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HIGHLIGHTS

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REVIEW

A few good reasons to use nanobodies for cancer treatment

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mAbs have been instrumental for targeted cancer therapies. However, their relatively large size and physicochemical properties result in a heterogenous distribution in the tumor microenvironment, usually restricted to the first cell layers surrounding blood vessels, and a limited ability to penetrate the brain. Nanobodies are tenfold smaller, resulting in a deeper tumor penetration and the ability to reach cells in poorly perfused tumor areas. Nanobodies are rapidly cleared from the circulation, which generates a fast target-to-background contrast that is ideally suited for molecular imaging purposes but may be less optimal for therapy. To circumvent this problem, nanobodies have been formatted to noncovalently bind albumin, increasing their serum half-life without majorly increasing their size. Finally, nanobodies have shown superior qualities to infiltrate brain tumors as compared to mAbs. In this review, we discuss why these features make nanobodies prime candidates for targeted therapy of cancer.

Keywords: Brain tumor · Cancer · Cancer therapy · Half-life extension · Nanobodies

Introduction

Cancer therapy relied for decades on approaches that are only poorly targeted to the tumor site and suboptimally discriminate between transformed and normal cells. A better understanding of the molecular makeup of cancer cells, allowing to discriminate subtypes of tumors within the same indication, and a more detailed knowledge of the tumor microenvironment (TME) encompassing various stromal cell types, such as immune cells, fibroblasts, and endothelial cells, opened the possibility for targeted therapies. The specific targeting of molecules on cancer cells or stromal cells, either for diagnostic or therapeutic purposes, has initially been achieved by monoclonal antibodies (mAbs), many of which are currently in clinical use. mAbs may have therapeutic benefits in an unconjugated form (e.g., immune checkpoint blockers), but often these mAbs have been used as vehicles to deliver therapeutic payloads or radioisotopes specifically to the cancer cells, as such avoiding systemic toxicity and allowing for a lower minimum effective dose and an overall wider therapeutic window [1]. Consequently, antibody–drug conjugates (ADCs) are heavily under investigation, with over 100 ADC candidates in clinical trials and 14 approvals for clinical use worldwide [2]. However, to be effective, these targeted agents need to efficiently penetrate the tumor and deliver the toxic drug homogenously within

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the TME, assets that are not evident for large molecules such as mAb-conjugates. This is especially the case when the aim is to target cancer cells or tumor-supporting stromal cells in regions further away from the vasculature, such as hypoxic regions.

Another barrier to the use of mAbs could be the anatomical location of the tumor. The blood–brain barrier (BBB) cannot easily be penetrated by mAbs, complicating the use of ADCs to treat brain tumors [3].

To solve these issues, smaller antigen-binding molecules are required. Nanobodies (Nbs), also termed single-domain antibodies or VHHs, are the antigen-recognition domains of camelid heavy chain–only antibodies and are with their 15 kDa 10× smaller than conventional antibodies [4]. Other discriminatory characteristics in favor of nanobodies are: (i) their ability to recognize hidden epitopes in cavities, due to their longer CDR3 loops [5]; (ii) their high stability and solubility, even under conditions of proteolytic activity and low pH, which clearly provides a benefit in the harsh conditions of the TME [6]; (iii) their weak immunogenicity in mice and men [7]; (iv) their fast elimination from the circulation via the kidneys, as they are smaller than the renal filtration threshold (∼40–50 kDa, 2–6 nm), leading to a rapid contrast between on-target binding and the circulation and avoiding the elevated overall nonspecific accumulation seen with mAbs [8]; and (iv) from a practical point of view, ease of production in prokaryotic or eukaryotic expression systems [9] and ease of conjugation and molecular engineering [10, 11]. The next sections will highlight some of the competitive edges that nanobodies have in the function of cancer therapy.

Nanobodies diffuse deeper into tumors

The ability of biomolecules to spread within the TME is heavily influenced by peculiar properties of solid tumors [12, 13], one of which is being the enhanced permeability and retention effect. The enhanced permeability and retention effect promotes the accumulation of molecules in the TME, owing to the leakiness of the tumor vasculature and the near absence of lymphatic tumor drainage [14]. However, the consequent accumulation of fluid generates an interstitial pressure that tends to push molecules out of the TME, creating two opposing forces [15]. The result is a limited or absent pressure gradient and hardly any fluid convection within tumors, meaning that molecules are mostly transported through diffusion. As the diffusion rate is inversely correlated to the size of a molecule [16], nanobodies evidently diffuse faster and bridge longer distances than conventional antibodies, attaining areas of the tumor that are out of reach for antibodies (Fig. 1).

Antibody bivalency limits tumor penetration

Another feature of conventional antibodies that works against their deeper penetration into tumors is their bivalent nature, resulting in a higher avidity for the target and, hence, their efficient trapping in regions of first antigen encounter, often in the immediate neighborhood of the vessels from which they extravasated. This so-called binding site–barrier effect results in a heterogenous intratumoral distribution of mAbs, which often do not surpass the first couple of cell layers surrounding blood vessels [17–22] (Figs. 1 and 2). This is especially problematic in case the ADC's drug only kills the targeted cell without bystander effect or in case the maximum tolerated dose of the drug is low. In those conditions, the ideal situation would be to deliver a threshold level of ADC to each individual target cell in the TME, requesting obviously a homogenous tumor distribution of the compound. One way to circumvent the binding site–barrier effect would be to dramatically increase the dosing of the ADC (also dramatically increasing cost and burden for the patient), thereby saturating the perivascular area, allowing subsequent doses of the ADC to diffuse beyond these cell layers [22]. However, this is unfeasible if the drug's maximum tolerated dose is low and would also lead to a potentially unacceptable a specific accumulation of the ADC in healthy organs.

All these theoretical considerations are now supported by solid experimental data. Debie et al. compared the whole-body biodistribution — in mice bearing subcutaneous xenografts of HER2$^+$ SKOV3 tumors — of monomeric and dimeric anti-HER2 nanobody constructs (2Rb17c and 2Rb17c-2Rb17c), monomeric and dimeric control nanobodies (R3B23 and R3B23-R3B23), a dimeric monovalent nanobody (2Rb17c-R3B23), and the clinically approved mAb trastuzumab [23]. Intravital imaging showed that monomeric anti-HER2 Nb tracers accumulated rapidly and distributed homogenously in the tumor only minutes after intravenous injection. This was not the case for the dimeric anti-HER2 nanobody, which remained closely associated with the blood vessels over 24 h. Conversely, the HER2-specific dimeric monovalent tracer achieved a more homogenous tumor distribution from 1 h postinjection onward; that is with a clearly retarded kinetics as compared to the monomeric Nb. Nonspecific tracers were not retained in the tumor. These data elegantly prove the notion that bivalency, resulting in a stronger binding to the target, restricts tumor penetration (even in the case of the small bivalent nanobodies), whereas only doubling the size to ∼30 kDa already delays tumor penetration. Trastuzumab extravasated much slower than all Nb variants, displayed the most heterogenous distribution of all compounds with a strong restriction to the perivascular space, but ultimately accumulated to the highest levels in the tumor after 24 h as a consequence of its long circulation time. This is in line with earlier findings by Wittrup et al, who reported that IgG-sized macromolecular constructs exhibit the most favorable balance between systemic clearance and vascular extravasation, resulting in maximal tumor uptake [24]. Though this latter feature of mAbs seems beneficial, the limited distribution within the tumor strongly counteracts this advantage and the therapeutic use. Nevertheless, it suggests that a longer $t1/2$ of nanobodies, without jeopardizing the size benefit, is warranted, as will be discussed later.

Related work by Erreni et al. provided microscopically detailed insights in the kinetics of monovalent versus bivalent anti-CD206 (macrophage mannose receptor) nanobodies in normal tissue, tumor tissue as well as the blood circulation [25]. CD206$^+$

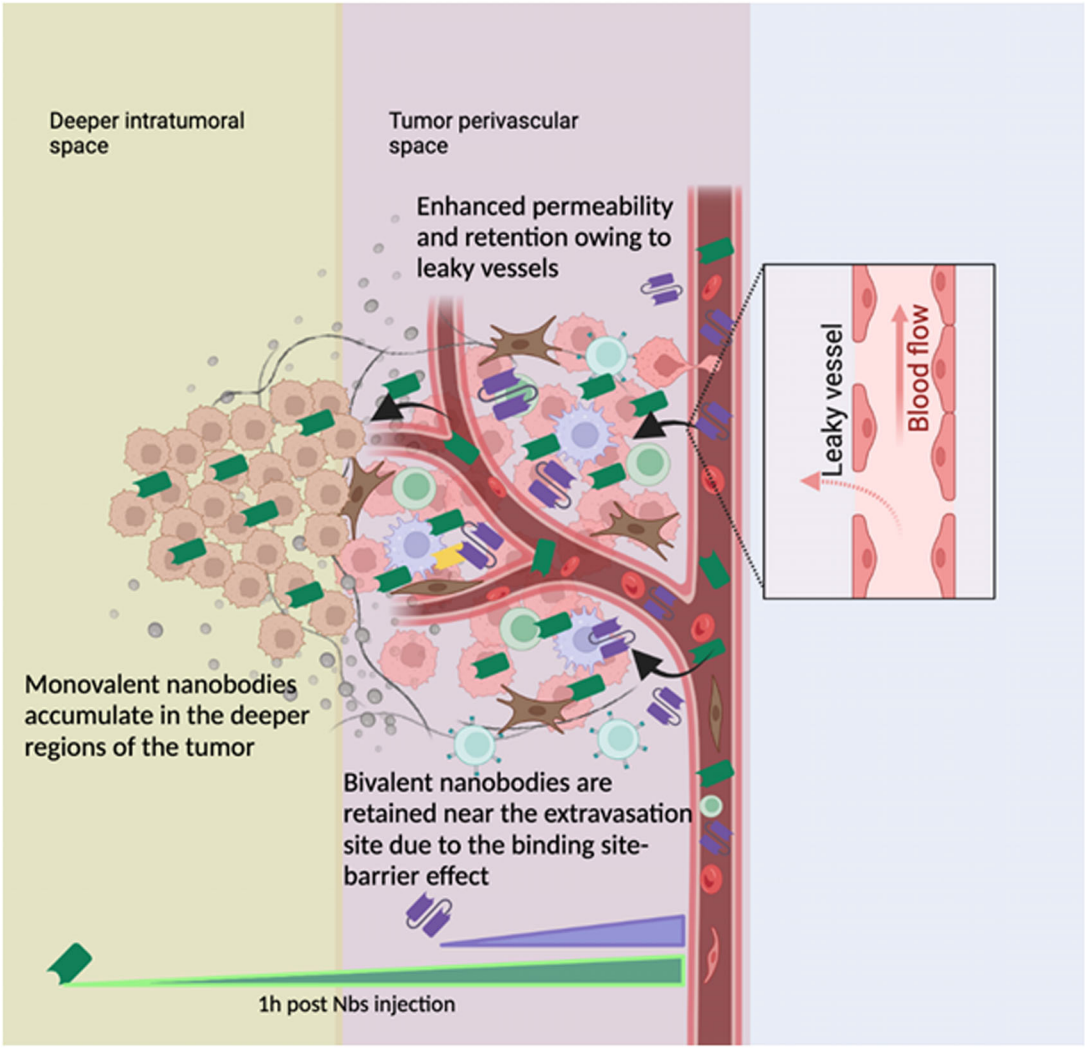

Figure 1. Nanobodies penetrate better the deeper regions of the tumor. Solid tumors exhibit areas that are characterized by minimal vascularization, accounting for their resistance to cancer therapy. Monovalent nanobodies have been shown to accumulate within these areas, such as hypoxic centers, even within 1 h following inoculation, whereas bivalent nanobodies and antibodies are retained within the perivascular layers of the tumor. *Source*: Figure generated via BioRender.

macrophages have been shown to exert a tumor-promoting role and can be found in hypoxic tumor areas [26–28], regions that are particularly challenging to be targeted by therapeutic compounds. The blood clearance rate of monovalent Nbs was clearly faster in healthy tissue, highlighting their size advantage for extravasating mature blood vessels. However, in tumors, the blood clearance rate of monovalent and bivalent Nbs was very comparable, suggesting that the more fenestrated and leaky tumor vasculature is easier to penetrate. In healthy tissue, where CD206 is mostly expressed by tissue-resident macrophages [29], monovalent Nbs reach these macrophages significantly faster than bivalent Nbs, a difference that is even much more pronounced in tumor tissue [25]. Moreover, even a strong molar excess of bivalent Nbs does not preclude the monovalent anti-CD206 Nbs from binding tumor-associated macrophages (TAMs), clearly highlighting that monovalent and bivalent Nbs can bind TAM in different locations [25, 30].

Nanobodies reach hypoxic areas, distant from the vessels

Besides the overall consideration that a homogenous distribution of therapeutic payloads maximizes the therapeutic effect, deeper tumor penetration is an absolute must when targeting molecules that are mostly expressed in regions distant from the vessels. Hypoxia is a strong driving force that promotes the tumor-supporting role of macrophages [27, 31], so either preventing macrophages from entering hypoxia or directly eliminating or repolarizing hypoxic macrophages has therapeutic benefit. Precluding macrophages from entering hypoxic areas have been achieved by the intratumoral expression of anti-Neuropilin-1 nanobodies [32], whereby neuropilin-1 is required to attract macrophages to the hypoxic areas [30]. A repolarization of hypoxic macrophages was accomplished by the use of anti-CD206 Nbs conjugated to a TLR7–8 ligand [33]. Because CD206$^+$ TAMs

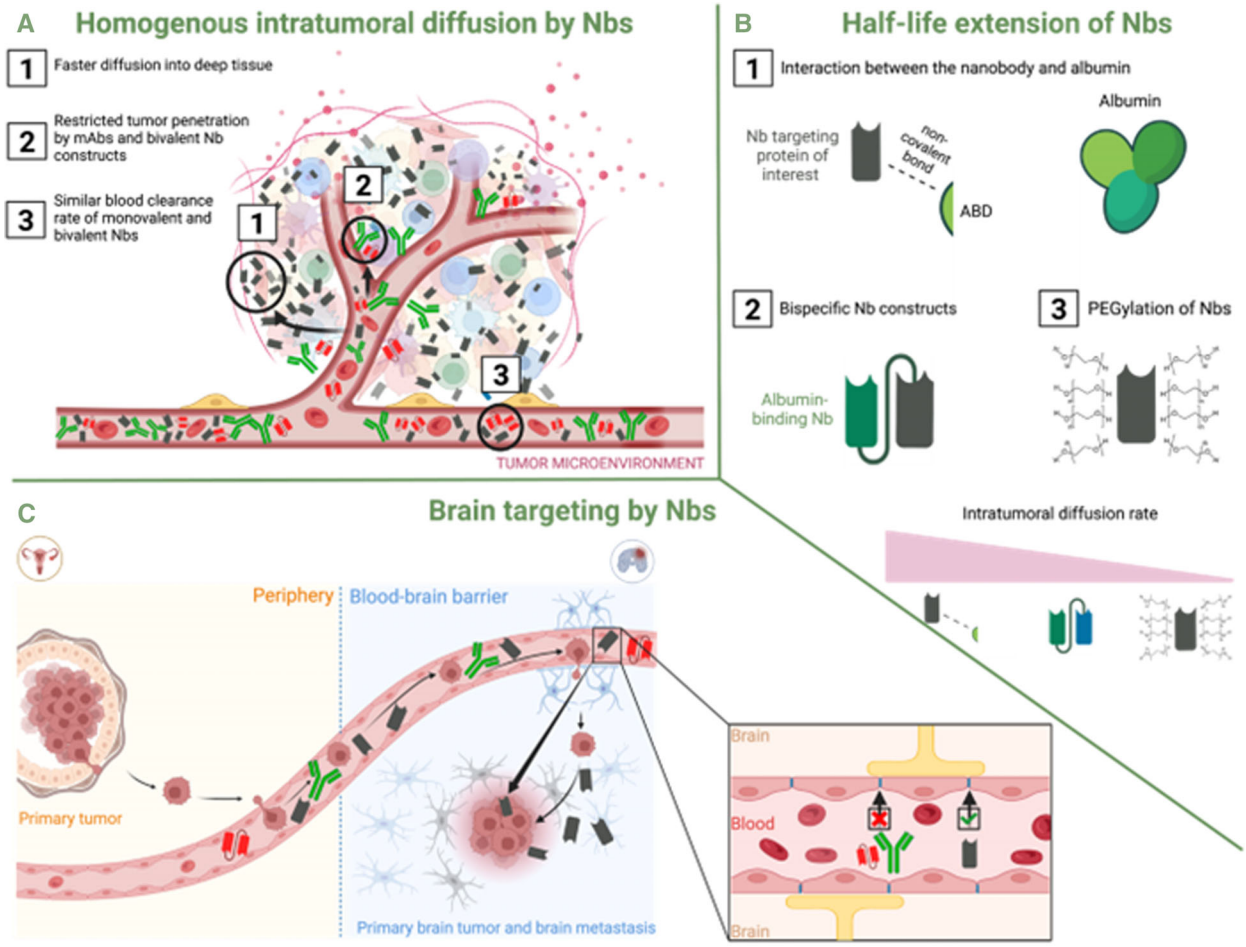

Figure 2. Three beneficial characteristics of nanobodies (Nbs) allowing them to (A) diffuse homogenously into the tumor microenvironment (TME) in comparison to mAbs and bivalent Nb constructs, (B) be engineered and adapted for longer retention and circulation in the body, and (C) cross the blood–brain barrier. *Source*: Figure generated via BioRender.

are found in relatively radioresistant hypoxic areas, CD206 has also been used as a target to bring high radiation doses to the hypoxic zones, by coupling ^{177}Lu to anti-CD206 Nbs [34]. Hypoxia induces the expression of VEGF, EMAP-II, endothelin, SEMA3A, SDF-1α, eotaxin, and oncostatin M, all of which have been implicated in the attraction of tumor-promoting immune cells [35–40]. Hypoxic TAMs promote tumor progression through the upregulation of growth factors, such as FGF2, PDGF, and VEGF, which support the growth of tumor cells in nutrient-deprived regions [41, 42]. Hence, nanobodies could be proposed as the most suitable compounds to block any of these factors that are majorly produced deep inside tumors.

Nanobodies with extended t1/2 in circulation but maintenance of size advantage

Nanobodies' small size results in their fast elimination via the kidneys. This property is ideal for imaging purposes, as high contrast between the target-expressing sites and the circulation is usually already achieved 1 h after injection of the nanobody-based tracer [30]. By now, Nbs have been conjugated to a multitude of radioisotopes for PET and SPECT imaging, as well as near-infrared fluorescent dyes for applications such as image-guided surgery [10, 43]. However, for therapeutic purposes, a longer circulation and retention time may be more suitable as it may allow a gradual accumulation of the compound within tumors in the function of time. An increase in size by creating multivalency or the coupling to an IgG Fc portion, resulting in neonatal Fc receptor–mediated recycling, strongly increases the *t*1/2 of nanobodies, but this obviously goes at the expense of their size-related tumor penetrating potential [44]. Hence, methodologies to increase the circulation *t*1/2 of nanobodies without a major size increase are in demand.

The use of albumin-binding domains

An elegant approach to achieve this goal is to establish a noncovalent interaction between the nanobody and albumin, the most

abundant protein in our blood plasma (Fig. 2). This interaction in the blood increases the size of the complex above the renal filtration threshold, whereas the noncovalent nature of the interaction allows for a reversal to unbound nanobody and deep tumor penetration in the TME. The longevity of human serum albumin in the circulation ($t1/2$ of ~19 days) is further due to its neonatal Fc receptor–mediated recycling, a feature that is also transferred to albumin-conjugated proteins [45]. Consequently, albumin binding has been known for at least two decades as a general strategy to improve the pharmacokinetics of therapeutic proteins and has been widely used in the clinic [45, 46]. Strategies that do not majorly affect the size of the protein are the coupling to either albumin-binding peptides or albumin-binding domains (ABD) [46–48]. Examples of the latter strategy are the Zag ABD of *Streptococcus zooepidemicus*, a 52 aa domain that was shown to increase the serum half-life of an anti-TNF nanobody ~39-fold [47], and the ABD of the streptococcal protein G that could strongly increase the $t1/2$ of a single-chain diabody [49]. Because nanobodies count on average 120 aas, the addition of these domains increases the size of the construct roughly by 45%, but the end result still is a small protein. The usefulness of this approach for nanobody-mediated cancer therapy has been demonstrated by Xenaki *et al.* [50]. The fusion of an anti-HER2 Nb to the streptococcal protein G ABD strongly increased the serum half-life and prolonged the accumulation of the compound in HER2-expressing xenografts, without compromising the homogenous intratumoral distribution. On top of that, the kidney retention of this construct was reduced, which can be important in the case of a toxic nanobody–drug conjugate. Finally, these authors could demonstrate that a single dose of an Nb–ABD–auristatin F construct yielded the long-term remission of the HER2-expressing xenografts in nude mice.

The use of an albumin-binding nanobody

An alternative to the use of ABD is simply to generate a bispecific Nb, with one arm consisting of an albumin-binding Nb and the other arm recognizing the target of interest (Fig. 2). This strategy has been used by Ablynx NV, now a Sanofi company, who brought the first EMA- and FDA-approved bivalent (not bispecific) nanobody, caplacizumab (bivalent anti-von Willebrand factor to treat acquired thrombotic thrombocytopenic purpura), to the market. For example, ALX-0061, a bispecific anti-IL-6R/anti–human serum albumin Nb of 26 kDa, demonstrated a serum half-life of 6.6 days and diminished pathology in a nonhuman primate model of arthritis [51]. In a mouse study, a trivalent construct consisting of two anti-MIF Nbs and one anti-mouse serum albumin Nb could strongly decrease mortality in a model of endotoxic shock [52]. Obviously, this strategy leads to at least a doubling of the compound's size, influencing the intratumoral diffusion rate. Although Debie *et al.* and Erreni *et al.* demonstrated that a dimeric monovalent Nb (i.e., one arm recognizing a target in the TME, the other arm being an irrelevant Nb) still homogenously distributes in the TME [23, 25], it is at present uncertain whether this is also the case if the second arm consists of an albumin-binding Nb that is bound to its target protein with (sub)nanomolar affinity. Future research will need to shed light on this aspect.

As a final note, PEGylation has also been used to increase the $t1/2$ of nanobodies [53] (Fig. 2). However, employing CEA-specific single-chain diabodies, Stork *et al.* recorded a twofold lower tumor accumulation of the PEGylated variants as compared to the ABD-conjugated variants, despite a similarly elongated serum half-life [49]. Moreover, the strong increase in molecular weight by PEG is likely to preclude deep intratumoral dissemination.

Nanobodies are superior to target brain tumors

The challenge to deliver biopharmaceuticals into tumors is arguably even greater for brain tumors due to the highly protected nature of the brain. In particular, the BBB is a major limiting factor for the transport of blood-borne molecules into the brain, as it is formed by tightly sealed endothelial cells that express multiple efflux pumps [3, 54, 55]. Especially, large hydrophilic molecules such as conventional antibodies experience difficulties to surpass the BBB [56]. This impermeability of the BBB potentially creates an additional hurdle to treat primary brain tumors, of which glioblastoma (GBM) is the most common, but also brain metastases from systemic tumors, which are even more prevalent than primary brain tumors. Indeed, although it has been argued that the BBB is more leaky in the case of brain malignancies, many of the targeted treatments that are effective against the systemic tumor fail to show any efficacy in the CNS.

Again, nanobodies could come to the rescue thanks to their smaller size and different physicochemical properties [57]. The group of Pierre Lafaye demonstrated that nanobodies are superior to conventional antibodies when it comes to diffusion into fixed brain tissues [58], or as BBB-permeable probes in vivo that are able to detect both intracellular and extracellular brain targets [59]. Puttemans *et al.* performed a side-by-side comparison of an anti-HER2 Nb or the clinically approved anti-HER2 mAb trastuzumab for their ability to infiltrate intracranially injected human ovarian cancer tumors (the trastuzumab-sensitive HER2$^+$ SKOV2-IP1 cell line) in nude mice [60]. Strikingly, this brain metastasis model was permissive for the infiltration by the anti-HER2 Nb but not at all for trastuzumab. Consequently, ^{131}I- or ^{225}Ac-labeled Nbs prolong survival, whereas trastuzumab has hardly any effect. In an orthotopic mouse GBM model, anti-SIRPα Nbs were used to image tumor-infiltrating myeloid cells [61]. Again, the size of the imaging tracers was of crucial importance, as only the monovalent Nb, but not its bivalent derivative, was able to reach these tumors. A physicochemical property of nanobodies that appears to influence accessibility to the brain is their isoelectric point, with basic nanobodies being more prone to cross the BBB [62]. However, a basic isoelectric point is not an intrinsic quality of nanobodies, as also basic antibodies can be designed. It

therefore seems more likely that the size advantage of nanobodies is the more decisive factor for brain penetration. The homogenous distribution of this myeloid cell–targeting Nb within GBM tumors will be of importance, considering the complexity of the macrophage compartment, including populations that clearly show a hypoxic signature in newly diagnosed or recurrent tumors [63]. Other GBM markers against which Nbs have been generated include ABCC3 [64], IGFBP7 [65], Trim28 [66], and US28 [67, 68].

Whether the advantages of Nbs in the context of brain tumors are inherent to a better capacity to cross the BBB or to a better behavior in the TME, as outlined in a previous section, is currently unclear.

Conclusion

Nanobodies have emerged as promising vehicles for in vivo molecular imaging and targeted therapy in cancer. When prolonging their $t1/2$ in the circulation, without compromising their size advantage and their ability to homogenously spread within the TME, nanobodies could become prime candidates to deliver therapeutic payloads deep inside tumors, even in the brain (Fig. 2). Such payloads could consist of therapeutic radiotracers and small immunomodulating agents that could be coupled to the nanobody carrier. Alternatively, the therapeutic benefit of nanobodies could be linked to their capacity to block molecules that are hard to reach for conventional antibodies, for example, in hypoxic tumor areas.

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Conflict of interest: JAVG, GR, and ND are co-founders of the company Abscint NV that specializes in the use of nanobodies for molecular imaging. The other authors declare no commercial or financial conflict of interest.

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References

1 **Beck, A., Goetsch, L., Dumontet, C. and Corvaïa, N.**, Strategies and challenges for the next generation of antibody-drug conjugates. *Nat. Rev. Drug Discov.* 2017. **16**: 315–337.

2 **Fu, Z., Li, S., Han, S., Shi, C. and Zhang, Y.**, Antibody-drug conjugate: the "biological missile" for targeted cancer therapy. *Signal Transduct. Target. Ther.* 2022. **7**: 93.

3 **Kumar, N. N., Pizzo, M. E., Nehra, G., Wilken-Resman, B., Boroumand, S. and Thorne, R. G.**, Passive immunotherapies for central nervous system disorders: current delivery challenges and new approaches. *Bioconjug. Chem.* 2018; **29**: 3937–3966.

4 **Schoonooghe, S., Laoui, D., Van Ginderachter, J. A., Devoogdt, N., Lahoutte, T., De Baetselier, P. and Raes, G.**, Novel applications of nanobodies for in vivo bio-imaging of inflamed tissues in inflammatory diseases and cancer. *Immunobiology* 2012; **217**: 1266–1272.

5 **Stijlemans, B., Conrath, K., Cortez-Retamozo, V., Van Xong, H., Wyns, L., Senter, P., Revets, H. et al.**, Efficient targeting of conserved cryptic epitopes of infectious agents by single domain antibodies. African trypanosomes as paradigm. *J. Biol. Chem.* 2004; **279**: 1256–1261.

6 **Kunz, P., Zinner, K., Mücke, N., Bartoschik, T., Muyldermans, S. and Hoheisel, J. D.**, The structural basis of nanobody unfolding reversibility and thermoresistance. *Sci. Rep.* 2018; **8**: 7934.

7 **Ackaert, C., Smiejkowska, N., Xavier, C., Sterckx, Y. G. J., Denies, S., Stijlemans, B., Elkrim, Y. et al.**, Immunogenicity risk profile of nanobodies. *Front. Immunol.* 2021. **12**: 632687.

8 **Vaneycken, I., D'huyvetter, M., Hernot, S., De Vos, J., Xavier, C., Devoogdt, N., Caveliers, V. et al.**, Immuno-imaging using nanobodies. *Curr. Opin. Biotechnol.* 2011. **22**: 877–881.

9 **de Marco, A.**, Recombinant expression of nanobodies and nanobody-derived immunoreagents. *Protein Expr. Purif.* 2020. **172**: 105645.

10 **Yang, E., Liu, Q., Huang, G., Liu, J. and Wei, W.** Engineering nanobodies for next-generation molecular imaging. *Drug Discov. Today* 2022. **27**: 1622–1638.

11 **Awad, R. M., Meeus, F., Ceuppens, H., Ertveldt, T., Hanssens, H., Lecocq, Q., Mateusiak, L. et al.**, Emerging applications of nanobodies in cancer therapy. *Int. Rev. Cell Mol. Biol.* 2022. **369**: 143–199.

12 **Tunggal, J. K., Cowan, D. S. M., Shaikh, H. and Tannock, I. F.**, Penetration of anticancer drugs through solid tissue: a factor that limits the effectiveness of chemotherapy for solid tumors. *Clin. Cancer Res.* 1999. **5**: 1583–1586.

13 **Jain, R. K.**, Vascular and interstitial barriers to delivery of therapeutic agents in tumors. *Cancer Metastasis Rev.* 1990. **9**: 253–266.

14 **Matsumura, Y. and Maeda, H.**, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agents Smancs. *Cancer Res.* 1986. **46**: 6387–6392.

15 **Chauhan, V. P., Stylianopoulos, T., Boucher, Y. and Jain, R. K.**, Delivery of molecular and nanoscale medicine to tumors: transport barriers and strategies. *Annu. Rev. Chem. Biomol. Eng.* 2011. **2**: 281–298.

16 **Thurber, G. M., Schmidt, M. M. and Wittrup, K. D.**, Factors determining antibody distribution in tumors. *Trends Pharmacol. Sci.* 2008. **29**: 57–61.

17 **van Osdol, W., Fujimori, K. and Weinstein, J. N.**, An analysis of monoclonal antibody distribution in microscopic tumor nodules: consequences of a "binding site barrier". *Cancer Res.* 1991. **51**: 4776–4784.

18 **Saga, T., Neumann, R. D., Heya, T., Sato, J., Kinuya, S., Le, N., Paik, C. H. et al.**, Targeting cancer micrometastases with monoclonal antibodies: a binding-site barrier. *Proc. Natl. Acad. Sci. USA* 1995. **92**: 8999–9003.

19 **Sato, J., van Osdol, W., Weinstein, J. N. and Perez-Bacete, M. J.**, Micropharmacology of monoclonal antibodies in solid tumors: direct experimental evidence for a binding site barrier. *Cancer Res.* 1992. **52**: 5144–5153.

20 **Lee, C. M. and Tannock, I. F.**, The distribution of the therapeutic monoclonal antibodies cetuximab and trastuzumab within solid tumors. *BMC Cancer* 2010. **10**: 255.

21 **Baker, J. H. E., Lindquist, K. E., Huxham, L. A., Kyle, A. H., Sy, J. T. and Minchinton, A. I.**, Direct visualization of heterogeneous extravascular distribution of trastuzumab in human epidermal growth factor receptor type 2 overexpressing xenografts. *Clin. Cancer Res.* 2008. **14**: 2171–2179.

22 **Rhoden, J. J. and Wittrup, K. D.**, Dose dependence of intratumoral perivascular distribution of monoclonal antibodies. *J. Pharm. Sci.* 2012. **101**: 860–867.

23 **Debie, P., Lafont, C., Defrise, M., Hansen, I., van Willigen, D. M., van Leeuwen, F. W. B., Gijsbers, R. et al.**, Size and affinity kinetics of nanobodies influence targeting and penetration of solid tumors. *J. Control Release* 2020. **317**: 34–42.

24 **Wittrup, K. D., Thurber, G. M., Schmidt, M. M. and Rhoden, J. J.**, Practical theoretic guidance for the design of tumor-targeting agents. *Methods Enzymol.* 2012. **503**: 255–268.

25 **Erreni, M., D'Autilia, F., Avigni, R., Bolli, E., Arnouk, S. M., Movahedi, K., Debie, P.**, et al., Size-advantage of monovalent nanobodies against the macrophage mannose receptor for deep tumor penetration and tumor-associated macrophage targeting. *Theranostics* 2023. **13**: 355–373.

26 **Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stangé, G., Van den Bossche, J., Mack, M.**, et al., Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* 2010. **70**: 5728–5739.

27 **Laoui, D., Van Overmeire, E., Di Conza, G., Aldeni, C., Keirsse, J., Morias, Y., Movahedi, K. et al.**, Tumor hypoxia does not drive differentiation of tumor-associated macrophages but rather fine-tunes the M2-like macrophage population. *Cancer Res.* 2014. **74**: 24–30.

28 **Van Overmeire, E., Stijlemans, B., Heymann, F., Keirsse, J., Morias, Y., Elkrim, Y., Brys, L. et al.**, M-CSF and GM-CSF receptor signaling differentially regulate monocyte maturation and macrophage polarization in the tumor microenvironment. *Cancer Res.* 2016. **76**: 35–42.

29 **Wang, X., Sathe, A. A., Smith, G. R., Ruf-Zamojski, F., Nair, V., Lavine, K. J., Xing, C. et al.**, Heterogeneous origins and functions of mouse skeletal muscle-resident macrophages. *Proc. Natl. Acad. Sci. USA* 2020. **117**: 20729–20740.

30 **Movahedi, K., Schoonooghe, S., Laoui, D., Houbracken, I., Waelput, W., Breckpot, K., Bouwens, L. et al.**, Nanobody-based targeting of the macrophage mannose receptor for effective in vivo imaging of tumor-associated macrophages. *Cancer Res.* 2012. **72**: 4165–4177.

31 **Casazza, A., Laoui, D., Wenes, M., Rizzolio, S., Bassani, N., Mambretti, M., Deschoemaeker, S. et al.**, Impeding macrophage entry into hypoxic tumor areas by Sema3A/Nrp1 signaling blockade inhibits angiogenesis and restores antitumor immunity. *Cancer Cell* 2013. **24**: 695–709.

32 **De Vlaeminck, Y., Bonelli, S., Awad, R. M., Dewilde, M., Rizzolio, S., Lecocq, Q., Bolli, E. et al.**, Targeting neuropilin-1 with nanobodies reduces colorectal carcinoma development. *Cancers (Basel)* 2020. **12**: 3582.

33 **Bolli, E., Scherger, M., Arnouk, S. M., Pombo Antunes, A. R., Straßburger, D., Urschbach, M., Stickdorn, J. et al.**, Targeted repolarization of tumor-associated macrophages via imidazoquinoline-linked nanobodies. *Adv. Sci. (Weinh)* 2021. **8**: 2004574.

34 **Bolli, E., D'Huyvetter, M., Murgaski, A., Berus, D., Stangé, G., Clappaert, E. J., Arnouk, S. et al.**, Stromal-targeting radioimmunotherapy mitigates the progression of therapy-resistant tumors. *J. Control Release* 2019. **314**: 1–11.

35 **Tripathi, C., Tewari, B. N., Kanchan, R. K., Baghel, K. S., Nautiyal, N., Shrivastava, R., Kaur, H. et al.**, Macrophages are recruited to hypoxic tumor areas and acquire a pro-angiogenic M2-polarized phenotype via hypoxic cancer cell derived cytokines Oncostatin M and Eotaxin. *Oncotarget* 2014. **5**: 5350–5368.

36 **Du, R., Lu, K. V., Petritsch, C., Liu, P., Ganss, R., Passegué, E., Song, H. et al.**, HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* 2008. **13**: 206–220.

37 **Leek, R. D., Hunt, N. C., Landers, R. J., Lewis, C. E., Royds, J. A. and Harris, A. L.**, Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J. Pathol.* 2000. **190**: 430–436.

38 **Grimshaw, M. J., Wilson, J. L. and Balkwill, F. R.**, Endothelin-2 is a macrophage chemoattractant: implications for macrophage distribution in tumors. *Eur. J. Immunol.* 2002. **32**: 2393–2400.

39 **Matschurat, S., Knies, U. E., Person, V., Fink, L., Stoelcker, B., Ebenebe, C., Behrensdorf, H. A. et al.**, Regulation of EMAP II by hypoxia. *Am. J. Pathol.* 2003. **162**: 93–103.

40 **Lewis, J. S., Landers, R. J., Underwood, J. C., Harris, A. L. and Lewis, C. E.**, Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. *J. Pathol.* 2000. **192**: 150–158.

41 **Kuwabara, K., Ogawa, S., Matsumoto, M., Koga, S., Clauss, M., Pinsky, D. J., Lyn, P. et al.**, Hypoxia-mediated induction of acidic/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. *Proc. Natl. Acad. Sci. USA* 1995. **92**: 4606–4610.

42 **Harmey, J. H., Dimitriadis, E., Kay, E., Redmond, H. P. and Bouchier-Hayes, D.**, Regulation of macrophage production of vascular endothelial growth factor (VEGF) by hypoxia and transforming growth factor β-1. *Ann. Surg. Oncol.* 1998. **5**: 271–278.

43 **Lwin, T. M., Turner, M. A., Amirfakhri, S., Nishino, H., Debie, P., Cosman, B. C., Hoffman, R. M. et al.**, Rapid tumor-labeling kinetics with a site-specific near-infra red anti-CEA nanobody in a patient-derived orthotopic xenograft mouse model of colon cancer. *J. Surg. Oncol.* 2021. **124**: 1121–1127

44 **Roopenian, D. C. and Akilesh, S.**, FcRn: the neonatal Fc receptor comes of age. *Nat. Rev. Immunol.* 2007. **7**: 715–725.

45 **Kontermann, R. E.**, Half-life extended biotherapeutics. *Expert Opin. Biol. Ther.* 2016. **16**: 903–915.

46 **Dennis, M. S., Zhang, M., Gloria Meng, Y., Kadkhodayan, M., Kirchhofer, D., Combs, D. and Damico, L. A.**, Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *J. Biol. Chem.* 2002. **277**: 35035–35043.

47 **Cantante, C., Lourenço, S., Morais, M., Leandro, J., Gano, L., Silva, N., Leandro, P. et al.**, Albumin-binding domain from *Streptococcus zooepidemicus* protein Zag as a novel strategy to improve the half-life of therapeutic proteins. *J. Biotechnol.* 2017. **253**: 23–33.

48 **Hopp, J., Hornig, N., Zettlitz, K. A., Schwarz, A., Fuß, N., Müller, D. and Kontermann, R. E.**, The effects of affinity and valency of an albumin-binding domain (ABD) on the half-life of a single-chain diabody-ABD fusion protein. *Protein Eng. Des. Sel.* 2010. **23**: 827–834.

49 **Stork, R., Campigna, E., Robert, B., Müller, D. and Kontermann, R. E.**, Biodistribution of a bispecific single-chain diabody and its half-life extended derivatives. *J. Biol. Chem.* 2009. **284**: 25612–25619.

50 **Xenaki, K. T., Dorrestijn, B., Muns, J. A., Adamzek, K., Doulkeridou, S., Houthoff, H., Oliveira, S. et al.**, Homogeneous tumor targeting with a single dose of HER2-targeted albumin-binding domain-fused nanobody-drug conjugates results in long-lasting tumor remission in mice. *Theranostics* 2021. **11**: 5525–5538.

51 **Van Roy, M., Ververken, C., Beirnaert, E., Hoefman, S., Kolkman, J., Vierboom, M., Breedveld, E. et al.**, The preclinical pharmacology of the high affinity anti-IL-6R Nanobody ALX-0061 supports its clinical development in rheumatoid arthritis. *Arthritis Res. Ther.* 2015. **17**: 135.

52 **Sparkes, A., De Baetselier, P., Brys, L., Cabrito, I., Sterckx, Y. G., Schoonooghe, S., Muyldermans, S. et al.**, Novel half-life extended anti-MIF nanobodies protect endotoxic shock. *FASEB J.* 2018. **32**: 3411–3422.

53 **Li, L., Zhu, Y., Liu, M., Jin, D., Zhang, L., Cheng, J. and Liu, Y.**, Conjugation of oxaliplatin with PEGylated-nanobody for enhancing tumor targeting and prolonging circulation. *J. Inorg. Biochem.* 2021. **223**: 111553.

54 **Banks, W. A.**, From blood-brain barrier to blood-brain interface: new opportunities for CNS drug delivery. *Nat. Rev. Drug Discov.* 2016. **15**: 275–292.

55 **Obermeier, B., Daneman, R. and Ransoho, R. M.** Development, maintenance and disruption of the blood-brain barrier. *Nat. Med.* 2013, **19**: 1584–1596.

56 **Stemmler, J., Schmitt, M., Willems, A., Bernhard, H., Harbeck, N. and Heinemann, V.**, Brain metastases in HER2-overexpressing metastatic breast cancer: comparative analysis of trastuzumab levels in serum and cerebrospinal fluid. *J. Clin. Oncol.* 2006. **24**: 1525.

57 **Zheng, F., Pang, Y., Li, L., Pang, Y., Zhang, J., Wang, X. and Raes, G.**, Applications of nanobodies in brain diseases. *Front. Immunol.* 2022. **13**: 978513.

58 **Perruchini, C., Pecorari, F., Bourgeois, J.-P., Duyckaerts, C., Rougeon, F. and Lafaye, P.**, Llama VHH antibody fragments against GFAP: better diffusion in fixed tissues than classical monoclonal antibodies. *Acta Neuropathol.* 2009, **118**: 685–695.

59 **Li, T., Vandesquille, M., Koukouli, F., Dudeffant, C., Youssef, I., Lenormand, P., Ganneau, C.**, et al., Camelid single-domain antibodies: a versatile tool for in vivo imaging of extracellular and intracellular brain targets. *J. Control Release* 2016, **243**: 1–10.

60 **Puttemans, J., Dekempeneer, Y., Eersels, J. L., Hanssens, H., Debie, P., Keyaerts, M., Windhorst, A. D. et al.**, Preclinical targeted α- and β^--radionuclide therapy in HER2-positive brain metastasis using camelid single-domain antibodies. *Cancers (Basel)* 2020. **12**: 1017.

61 **De Vlaminck, K., Romão, E., Puttemans, J., Pombo Antunes, A. R., Kancheva, D., Scheyltjens, I. et al.**, Imaging of glioblastoma tumor-associated myeloid cells using nanobodies targeting Signal Regulatory Protein Alpha. *Front. Immunol.* 2021. **12**: 777524.

62 **Li, T., Bourgeois, J. P., Celli, S., Glacial, F., Le Sourd, A. M., Mecheri, S., Weksler, B. et al.**, Cell-penetrating anti-GFAP VHH and corresponding fluorescent fusion protein VHH-GFP spontaneously cross the blood-brain barrier and specifically recognize astrocytes: applications to brain imaging. *FASEB J.* 2012. **26**: 3969–3979.

63 **Pombo Antunes, A. R., Scheyltjens, I., Lodi, F., Messiaen, J., Antoranz, A., Duerinck, J., Kancheva, D. et al.**, Single-cell profiling of myeloid cells in glioblastoma across species and disease stage reveals macrophage competition and specialization. *Nat. Neurosci.* 2021. **24**: 595–610.

64 **Ruiz-Lopez, E., Jovcevska, I., Gonzalez-Gomez, R., Tejero, H., Al-Shahrour, F., Muyldermans, S. and Schuhmacher, A. J.**. Nanobodies targeting ABCC3 for immunotargeted applications in glioblastoma. *Sci. Rep.* 2022. **12**: 22581.

65 **Iqbal, U., Albaghdadi, H., Luo, Y., Arbabi, M., Desvaux, C., Veres, T., Stanimirovic, D. et al.**, Molecular imaging of glioblastoma multiforme using anti-insulin-like growth factor-binding protein-7 single-domain antibodies. *Br. J. Cancer* 2010. **103**: 1606–1616.

66 **Porčnik, A., Novak, M., Breznik, B., Majc, B., Hrastar, B., Šamec, N., Zottel, A. et al.**, Trim28 selective nanobody reduces glioblastoma stem cell invasion. *Molecules* 2021:**26**: 5141.

67 **De Groof, T. W. M., Mashayekhi, V., Fan, T. S., Bergkamp, N. D., Sastre Toraño, J., van Senten, J. R., Heukers, R. et al.**, Nanobody-targeted photodynamic therapy selectively kills viral GPCR-expressing glioblastoma cells. *Mol. Pharm.* 2019. **16**: 3145–3156.

68 **Heukers, R., Fan, T. S., de Wit, R. H., van Senten, J. R., De Groof, T. W. M., Bebelman, M. P., Lagerweij, T. et al.**, The constitutive activity of the virally encoded chemokine receptor US28 accelerates glioblastoma growth. *Oncogene* 2018. **37**: 4110–4121.

Abbreviations: **ABD**: albumin-binding domains · **ADCs**: antibody–drug conjugates · **BBB**: blood–brain barrier · **GBM**: glioblastoma · **Nbs**: nanobodies · **TAMs**: tumor-associated macrophages · **TME**: tumor microenvironment

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