therapy, which have attracted considerable interest and opened up a wide range of applications. After specifying the different therapeutic approaches in which nanobodies have been involved, the subsequent sections will describe factors that affect the performance of nanobody-based therapeutics at the three major phases that are essential for cancer therapy: during their systemic circulation upon intravenous injection and renal, hepatic or splenic filtration; their extravasation and tumor penetration; and, finally, their interaction with cancer targets.

Nanobodies: characteristics & therapeutic strategies

The molecular biology of nanobodies has recently been explained in detail in an excellent review by Muyldermans [11]. Clear advantages of nanobodies over conventional antibodies include their size [12], stability [13,14] and solubility [15]. Because of these characteristics, nanobodies can be formulated as a long shelf-life, ready-to-use solution [16]. Furthermore, nanobodies are relatively easy to produce in bacteria, yeast or mammalian cells, enabling large-scale production at reasonable costs. The VHH is very similar to the human VH framework of family III and data from Phase I trials performed by Ablynx NV (Belgium) support the notion that nanobodies are associated with very low immunogenic potential [17,18]. In addition, when it becomes necessary, additional procedures can be taken to humanize nanobodies [17]. On the other hand, the small interaction surface of the paratope may result in lower affinities. Therefore, careful selection is required to obtain nanobodies with sufficient affinity. Moreover, the effect of conjugation to random lysine residues in the nanobody may affect the binding properties of the nanobody, a problem that has been solved by the site-directed conjugation to a C-terminal cysteine [18].

The single-domain property of the nanobody allows for selections based on phage display, which, in principle, enables selection of nanobodies that specifically recognize any protein of interest. This aspect makes the nanobody technology very versatile. In the context of cancer therapy, nanobodies can be used to target cancer cells and/or tumor vasculature by binding to specific targets. As nanobodies do not freely cross membranes, possible cancer targets are either extracellular targets, such as receptor ligands or transmembrane proteins that are solely expressed or overexpressed as compared with normal cells. In order to develop nanobodies that specifically bind, three essential tools are needed: first, the phage display library, obtained from immunized animals. The immunization of animals from the Camelidae family can be carried out with the protein of interest (e.g., [19]), with cells (e.g., [20]) or even purified cell compartments (e.g., [20]) expressing this protein. From the peripheral blood lymphocytes of these animals, a library can be constructed following standard protocols [20,21]. The phage display technology allows for different selection protocols aimed at obtaining nanobodies with high affinity or for targets that are differentially expressed, such as subtractive panning. Second, the purified proteins of interest, which are in many cases available through purchase and/or cell lines expressing these proteins, while having a nonexpressing cell line as a negative control, are essential for panning. Third, tools to detect the selected nanobodies during the screening procedures are required.

Nanobodies binding to transmembrane proteins

Nanobodies have been selected to bind to transmembrane proteins that can be used as cancer targets. These include growth factor receptors such as the EGFR1 or EGFR2 (or HER1 and HER2, respectively), VEGFR2, c-Met and CXCR7. EGFR, HER2 and c-Met belong to the family of receptor tyrosine kinases. Nanobodies targeted to EGFR have been developed by Roovers *et al.* through phage display selection, combined with competitive elution with EGF or cetuximab, to select antagonistic anti-EGFR nanobodies (among which are the Ia1 or EGa1 nanobodies) [20]. Several nanobodies targeting HER2 were selected through phage display on immobilized HER2 [18,22] or on cells possessing high HER2 expression [18], and one HER2-targeted nanobody has been investigated as a targeting moiety for therapeutic application *in vitro* [23]. VEGFR2 belongs to the family of human VEGFR receptors 1–3 [24] and has an important role in embryogenesis and angiogenesis. This receptor has been described to be overexpressed in many types of cancers, among which include lung and colon cancers [25]. The nanobody targeting VEGFR2, which inhibits capillary tube formation *in vitro,* has been obtained through phage display selection on immobilized recombinant extracellular domains of this receptor [26].

The c-Met receptor is activated upon HGF binding and is involved in the regulation of cell proliferation, motility and morphogenesis [27]. This receptor has been implicated in a variety of human malignancies, such as colon, breast, ovarian and hematological malignancies [28]. The anti-c-Met nanobody was shown to compete with HGF, thereby inhibiting c-Met activation, cell proliferation and migration *in vitro* [29,30]. Different c-Met-targeted nanobodies, as well as HGF competitors, were obtained through selections on immobilized c-Met, and the final selection was based on the maintenance of the binding affinity upon modification for conjugation to nanoparticles [30]. The chemokine receptor CXCR7 has recently been described as the receptor for chemokine CXCL11 and CXCL12, which, upon activation, stimulates other downstream signaling pathways [31]. Several tumors have been associated with CXCR7 overexpression, among which are breast and lung tumors [32]. The nanobodies targeting CXCR7 were selected on CXCR7 virus-like lipoparticles, followed by confirmation of their binding specificity on CXCR7-expressing cells. Further refinement was obtained by a selection based on competition with CXCL12.

In addition to growth factor receptors, other transmembrane proteins can also be targeted with nanobodies, such as extracellular cancer-specific glycoproteins. Overexpression of MUC-1 (a high-molecular-weight glycoprotein) at the transcriptional level is found in epithelial tumors such as breast and colon. An anti-MUC-1 nanobody was selected by Sadeqzadeh *et al.* by phage display panning against whole protein purified from the ascetic fluid of a patient with lung carcinoma [33]. Carcinoembryonic antigen (CEA) is a glycoprotein that is highly overexpressed in epithelial tumors, while its expression in normal tissue is restricted to low-level gastrointestinal expression. Several nanobodies were selected by Cortez-Retamozo and coworkers by phage display panning against complete CEA protein [34].

Nanobodies binding to extracellular targets

An alternative approach includes nanobodies that bind directly to extracellular targets that are functional within the tumor. In this context, nanobodies have been developed for HGF and diverse chemokines. Anti-HGF nanobodies were obtained after selection of binders to immobilized HGF and the assessment of HGF/c-Met interaction inhibition. Chemokines generally play a role in immune responses and inflammatory processes, which may also be involved in cancer development [32]. Neutralizing nanobodies targeting diverse chemokines were obtained from a library constructed after llamas had been immunized with a mixture of recombinant chemokines, followed by phage display selections on immobilized chemokines and further selection for receptor antagonists and inhibition of cellular migration [35]. These neutralizing antichemokine nanobodies have not yet been evaluated *in vivo*.

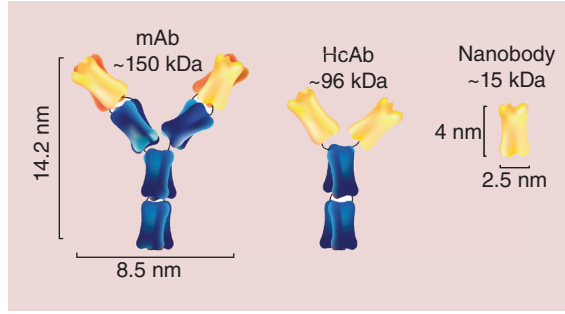

Figure 1. Schematic overview of different antibody formats. Conventional mAb, HcAb and its derivative (i.e., nanobody, also referred to as the variable domain of the heavy chain of a HcAb or VHH). The molecular weights and sizes of these antibodies are also depicted.
HcAb: Heavy-chain antibody; mAb: Monoclonal antibody.

Nanobodies & cancer therapy

In the context of cancer therapy, nanobodies have first been tested as antagonists (described below), which prevent ligand binding and thereby conformational changes that lead to the activation of signaling cascades (Table 1). Examples are nanobodies against EGFR and c-Met [19,20,29,36,37]. Both types of nanobodies were acting as effective antagonists for either EGF or HGF signaling *in vitro*. Inhibition of tumor growth *in vivo* was obtained with the trivalent biparatopic anti-EGFR nanobody 7D12-9G8-Alb [36]. In this latter study, the ultrafast clearance of nanobodies was circumvented by fusion of the anti-EGFR nanobodies with a nanobody targeting albumin that could prolong the half-life from 1–2 h to 2–3 days [36]. Eradication of the tumors was not observed, despite the fact that different combinations of anti-EGFR nanobodies were used (either bivalent or biparatopic). In this case, the absence of the Fc domain, which is necessary to trigger ADCC and complement-dependent cytotoxicity upon antigen binding, was certainly one of the reasons for the limited antitumor effect [8,36].

Besides antagonistic nanobodies, nanobodies can also be developed as allosteric inhibitors that are able to modulate the enzymatic activity of their target protein (CAIX) [48]. Alternatively, nanobodies can be used to directly bind to ligands, such as HGF, thereby preventing its binding to the c-Met receptor. This approach can only be successful when just one ligand is responsible for the activation of the corresponding receptor. In a study by Vosjan *et al.*, nanobodies targeting HGF and fused to an albumin binding domain were indeed capable of inhibiting U87 MG tumor outgrowth, and curative

Table 1. Nanobodies in cancer therapy: the targets nanobodies bind to and the therapeutic platforms they have been used in, as evaluated in preclinical *in vivo* studies.

Target	Platform(s)†	*In vivo*	Ref.
Transmembrane proteins			
– EGFR	A, B, C	Yes	[20,36–44]
– HER2	B	Yes	[23,45]
– VEGFR2	A, B	No	[26,46]
– HGFR or c-Met	A, C	No	[29,30]
– CXCR7	A	Yes	[47]
– Carcinoembryonic antigen	B	Yes	[34]
– MUC-1	C	No	[33]
Extracellular proteins			
– HGF	A	Yes	[19]
– Diverse chemokines	A	No	[35]

A: naked nanobodies acting as antagonists; B: targeting moieties of effector domains; C: targeting moieties of drug delivery systems.

responses were even observed [19]. These studies show that the therapeutic effect of nanobodies is certainly dependent on the target and tumor model employed.

These results stimulated further investigations into different applications of nanobodies in cancer therapy. On the basis of these developments, we have categorized the anticancer nanobodies into three platforms: the naked nanobodies (platform A); nanobodies fused to effector domains (platform B); and nanobodies decorating the surface of nanoparticles, such as liposomes, micelles, polyplexes or albumin nanoparticles, which, in turn, encapsulate drugs (platform C; Figure 2). The conjugation of the nanobody to the nanoparticles is obtained via N-succinimidyl-S-acetyl-thioacetate (referred to as 'SATA') modification of the nanobodies that, after de-protection, react with the maleimide or sulfhydryl groups available on the nanoparticles [38,38,49]. *In vitro* experiments employing nanobodies as targeting moieties of nanoparticles have shown improved binding to the target cells [34,38–40].

An important prerequisite for these latter two platforms is the restriction of drug toxicity to the tumor. For platform B, effector domains include such as the Fc domain, soluble TRAIL, the *Pseudomonas* exotoxin A (variant PE38), therapeutic radionuclides, enzymes for prodrug activation or domains conjugated to photosensitizers (PSs). The nanobody fused with an effector domain such as an Fc fragment provides the nanobody with the typical ADCC activity restricted to the target cell [50]. The nanobody–drug conjugates can be designed with cleavable linkers to be cut by proteases (Table 1) within the tumor stroma or inside the tumor cell. Of interest in this respect is the recent development of biparatopic nanobodies that stimulate internalization via the clathrin-dependent pathway [51]. Alternatively, nanobody–enzyme conjugates can be employed to render a drug solely active on the targeted site [34]. In the constructs of platform C, the protection of drugs is carried out by the attachment or encapsulation of the drugs onto or inside the nanoparticles, or through charge interaction in case of polyplexes [33]. Release of these drugs from the particles can be achieved by leakage or by mechanical destruction by ultrasound or intracellular degradation. The first liposomes that were decorated with EGa1 anti-EGFR nanobodies were indeed shown to become internalized into the target cell [39]. Moreover, nanobody-targeted polymeric micelles (also EGa1 nanobodies) containing doxorubicin were significantly more effective at inhibiting tumor growth and prolonging the survival of animals compared with the untargeted formulation [43]. In this context of nanobody-targeted nanoparticles, the absence of the Fc domain may be preferential, as it could decrease the chance of immunogenic responses and delay the clearance of these nanoparticles [52].

Three steps leading to therapy

For each of these platforms, the distribution through the body and the delivery into the tumor tissue is different, and the efficiency of this process contributes strongly to the efficacy of the treatment. A therapeutic formulation that is very effective in a 2D cell culture set-up is not necessarily effective in an *in vivo* preclinical model. In this section of the article, we will discuss the steps that the nanobody-based therapeutic molecules go through before reaching their therapeutic target *in vivo*.

Delivery of therapeutic agents can be conducted in different ways: oral, intravenous, intraperitoneal or intratumoral. Nanobody technology is applicable to all of the aforementioned administration routes, although the first three have been used previously (e.g., [46,48,53]). Each

pathway has different demands for the nanobody-based formulation. For instance, with the oral application or the intraperitoneal injection, the nanobody requires resistance to extreme conditions (i.e., proteases and/or acidic pH). Nanobodies can be made resistant to proteases by adaptation of the sequence or by the introduction of an additional disulfide bond in order to improve resistance to pepsin and chymotrypsin [54]. For intravenous injection, stability in serum is essential. Although most nanobodies have been described as very stable, when combined with effector domains or nanoparticles, the stability of these systems might be different. Instability of nanobody-based formulations may give rise to an early release of the drug before reaching the cancerous mass, which could result in severe side effects and decreased therapeutic benefits. As nanobodies are usually sufficiently stable for intravenous injection, this method of administration is currently the most frequently used method for *in vivo* nanobody-based cancer therapy studies.

Systemic circulation upon intravenous injection

Intravenous administration of therapeutics is not always performed in close proximity to the tumor mass [53]. Consequently, the injected material needs to travel for a sufficient period of time along the circulatory system in order to reach the tumor. Sufficient tumor accumulation therefore requires a sufficient residence time of the nanobody in the blood stream, which differs for the type of nanobody platform. Naked nanobodies are rapidly cleared from the bloodstream, which reduces the time interval to bind to their target molecule. On the other hand, efficient clearance also decreases the risk of unwanted toxic side effects. Therefore, an appropriate balance between these factors might be essential for successful therapy. In case of larger nanobody drug formats, opsonization and subsequent recognition and uptake by the reticuloendothelial system (RES) may occur, leading to hepatic clearance of these therapeutic compounds.

Renal clearance

Renal clearance is a multifaceted process involving glomerular filtration, which depends on the size of the molecule [55]. Molecules with an *in vivo* hydrodynamic diameter (HD) <6 nm in size are filtered by fenestrations in the endothelial cell layer, in contrast to molecules with a HD >8 nm. In general, the average weight cutoff for renal clearance is approximately 60 kDa [56,57]. For intermediate-sized molecules, the filtration is further dependent on their charge. Positively charged molecules are more likely to be filtered due to the negative charge of the globular membrane [55,58]. Besides this, the charge of a molecule may provide interactions with plasma proteins, increasing the HD and preventing renal clearance [59]. However, in the case of nanobodies, there is no general rule for the net charge *in vivo*. Due to the small size of these molecules, their isoelectric point is mainly determined by the different amino acid composition of the complementary determining region (CDR) regions. It is important to note that, in general, an extremely high or low isoelectric point will render some of the nanobodies unsuitable for *in vivo* use [60]. The size (2.5 nm diameter and 4 nm height) and prolate shape of nanobodies predict rapid renal clearance [61]. This prediction has already been confirmed by several *in vivo* studies [18,62]. Importantly, the renal clearance and/or retention of the nanobody–toxin conjugates in the kidney may lead to renal toxicity. Whether these compounds are retained in the kidneys depends on the added size, change in charge and/or overall HD due to the coupled effector domain. For instance, a PS such as IRDye700DX results in the addition of only 2 kDa [41], which, in this case, will result in clearance of the nanobody–PS construct through the kidneys. Nevertheless, in this particular context, nephrotoxicity is minimized due to the fact that the PS only leads to toxicity when it is activated through specific illumination.

Although no nanobody–effector domain platform for cancer therapy has been characterized *in vivo* so far, pharmacokinetic toxicology studies of other immunotoxins, such as B43-pokeweed antiviral immunotoxin, have already showed dose-dependent kidney toxicity due to renal retention [63,64]. To avoid toxicity, renal retention of nanobody–drug conjugates should be minimized. As the renal retention relies on the endocytic pathway, the coinfusion of gelofusine and/or lysine in order to compete with megalin may lower the retention [65]. In addition to this, substitution of negative or positive residues of the nanobodies could affect renal retention [17,66–67]. Since the nanobody scaffold can be engineered to a certain extent, it can be designed to ensure lower renal retention [17,56]. A different method for reducing renal accumulation and retention is to lower the renal filtration rate [57], resulting

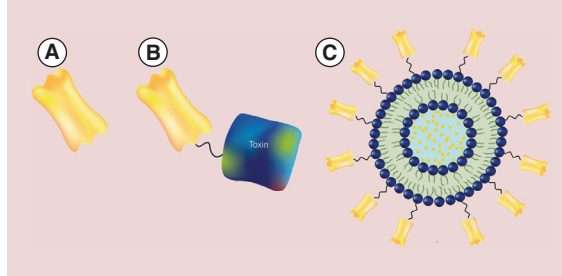

Figure 2. Schematic representation of nanobody-based therapeutic platforms. (A) Platform A: receptor antagonists that interfere with receptor activation and signaling; **(B)** Platform B: targeting moieties of effector domains, such as toxic peptides or drugs; and **(C)** Platform C: targeting molecules on the surface of nanoparticles, such as liposomes.

in an increase in half-life and the chance of improved tumor uptake [68]. Increasing the size by, for instance, glycosylation [69], PEGylation [70] or noncovalent binding to circulating serum proteins (albumin), such as the fusion with an albumin-binding nanobody [19], can prolong the half-life and thus lower renal retention [68].

In contrast to the described kidney clearance for antagonistic nanobodies and targeting nanobodies with effector domains, nanoparticles decorated with nanobodies are, due to their size, not eliminated through the kidneys. These larger types of nanobody platforms (nanoparticles) are cleared by the liver.

Hepatic clearance

The hepatobiliary system is the primary route of excretion for drugs that are too large for renal filtration [55]. Compounds and particles that undergo hepatic clearance are catabolized by hepatocytes [71]. Kupffer cells and hepatocytes are parts of the biliary system and particles endocytosed by these cells are excreted into the bile. Kupffer cells have a much higher phagocytotic capacity than hepatocytes and form the RES or mononuclear phagocyte system. Particles taken up by Kupffer cells rely exclusively on intracellular degradation; however, particles that are not broken down will be retained inside the cells. Hepatic clearance has a preference for the removal of particles with a HD of 10–20 nm, as their primary task is filtration of, for instance, viruses [59]. In addition to the liver, phagocytic cells of the RES also reside in the spleen, making this organ another target of the clearance of nonglomerular-cleared compounds.

Nanobody-based drug delivery systems, such as albumin nanoparticles, liposomes or micelles, are spherical with a large HD [55]. A biodistribution study of untargeted liposomes indeed showed an accumulation in liver and spleen [72]. Of interest is that saturation of the liver accumulation results in a shift of the liposome distribution to the spleen [73], appointing the liver as the main clearance organ of liposomes. Moreover, accumulation of liposomes into the tumor might also occur after saturation of the liver. Modifications of liposomes can be carried out in order to avoid 'first-pass' hepatic clearance, at least to a certain extent. For instance, PEGylation of liposomes lowers opsonization by plasma proteins and increases the circulation time by avoiding phagocytosis by the RES components [74]. Although accumulation at the tumor depends on the circulation time of liposomes, an increase in circulation time does not directly translate into increased tumor uptake [75].

Nanobody extravasation & tumor penetration

The second phase of drug delivery into a solid tumor, once the drug has reached the tumor blood circulation or the nearby blood supply, is the extravasation from the bloodstream and retention at the tumor site in order to allow interaction with cancer cells, resulting in accumulation in the tumor. Transport of nanobody-based therapeutics across the vessel wall is mediated by diffusion and fluid transport. In normal tissue, a net negative pressure between blood vessels and the interstitial space exists, resulting in fluid movement towards the interstitial space and further to lymphatic ducts. In tumors, the interstitial fluid pressure (IFP) is higher than that of surrounding tissue. This elevated IFP limits the transport of large molecules (e.g., mAbs) and particles into the interstitial matrix, and the tumor penetration becomes more dependent upon diffusion [76]. Targeted therapeutics are aimed at binding to receptors present on tumor cells. However, the binding site barrier effect, first suggested by Fujimori *et al.* [77], was described as a limiting factor of high-affinity binding mAbs that, due to their large size, hampers the diffusion of other mAb molecules into the tumor tissue [76]. Importantly, this effect was not observed with small molecules such as affibodies and nanobodies, which are able to distribute throughout tumors in a more homogenous manner [12,68]. In a study by Oliveira and coworkers, the tumor distribution of 15-kDa nanobodies was compared with a 150-kDa mAb after conjugation to the fluorophore IRDye800CW (IR) [12]. The EGFR-specific 7D12-IR nanobody showed a homogeneous distribution of the probe in A431 human tumor xenografts at 30 min to 2 h postinjection, which led to a relatively high tumor uptake, whereas the negative control R2-IR did not accumulate in the tumors. An irregular distribution of cetuximab-IR in the tumor stroma was observed, possibly due to the binding site barrier effect [78]. Similar results were obtained for the anti-EGFR affibody, a small binding scaffold based on protein A, conjugated to IR, in contrast to cetuximab-IR, which was confined to the center of the tumor [79]. Homogenous distribution of the drug throughout the tumor mass is essential for successful treatment. If only part of the tumor mass os exposed to the drug, complete tumor eradication will not be achieved, leading to eventual tumor regrowth [69]. In this respect, nanobodies are expected to outperform mAbs.

As molecular size is an important factor for diffusion, the diffusion capacity of nanobody-targeted nanoparticles (platform C) is dependent on their size, in that the smallest will have a better chance of diffusing into the tumor (Figure 3). Importantly, the blood and lymphatic vasculature differ substantially in tumors and normal tissues. Blood vessels of healthy tissues are normally well sealed and continuous, which prevents extravasation of therapeutic compounds. By contrast, immature, dilated tumor vessels are leaky due to the presence of much larger pores in postcapillary venules, often exceeding 100 nm in size [71]. This hyperpermeability of the

tumor vasculature allows leakage of macromolecules and nanoparticles into the tumor. This phenomenon is referred to as the enhanced permeability and retention effect, and was first described by Matsumura and Maeda in 1986 [80]. High levels of doxorubicin were delivered to the tumor site through cross-linked polymeric micelles (with a diameter of ~70 nm) decorated with EGFR-targeting nanobodies (EGa1), due to the enhanced permeability and retention effect [39]. On the other hand, no significant effect on tumor growth inhibition of a 14C tumor xenograft model was observed with liposomes encapsulating the small tyrosine kinase inhibitor AG538 and decorated with anti-EGFR nanobody (EGa1) [42], despite the fact that a clear inhibitory effect on cell proliferation was observed *in vitro*. This lack of toxic effect can be attributed to the electrostatic interactions between this cationic liposome formulation with serum proteins, thereby affecting the circulation time and subsequent accumulation at the tumor site. On the other hand, active targeting by the surface-bound nanobodies does not contribute to significant accumulation of nanoparticles in solid tumors, but has a vital contribution to the subsequent step.

Nanobody interaction with targets

For all of the mentioned nanobody platforms, the final step before the therapeutic mechanism of action, is the actual binding to the target molecule, which is mediated by the nanobody. The specificity of this last stage is essential for the therapy to occur with minimal side effects. For antagonistic nanobodies, a high binding affinity is essential, as these nanobodies are expected to compete with the natural ligands, which normally bind with high affinity themselves to their receptor. Phage display selections can specifically be aimed at the retrieval of high-affinity binders. In addition, the improvement of binding affinity can be obtained by preparing a family library based upon the CDR3 sequence of an already-selected nanobody [81]. A disadvantage of the nanobody technology is that the conjugation to an effector domain might have a severe effect on the binding properties of the nanobody. Crystal structures of nanobodies have shown that the N-terminus is positioned close to the site of the CDR sequences and conjugation to this site of the protein might affect antigen binding.

Although not in all cases, random conjugation to the primary amines (lysines and the N-terminus of

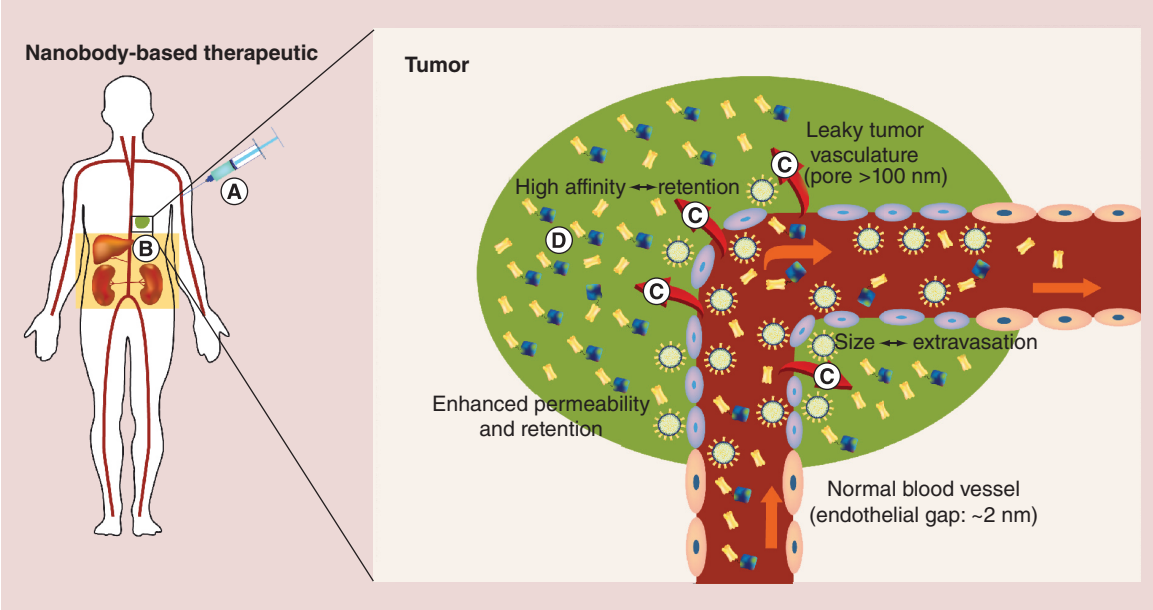

Figure 3. Schematic representation depicting the journey of nanobody-based therapeutics upon administration. Upon intravenous injection **(A)**, the nanobody-based therapeutic circulates in the bloodstream for different times, which strongly depends on the size of the employed platform. **(B)** With the blood flow, it reaches the main clearance organs (i.e., kidney and liver, but also the tumor site). **(C)** To exert its cytotoxic activity, nanobody-based therapeutics need to extravasate from the circulation into the tumor mass. This extravasation is enabled due to leaky vasculature of the tumor. Nanobodies belonging to platform C (targeting moieties of drug delivery systems) accumulate at the tumor due to the enhanced permeability and retention effect and are not capable of homogenous tumor penetration; therefore, they localize in close proximity to the blood vessels. On the other hand, nanobodies of platform A (naked nanobodies acting as antagonists) and platform B (targeting moieties of effector domains) may homogenously diffuse throughout the tumor mass due to their small size, and their high binding affinity is essential for associating with their targets. **(D)** The specificity of the interaction of each of the platforms with target cells depends strongly on the nanobody employed.

the protein) was found to affect the binding properties of the nanobody [18]. In this study, the conjugation of the fluorophore IR was shown to completely prevent the binding of a HER2-directed nanobody to its target *in vivo*. Importantly, affinity was retained after conjugation of this fluorophore to a C-terminal cysteine [18]. Thus, the best solution for the conjugation of effector domains to the nanobody appears to be via the C-terminus. Two nanobodies fused to effector domains have been described and both have been fused to the C-terminus: the anti-VEGFR2 has been fused to PE38 [46] and an anti-EGFR nanobody to soluble TRAIL [26]. In the latter case, a drop in affinity was observed, but in this particular set-up, it did not hamper the efficacy in killing cancer cells. In addition, other examples have documented site-directed conjugations using a C-terminal cysteine [33,82–83]. Alternatively, click chemistry and intein- and sortase-based conjugation systems are in development and may contribute to further functionalization of the nanobodies [84–88].

Recently, we have randomly conjugated EGFR-targeted nanobodies (named 7D12 and 7D12-9G8) to a traceable PS for photodynamic therapy (PDT) [41]. The binding affinities of these EGFR-targeted nanobody–PS conjugates remained in the low-nanomolar range and these conjugates are expected to behave *in vivo* very similarly to what has been observed in molecular imaging studies [12]. After the preclinical testing, more will be known of the feasibility of the approach in which the fluorescent nanobody–PS conjugate can be detected through optical imaging, enabling guidance of the actual treatment (i.e., PDT). After binding of the nanobodies to their target receptor (e.g., EGFR), the nanobodies undergo a very slow internalization (one round of internalization is completed after 24 h). For a more rapid internalization of the cargo, the use of biparatopic nanobodies (e.g., 7D12–9G8) was recently introduced [51]. These biparatopic nanobodies consist of two different nanobodies binding to the same target protein (EGFR), but on different, nonoverlapping sites. As a result, these nanobodies stimulate receptor clustering, which induces receptor internalization and subsequent degradation in the lysosomes. Similar results were shown for antibody constructs [89]. This method allows specific binding to the target cells, followed by internalization, enabling the reversible conjugation of drugs that are sensitive to intracellular proteases, such as cathepsin B, to then be released for their action.

When nanobodies are employed as targeting moieties of long-circulating nanoparticles, such as PEGylated liposomes or branched gold nanoparticles, affinity becomes less critical. This is mainly because the affinity will be sufficient as a result of avidity, as several nanobodies are present on the same particle. Mamot *et al.* have shown that the targeting moiety has a function in the cellular uptake of the particles [75,90–91]. This has been demonstrated with nanobodies binding to cell membrane proteins, such as anti-EGFR nanobodies (EGa1) conjugated to liposomes [39,39,42], polymeric micelles [38,43] or to albumin nanoparticles [44]. Another example is the binding of the anti-c-Met nanobodies conjugated to albumin nanoparticles to the human ovarian carcinoma cell line TOV, stably expressing c-Met [30]. In this case, the presence multiple nanobodies on the surface of those particles also results in clustering of their target receptor at the membrane, causing their internalization. The c-Met-targeted albumin nanoparticles clearly entered by the route of early endosomes, late endosomes and lysosomes, where degradation of both nanoparticle and c-Met took place [30]. These nanoparticles were also able to induce phosphorylation of c-Met. However, this activation of c-Met was insufficient to induce complete c-Met signaling, as cell migration was not induced. Similar observations were previously reported for agonistic antibodies against c-Met as well as EGFR [92,93]. The most prominent effect of the anti-c-Met nanoby-albumin nanoparticles (NANAPs) seems to be the degradation (i.e., downregulation) of the c-Met receptor. By contrast, EGFR-targeted liposomes were able to inhibit EGF-induced activation and also induced EGFR receptor downregulation [39]. Interestingly, scFv liposomes also targeting EGFR were unable to induce the same EGFR downregulation effect, which is believed to be related to the fact that nanobodies dissociate from their targets only at very acidic pH levels (below the pH of late endosomes) [39]. Thus, by remaining attached to the nanobody, EGFR is unable to recycle to the cell membrane, and therefore the EGFR–nanobody–liposome is directed to lysosomes for degradation.

Lysosomal routing and subsequent degradation is very valuable as it leads to the downregulation of receptors that play an active role in tumor proliferation. This routing also opens up the possibility of incorporating sensitive linkers (pH and protease), enabling the release of cargos from the nanoparticle in the endolysosomal system. For instance, the EGFR-targeted nanobody liposomes containing IGF-1R-targeted kinase inhibitors were clearly able to release the kinase inhibitor, which then reached the target site of action (i.e., cytoplasm) [40,42]. Similarly, the EGFR-targeted nanobody albumin nanoparticles that trafficked to the lysosomes were also able to release the multikinase inhibitor from its linker, as this could carry out its mechanism of action (in an *in vitro* study) [44]. In case of the EGFR-targeted nanobody–micelles containing doxorubicin as the payload [38,43], doxorubicin was coupled with the polymer via a hydrolytically degradable linker (at

pH 5), enabling the release of doxorubicin from the polymer upon trafficking of the nanoparticles to the late endosomes/lysosomes [94]. In these examples, cargos were very-low-molecular-weight drugs that could diffuse out of the late endosome/lysosome compartments. More complex will be the situation in which the cargo cannot cross the cell membrane, unless binding of the nanobody to the target protein is sufficient for the mechanism of action. This seems to be the case for the HER2-targeted branched gold nanoparticles for photothermal therapy [23] and the HER2-targeted nanobodies for radionuclide therapy [45], as well as for EGFR-targeted nanobodies (7D12 and 7D12-9G8) conjugated to PSs for PDT [41]. Nevertheless, in the latter case, enhanced toxicities were documented upon increased internalization of the conjugates [41].

Conclusion & future perspective

Research into the application of nanobodies in cancer therapy has resulted in the development of three distinct nanobody-based therapeutic platforms: platform A, naked nanobodies; platform B, nanobodies as targeting moieties transporting effector domains; and platform C, nanobodies as targeting molecules on the surface of nanoparticles encapsulating a drug. These platforms increase in size from platforms A to C and as a result, their tumor penetration decreases. On the other hand, the toxic payload increases from platforms A to C, which could make the latter platform more effective. Furthermore, the residence time of the different platforms in the bloodstream differs: while platforms A and B are generally more rapidly cleared, platform C can remain for longer in the bloodstream, but generally accumulates in the liver and spleen and not sufficiently in the tumor. The current question related to which improvements are needed and which will be sufficiently effective to make the respective platform optimal for the application of choice.

In general, the reviewed studies demonstrate that nanobody-based platforms may overcome some of the obstacles that hamper successful therapy at different stages upon intravenous injection of mAbs. The main advantage of nanobodies over mAbs is the simplicity in functionality: their function is binding. Their single-chain property facilitates their cloning and production. The nanobodies are an excellent system for making multivalent constructs. Bivalent nanobodies can be designed to improve affinity, which could lead to a reduced off rate and, consequently, a reduced release of the nanobodies from their target. To increase the *in vivo* half-life, different solutions exist, such as the introduction of a nanobody binding to human serum albumin [68]. Biparatopic nanobodies can be used for the induction of internalization [51], and bi- or tri-specific nanobodies for the targeting of different markers by one single molecule. In this respect, nanobodies offer a great advantage over mAbs, for which the production of bispecific antibodies is not easy, as this involves the specific combination of four different proteins. However, such novel nanobody constructs need to be carefully evaluated in *in vivo* studies concentrating on the immunogenicity of nanobody-based formulations, as most cancer therapies require repeated drug administrations. Once these issues are clarified, clinical trials may be initiated in order to further establish the most effective treatment platform.

With respect to platform A, nanobodies as receptor antagonists do not seem to be sufficiently effective, as they lack an effector function (i.e., a Fc tail). On the other hand, nanobodies targeting extracellular proteins such as HGF are very successful [19]. With respect to platform B, currently introduced nanobody effector domains are promising, and further developments may contribute to a higher efficacy with respect to full tumor eradication. Of interest are activatable systems, in which drugs only become toxic upon light activation (e.g., PDT [41]), changes in pH or after enzymatic cleavage, specifically within tumor cells [95]. In this context, nanobody–drug conjugates may benefit from internalization mediated by biparatopic nanobodies. In platform B, similarly to the other platforms, specificity is governed by the nanobody targeting to the tumor marker. Although a few tumor markers have been described, the future challenge will be the further development of tumor-specific markers. In fact, a small number of studies have thus far involved nanobodies targeting other cancer cell markers besides transmembrane receptors, such as CEA [34] and MUC-1 [33]. More available nanobodies against different tumor targets will also stimulate the production of bispecific constructs, and it will be interesting to see how effective these constructs can be.

The tumor targeting of nanobodies (platform A) and nanobody conjugates (platform B) are very promising, as their relatively small sizes allow for better distributions through solid tumors. Nevertheless, future research should be aimed at preventing the rapid clearance of these small therapeutic molecules through increases of their half-lifes in patients, although while maintaining their minimal side effects. Most of the reviewed literature focuses on the intravenous delivery of nanobody-based platforms; however, it is known that heterogeneous vascularization and limited diffusion due to increased IFP hamper uniform drug distribution, especially in large tumors. Although the existing nanobody platforms offer possibilities to circumvent this, future studies should investigate alternative methods of therapeutic nanobody delivery. One of the directions for further research could be the potential of

engineered stem cell-based therapeutics, as initiated by van de Water *et al.* [96]. Constructs encoding bivalent anti-EGFR nanobodies were packaged into lentiviral virions and used to create neural stem cells secreting nanobodies. These cells were then implanted into mice bearing tumors in a dorsal skinfold window chamber. The authors reported that nanobodies (7D12-38G7 and 7D12-9G8) secreted by stem cells efficiently localized to brain tumors and that a continuous release of nanobodies inhibited EGFR signaling and reduced brain tumor growth [96].

With respect to platform C, this platform remains a very potent strategy, as a large payload of drugs can be encapsulated in a nanoparticle, thereby protecting normal tissues, although the distribution through the tumor may be less effective due to their size. The design of smaller (<100 nm) and biodegradable nanoparticles that are still able to encapsulate a sufficient amount of drugs that are released upon a particular stimulus at the tumor site might improve distribution and thus the efficacy of these systems. Tumor heterogeneity in terms of receptor expression is one of many challenges to be overcome in order to provide successful therapy. In this respect, novel tumor targets are necessary. Personalized medicine will require the initial analysis of the expression levels of tumor markers. This can be achieved by analysis of a biopsy using a cocktail of nanobodies with different specificities and detection either by microscopy or by the analysis of the F?rster resonance energy transfer (FRET) between the different markers [97]. Subsequently, the appropriate nanobody-based therapy could be selected. This approach will greatly improve the attempts at personalized medicine in the near future.

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Executive summary

Nanobodies as solid cancer therapeutics

- Nanobodies, even though they are ten-times smaller than conventional monoclonal antibodies, retain high target specificity and affinity.
- Due to the ease of nanobody engineering, they can be employed in three nanobody-based therapeutic platforms: platform A, receptor antagonists to interfere with or block the activation of a certain signaling pathway; platform B, targeting moieties that transports effector domains, such as toxic peptides or drugs; or platform C, targeting molecules on the surface of nanoparticles.
- So far, nanobodies targeting receptors such as EGFR, HER2, VEGFR2, c-Met, CXCR7 or ligands (HGF and chemokines) have been developed.

Systemic circulation upon injection

- Residence time in the bloodstream upon intravenous injection is an important factor that depends upon the type of nanobody platform and affects nanobody-based therapeutic accumulation at the tumor.
- The size and shape of the molecule are the main determinants of the secretion route. In general, an average weight cutoff for renal clearance is approximately 60 kDa, with a preference for particles with a hydrodynamic diameter of <8 nm.
- Therapeutics that do not undergo renal clearance are removed by hepatic clearance, which preferentially removes particles with a hydrodynamic diameter of 10–20 nm.
- Several strategies can be employed to prolong the residence time of nanobodies in the circulation, such as coupling with antialbumin nanobodies in case of platform A or PEGylation in case of platform C.

Nanobody extravasation & tumor penetration

- Due to elevated interstitial pressure, penetration of therapeutics into the tumor is mediated by diffusion. As this depends on size, nanobodies in platforms A and B penetrate tumors much better than monoclonal antidbodies.
- The hyperpermeability of chaotically organized tumor blood vessels and the lack of lymphatic drainage lead to passive accumulation of platform C therapeutics at the tumor site.

Nanobody interaction with targets

- Specific interaction with target cells in all platforms is mediated by the nanobody.
- The high affinity of the nanobodies allow them to remain at the tumor and escape renal clearance in platforms A and B.
- The high affinity of nanobodies does not play a major role when nanobodies are employed as targeting moieties of liposomes, micelles or nanoparticles (platform C), which accumulate at the tumor as a consequence of the enhanced permeability and retention effect.

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