Camelid single-domain antibody-fragment engineering for (pre)clinical in vivo molecular imaging applications: adjusting the bullet to its target

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EXPERT OPINION

Camelid single-domain antibody-fragment engineering for (pre)clinical *in vivo* molecular imaging applications: adjusting the bullet to its target

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Introduction: Molecular imaging is a fast developing field and there is a growing need for specific imaging tracers in the clinic. Camelid single-domain antibody-fragments (sdAbs) recently emerged as a new class of molecular imaging tracers.
Areas covered: We review the importance of molecular imaging in the clinic and the use of camelid sdAbs as *in vivo* molecular imaging tracers. Interest in imaging tracers based on antibody fragments or man-made protein scaffolds expanded over the last years. Camelid sdAbs are small, monomeric binding fragments that are derived from unique heavy-chain-only antibodies. *In vivo* imaging studies with sdAbs targeting various cell membrane receptors in different disease models have been reported and more sdAb imaging tracers are under development. The first clinical trial with a camelid sdAb as a molecular imaging tracer targeting the breast cancer marker Human Epidermal growth factor Receptor 2 is currently ongoing.
Expert opinion: We expect that the development and use of sdAbs as tracers for both preclinical and clinical molecular imaging applications will become widespread.

Keywords: biodistribution, blood-brain-barrier permeability, camelid single-domain antibody-fragment, clinical molecular imaging, cross-reactivity, diagnosis, nanobody, probe, radionuclide labeling, tracer

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1. Introduction

1.1 The need and importance of preclinical molecular imaging

Molecular imaging is defined as 'the visualization, characterization, and measurement of biological processes at molecular and cellular levels in humans and other living systems' [1]. A convenient approach to visualize molecular events *in vivo* consists of injecting and following a molecular imaging tracer. In essence, a molecular imaging tracer comprises a targeting vehicle joined to a detection label. Various kinds of compounds are used as targeting vehicles including small organic compounds, peptides, proteins, protein scaffolds, antibodies, and antibody-derived fragments [2-4]. In recent years, a variety of molecular imaging tracers has been developed for (pre)clinical research fields such as oncology, cardiology, neurology, or rheumatology [2,5]. It has been argued that molecular imaging probes are merely a novel tool for fundamental research, rather than a clinical tool [5]. However, to date, molecular imaging is becoming complementary to current diagnostic methods and we hypothesize that an increasing number of molecular imaging tracers will soon be an integral part of the clinical toolbox.

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Article highlights.

- The rapidly growing field of *in vivo* molecular imaging has inspired the development of a plethora of imaging tracers, each with its own advantages and limitations.
- An optimal *in vivo* imaging tracer has specific characteristics that in general differ from those of a labeled therapeutic compound.
- Camelid sdAbs are the small monomeric variable domains of HCAbs from *camelidae* and are perfect tools for *in vivo* imaging.
- Cross-reactivity and off-site uptake of tracers have important functional implications for *in vivo* imaging applications.
- Camelid sdAbs targeting various cellular receptors are being generated and these make a wide range of preclinical and clinical imaging applications possible.

This box summarizes key points contained in the article.

Currently, 2-^{18}F-fluoro-2-deoxy-D-glucose (^{18}F-FDG) is the most widely used molecular tracer in the clinic. ^{18}F-FDG is a glucose analog taken up and accumulating in metabolically active cells. The tracer is approved for clinical tumor detection and has also been assessed as an imaging agent in other pathologies, including atherosclerosis. Highly metabolic cells take up the glucose analog that cannot be metabolized, leading to a signal that is detected with a positron emission tomography (PET) scan. However, high ^{18}FDG uptake in many metabolically active tissues such as brain, heart, brown adipose, or inflamed tissue leads to high background signals and forms a severe limitation for imaging. Hence, a target signal in these tissues is hard to distinguish from surrounding tissue due to the lack of specificity of the ^{18}FDG uptake [6,7]. In view of that, a number of other tracers are being tested or have recently been approved for clinical use [8].

The most straightforward approach to diagnose a particular molecular event or visualize a biomarker in patients consists of labeling an already clinically approved therapeutic compound that targets this event or biomarker and using it as an imaging tracer. However, most therapeutic compounds (e.g., antibodies) possess characteristics that conflict with those of ideal imaging tracers (**Table 1**). Consequently, many antibody-derived fragments and protein scaffolds such as Fabs, single-chain variable fragments (scFvs), minibodies, diabodies, affibodies, single-domain antibody-fragments (sdAbs), small immunoproteins (SIPs), knottins, designed ankyrin repeat proteins (DARPins), and adnectins emerged as molecular imaging agents (**Figure 1**) [9-18]. These probes clear faster from blood compared to full-size monoclonal antibodies due to their smaller size and lack of an Fc part. Faster blood clearance allows faster imaging after injection and results in higher signal-to-background ratios. Ever since the first molecular imaging studies, the quality of the images has improved significantly due to improvements in tracer design, labeling procedures, and camera sensitivity and resolution [19-21]. Some of these small tracers have already been employed in clinical imaging studies [22-26].

1.2 Imaging vs therapy: different compound engineering

Noninvasive, whole-body imaging studies with radiolabeled therapeutic antibody-derived probes are common practice [27]. This approach is valuable to evaluate the pharmacokinetics and biodistribution of the compound *in vivo*, mainly to find out whether it reaches the target and to assess background uptake. However, these radiolabeled therapeutic probes are far from ideal imaging tracers (**Figure 1** and **Table 1**). Most therapeutic antibody-based compounds have a prolonged blood retention time. The slow blood clearance is due to its molecular weight (150 kDa) well above the renal cutoff of glomerular filtration (60 kDa) and the presence of an Fc-effector part that increases retention through the interaction with the neonatal Fc receptor. This receptor protects Fc-bearing molecules from a default degradation pathway [28-30].

A slow blood clearance rate is critical to reach high uptake values in the target tissues and it enables a long-lasting therapeutic effect. However, the use of such molecules as a probe for imaging purposes requires extensive waiting times, up to a week after administration, before image acquisition of reasonable signal-to-background ratios can be initiated. As a consequence these probes need to be labeled with long-lived isotopes to obtain a sufficiently high signal after the long incubation period [27].

To be an ideal imaging tracer it is essential to reach and bind the target as fast as possible, with a minimal uptake in non-targeted organs and to clear rapidly the unbound fraction from blood. Imaging within a few hours instead of days after injection has major practical advantages for routine clinical use. In addition, the imaging soon after administration enables labeling with short-lived radioisotopes, having beneficial effects on radioprotection and dosimetry.

Aside from the specific tumor-antigen targeting, most therapeutic anticancer compounds also accumulate into tumors through a phenomenon known as 'enhanced permeability and retention' (EPR). In this phenomenon, the immature and leaky nature of tumor vasculature and lack of efficient lymph drainage allow macromolecules > 40 kDa to accumulate inside tumor tissue [31]. However, this EPR effect is antigen-unrelated and merely depends on compound size, where it is less pronounced for small, rapidly cleared compounds [31]. Hence, when using full antibodies as imaging tracers, tracer accumulation inside tumors might lead to false-positive interpretations.

An inherent property of a therapeutic is to raise a functional biological effect. However, this is to be avoided for an imaging tracer in order to circumvent possible adverse side effects. Probes for imaging or therapy also differ widely in the frequency and dosage of administration: diagnostic

Table 1. Characteristics of imaging tracers and therapeutic compounds.

Imaging tracer	Therapeutic compound
High contrast between target and background	High uptake in target tissue
Fast pharmacokinetics/blood clearance	Slow pharmacokinetics/blood clearance
Labeling with short-lived isotopes	Labeling with long-lived isotopes
No functional biological effect	Functional biological effect
Low dose	High dose
Single/limited administration	Repeated administration
High affinity for target	
Specific targeting	

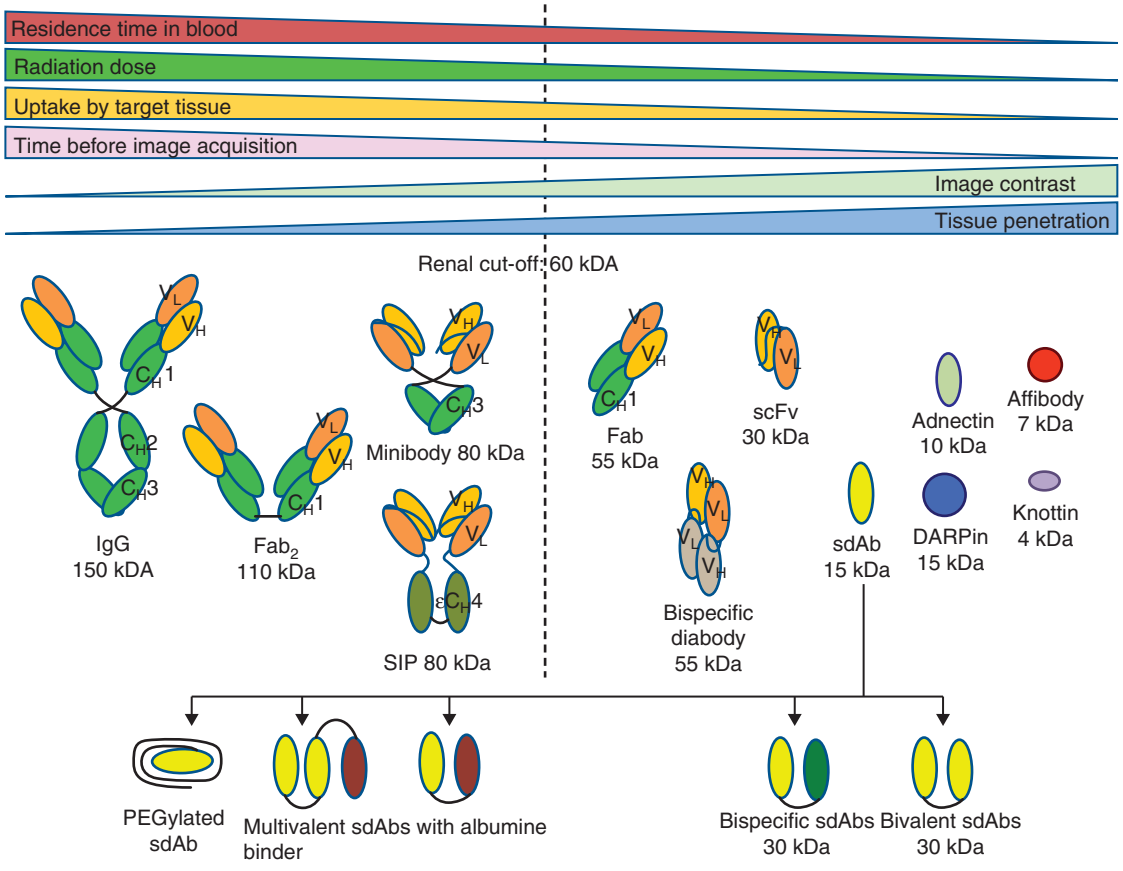

Figure 1. Different characteristics of tracers used for *in vivo* imaging. Relationship between tracer size and residence time in blood, radiation dose, uptake by target tissue, time to image acquisition, image contrast, and tissue penetration.

CH: Constant domain of a heavy chain of conventional antibody; DARPins: Designed Ankyrin Repeat Proteins; εCH4: CH4 domain of the human IgE secretory isoform IgE-S2; IgG: Immunoglobulin G; scFv: Single chain Variable Fragment; sdAb: Single-domain antibody-fragment; SIP: Small ImmunoProtein; VH: Variable domain of a heavy chain of conventional antibody; VL: Variable domain of a light chain.

imaging requires fewer repeated injections of tracers, thereby limiting the risk of immune reactions against the probe. Besides the infrequent administrations, also the low dosage compared to a higher therapeutic dosage lowers the possibility of any adverse events.

Specificity of targeting is also a critical feature to reduce the likelihood of adverse effects. Although both imaging and therapeutic probes will benefit from high specificity and affinity to associate to their target, for imaging purposes it is crucial to prevent false-positive images. Hence, imaging tracers must have maximized specificity features.

In conclusion, ideal imaging tracers and therapeutic compounds each have their individual optimal characteristics, some of which are contradictory to each other (**Table 1**).

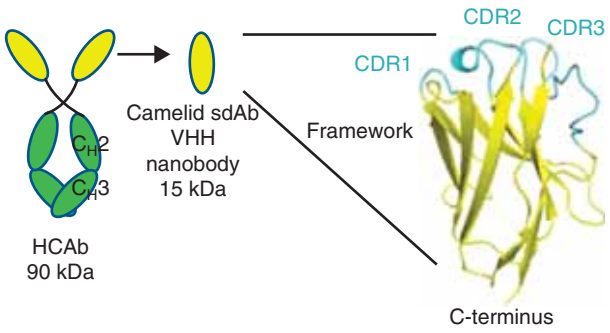

Figure 2. A camelid sdAb. Camelid sdAb, also referred to as VHH or nanobody, is the recombinantly produced antigen-binding domain of HCAbs that are naturally occurring in *camelidae*. The framework region (yellow) and the three CDR antigen-binding loops (cyan) are indicated in the modeled three-dimensional structure of an sdAb.
CDR: Complementary determining region; sdAb: Single-domain antibody-fragment.

2. Camelid sdAbs as *in vivo* molecular imaging tracers

2.1 Single-domain antibody-fragments

Camelidae possess besides conventional antibodies also heavy-chain-only antibodies (HCAb) that lack light chains (**Figure 2**) [32]. These latter antibodies bind their antigen through a single domain known as the Variable domain of a Heavy chain of an HCAb (VHH). The single VHH domain is a type of sdAb that can be produced recombinantly as an autonomous soluble binding entity. It interacts to its cognate antigen with a high affinity and specificity through its three complementary determining region (CDR) loops (**Figure 2**). VHHs are also referred to as nanobodies due to their dimensions in the nanometer range (2.5 nm in diameter by 4 nm) [33,34]. Some species of cartilaginous fish have antibodies, called immunoglobulin new antigen receptor (Ig-NAR), with a similar single-domain antigen-binding fragment. sdAbs isolated from Ig-NARs are referred to as variable domain of Ig-NAR (VNAR) [35]. Although size and shape of VNAR and VHH are similar, the camelid sdAbs share a higher degree of sequence identity with human VHs. Therefore, introducing camelid sdAbs as imaging probes in human might be favored.

Camelid sdAbs have a molecular weight of < 15 kDa, which is far below the renal cutoff for glomerular filtration (60 kDa). As a consequence, they are cleared much faster from blood than full-sized antibodies (150 kDa) (**Figure 1**). When labeling these fragments with a radioisotope they can be used as highly sensitive and specific probes for *in vivo* imaging [36,37]. The unmodified variable domain of a heavy chain of conventional antibodies (VH) is not suited as an autonomous binding entity due to the hydrophobic region that is exposed in absence of the variable domain of a light chain (VL) domain. The exposure of the hydrophobic patches on the surface of the domain to the aqueous solution leads to aggregation of the VH domain. Therefore, VH and VL domains must be linked together in larger constructs such as scFvs or diabodies (**Figure 1**) to be practical as imaging probes. Alternatively, it is feasible to introduce mutations in the VH domain at the VH/VL interface to generate a 'camelized' sdAb with increased stability and solubility [38]. However at present, neither VH nor VNAR fragments have been reported for non-invasive *in vivo* imaging applications.

Camelid sdAbs do not trigger any immunogenic response in mice [39]. Several clinical studies have been performed using camelid sdAbs as therapeutics and no immune adverse event has been reported in human either [40]. This is explained by the high sequence identity between VHH and human VH domains [34]. In addition, a 'humanization' strategy for camelid sdAbs without loosing their functional properties to recognize their cognate target *in vitro* and *in vivo* has been proposed [41,42].

2.2 Comparison to other *in vivo* imaging tracers

Several other man-made (human) proteins have comparable favorable properties to camelid sdAbs for non-invasive *in vivo* imaging (**Figure 1**). These include affibodies (protein-A-derived triple α-helix), DARPins, knottins, adnectins, or monobodies (derived from fibronectin domain) and are mostly proteins without an immunoglobulin fold [16-18,20]. These protein scaffolds are all well produced by microorganisms, are stable, soluble, and their amino acids (AAs) can be randomized at one side of the domain so that a large repertoire of variants can be generated from which to select the best binder recognizing the biomarker. Although the binders retrieved from these large libraries are very specific for the target and the affinity can be quite good as well, it is preferred to further improve the affinity by intensive *in vitro* maturation processes that mimic the natural affinity maturation of antibodies [43-45]. These additional engineering tasks retard the process to identify the optimal lead for imaging or any other application. The sdAbs that are retrieved from camelids that were immunized with the target benefit from the *in vivo* affinity maturation in the animal, so that additional *in vitro* maturation seems unnecessary. So, the average time to identify an sdAb as a lead is shorter than finding any other lead compound based on man-made protein scaffolds. Consequently, the number of possible lead sdAbs for cancer or inflammation *in vivo* imaging is expected to outnumber rapidly those derived from other protein scaffolds (**Table 2**).

Another major difference between sdAbs and other man-made protein scaffolds resides in the antibody nature of the former. Scientists and doctors are more familiar with this protein format as it is the natural defense molecule for any disease, and therefore, they are more open for its employment.

With respect to additional engineering (e.g., for advanced labeling) there is hardly any difference between sdAbs and

Table 2. Multiple sdAb-labeling strategies for different *in vivo* applications.

Application	Label (half-life)	Coupling method	Coupling AA	Target	Refs.
SPECT imaging	^{99m}Tc (6 h)	Tricarbonyl	His	CEA, EGFR, HER2, DCs, VCAM-1, MMR	[14,21,42,50-54,59,82,83]
PET imaging	^{68}Ga (68 min)	Df-Bz-NCS, NOTA derivative	Lys	EGFR, HER2	[26,84]
	^{64}Cu (12.7 h)	DOTA derivative	Lys	EGFR	[85]
	^{89}Zr (3.3 d)	Df–Bz–NCS	Lys	HGF	[86]
Radionuclide therapy	^{177}Lu (6.7 d)	DTPA, DOTA derivatives	Lys	EGFR, HER2	[55,87]
	^{131}I (8 d)	IB-Mal-D-GEEK	Lys	HER2	[67]
NIR imaging	IRDye800CW	-	Lys	EGFR	[56]
Ultrasound imaging	Microbubble	Biotin-Streptavidin	Biotin acceptor sequence	VCAM-1	[88]

CEA: CarcinoEmbryonic Antigen; DCs: Dendritic Cells; DOTA: 1, 4, 7, 10-Tetraazacyclododecanetetraacetic acid; DTPA: Diethylenetriamine Pentaacetic Acid; EGFR: Epidermal Growth Factor Receptor; HER2: human epidermal growth factor receptor 2; HGF: Hepatocyte Growth Factor; MMR: Macrophage Mannose Receptor; NIR: Near InfraRed; NOTA: 1,4,7-Triazacyclononane-1,4,7-Triacetic acid; SPECT: Single Photon Emission Tomography; PET: Positron Emission Tomography; VCAM-1: Vascular Cell Adhesion Molecule-1.

other man-made protein scaffolds. They all exist as a single-domain fragment and are easily tagged or tailored in bispecific or bivalent constructs. The generation of constructs involving multiple binding domains increases avidity and creates multi-specific binders (**Figure 1**). This has been achieved equally well with sdAbs, affibodies, DARPins, or Adnectins [46-49].

Conventional Ab-derived constructs (e.g., Fab_2, minibody) with molecular weights that remain above the renal cutoff have a longer blood half-life and imaging can only take place a longer time after injection to obtain good signal-to-background ratios [27]. Other conventional Ab-derived fragments such as scFvs (consisting of paired VH and VL domains connected with a linker) with molecular weight below the renal cutoff seem to be more difficult to produce in large amounts in a stable format. To increase the avidity, diabodies have been generated that contain two paired scFv constructs (**Figure 1**). Such 55-kDa molecules are at the borderline of renal clearance cutoff but seem to be preferred over scFvs for imaging [27].

Table 3 summarizes a selection of reported mouse xenograft imaging studies in which the breast cancer marker human epidermal growth factor receptor 2 (HER2) is targeted by various tracer types, including man-made protein scaffolds, sdAbs, conventional antibodies, and their engineered fragments. Of note, such comparisons should be interpreted with caution, since both tumor uptake levels and tumor-to-blood ratios are critically influenced by a wide number of parameters, including the type of labeling, tracer specific activity and affinity, the time-point of assessment, and the type of xenografted tumor. In this table we assembled representative studies for each individual tracer type and we focus, whenever possible, on early time-points after injection since these are clinically the most relevant.

Camelid sdAbs only show mediocre tumor uptake levels but generate excellent tumor-to-blood levels early after tracer administration, resulting in the generation of specific and high-contrast whole-body images of HER2 tumor-targeting (**Figure 3**). Tumor and tumor-to-blood values are equalized or even improved only by affibodies and DARPins. Antibodies in general efficiently accumulate in tumors but generate poor contrast, even at time-points much later than those used for smaller tracer types. Tracers based on Fab, Fab_2, scFv, diabody, and minibody, which are all engineered fragments from conventional antibodies, generate only poor contrast values at early time-points post-injection. These selected studies support the statements that are schematically shown in **Figure 1**.

2.3 Cross-reactive sdAbs for preclinical and clinical studies

A number of tumor imaging studies with sdAbs have been performed by growing human cancer cells in mice, the so-called mouse xenograft model (**Figure 3**) [14,42,50-56]. It should be realized that this puts severe limitations on the optimization of the imaging strategy and its translation into the clinic. If the sdAb does not recognize the endogenous mouse homolog of the human target, no uptake in off-site mouse organs will be present. However, using the same sdAb in a clinical setting where the human target antigen might also be expressed (at lower levels) in off-site organs will lead to background signals. Moreover, the expression of the target biomarker in other organs might prevent or reduce the uptake of the sdAb tracer at the target site. This so-called 'sink effect' has been described recently by imaging a mouse target antigen [21]. Since the sdAb described in this study fails to recognize the human homolog of the target biomarker, it needs to be substituted by another sdAb that targets the human protein for clinical testing.

As a consequence, it is preferred to select from the very beginning an sdAb that recognizes both the human and the animal homolog of the target biomarker. The performance

Table 3. Non-exhaustive overview of different imaging tracers targeting HER-2-positive tumors.

Tracer	Molecular weight (kDa)	Labeling type	Tumor model	Tumor uptake, %IA/g (h p.i.)	Tumor-to-blood, %IA/g (h p.i.)	Refs.
Affibody	7	^{99m}Tc/His_6	LS174T	8.7 (1 h)	9.1 (1 h)	[89]
		^{68}Ga/NOTA	SKOV3	6* (1 h)	8* (1 h)	[20]
DARPin	15	^{99m}Tc/His_6	SKOV3	9.12 (1 h)	12.67 (1 h)	[17]
Camelid sdAb	15	^{99m}Tc/His_6	SKOV3	4.19 (1.5 h)	16.4 (1.5 h)	[54]
		^{68}Ga/NOTA	SKOV3	3.13 (1 h)	9.51 (1 h)	[26]
scFv	30	^{99m}Tc/His_6	SKOV3	1.06 (1 h)	1.2 (1 h)	[90]
Diabody	55	^{18}F/SFB	MCF-7/HER2	2.87 (6 h)	1.83 (6 h)	[19]
Fab	55	^{99m} Tc/HYNIC	BT-474	10.4 (24 h)	3.2 (24 h)	[91]
Minibody	80	^{131}I/Iodogen	MCF-7/HER2	5.59 (12 h)	1.10 (12 h)	[92]
Fab_2	110	^{68}Ga/DOTA	BT-474	12* (3.5 h)	0.8* (3.5 h)	[93]
Antibody	150	^{89}Zr/N-SucDf	SKOV3	15.7 (24 h)	0.77 (24 h)	[94]

*Data derived from graphs presented in papers.

DOTA: 1, 4, 7, 10-Tetraazacyclododecanetetraacetic acid; HER2: Human epidermal growth factor receptor 2; HYNIC: Hydrazinonivotinamide; NOTA: 1,4,7-Triazacyclononane-1,4,7-Triacetic acid; N-SucDf: N-succinyldesferal-Fe; SFB: N-succinimidyl 4-[^{18}F]fluorobenzoate.

of such cross-reactive sdAb can be monitored and optimized preclinically in appropriate animal models to identify the best lead compound to translate into the clinic.

The preferential specificity of sdAbs to recognize conformational epitopes [57,58] makes it difficult to obtain cross-reactive sdAbs that associate equally well with the mouse and human biomarker. To maximize the chances of finding an sdAb that recognizes mouse-human homologs of a protein biomarker, special immunization and selection procedures are employed. Broisat *et al.* obtained cross-reactive sdAbs that recognize both the human and mouse vascular cell adhesion molecule 1 (VCAM-1) homolog by immunizing the camel and selecting the sdAb library from the immunized animal with both proteins. These sdAbs were successfully used in a mouse model of atherosclerosis to detect atherosclerotic plaques (**Figure 4**). In the future, the same cross-reactive sdAb can be translated to clinical settings [59].

In the absence of a human-mouse target cross-reactive sdAb, an alternative approach can be followed in which a human protein knock-in animal is generated. However, this is expensive and tedious. Conversely, for sdAbs that cross-react with both human and mouse target homologs, it might be expected that they also cross-react with homologs from other animals. Therefore, preclinical testing in multiple animal models is immediately offered without having to generate additional knock-in animal models.

2.4 Imaging techniques require dedicated labelings

It is well established that sdAbs are very stable in serum, and resistant to unfolding at elevated temperatures (typically, melting temperatures of sdAbs are between 60 and 80°C), in various solutions (e.g., stable in 6 – 8 M urea), and under stress conditions (pH ranging from 3 to 9; pressure at 500 – 750 MPa) [53,54,59-62]. Hence, the robust sdAbs tolerate numerous labeling strategies to meet the requirements for the various *in vivo* imaging modalities (**Table 2**). New labeling methods for nuclear imaging, radio-immunotherapy, and near-infrared (NIR) imaging are being developed for future clinical applications.

The sdAbs are labeled either directly on an AA residue or indirectly via a bifunctional chelator. Both direct and indirect labelings are performed randomly on naturally occurring AA residues of the sdAb. A typical example is the coupling of a bifunctional chelator (for radiometals) or a prostethic group (for radiohalogens) to lysines in the framework region. According to these approaches, the sdAbs are labeled with both short-lived isotopes for radio-imaging and long-lived isotopes for radio-therapy (**Table 2**). However, in cases where the reactive AA residue also occurs in or near the antigen-binding loops (i.e., the CDRs), random labeling might affect the binding capacity of the sdAb probe (unpublished data).

The introduction of a tag for site-specific labeling at the C-terminal end of the sdAb is a valid alternative to obtain a better, more controlled probe. The C-terminal end and the CDR loops are located on opposite sides of the domain so that the labeling at the tag prevents interference with target binding (**Figure 2**). Van de Broeck *et al.* describe a site-specific coupling method of an sdAb using maleimide chemistry on a cysteine residue that is introduced C-terminally [63].

Most sdAb-imaging studies have been performed with ^{99m}Tc-labeled probes and single photon emission computed tomography (SPECT). ^{99m}Tc labeling and subsequent SPECT imaging are straightforward and very useful to identify a lead compound out of different sdAb candidates [54,59]. In this labeling method a ^{99m}Tc-tricarbonyl precursor is bound on the imidazole groups of a hexahistidine tag via tricarbonyl chemistry. The hexahistidine stretch at the C-terminal end of the sdAb is also employed as tag to purify the protein by chromatography by immobilized metal ion affinity [64].

A recent study by Oliveira *et al.* describes *in vivo* optical imaging of a tumor with an NIR-fluorophore-labeled sdAb [56]. Further development of such optical tracers could lead to specific, intraoperative surgery tools that visualize the target region [65].

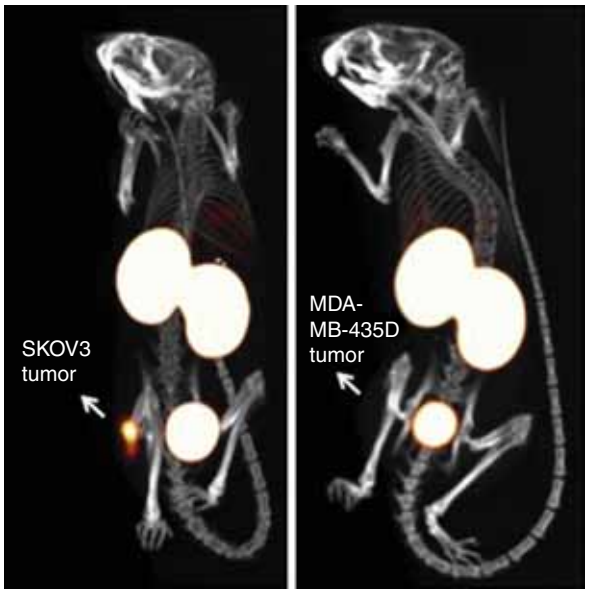

Figure 3. ^{99m}Tc-labeled camelid sdAb targeting tumors expressing the HER2 in a mouse xenograft model, 1 h p.i. The human cancer cell lines SKOV3 (HER2-positive) and MDA-MB-435D (HER2-negative) were injected subcutaneously in the right hind limb.

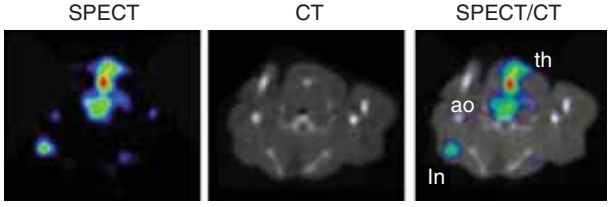

Figure 4. *In vivo*, non-invasive imaging of a mouse/human -cross-reactive sdAb targeting mouse VCAM-1 in an ApoE$^{-/-}$ mouse model of atherosclerosis, 3 h p.i. The fusion of SPECT and CT images localizes the molecular SPECT signal from the ^{99m}Tc-labeled sdAb on the anatomical CT image. On this transversal image, signals are visible in all tissues expressing VCAM-1: the aorta (ao) containing atherosclerotic plaques, the thymus (th), and the lymph node (ln).

2.5 Limitations for sdAb imaging

As discussed above, sdAbs show favorable pharmacokinetics as *in vivo* tracers for non-invasive imaging. However, sdAbs are not suitable for all possible imaging applications. For instance, high kidney signals with sdAbs require further attention and molecular targets located behind the intact blood-brain barrier (BBB) still remain a challenge for sdAbs as well.

2.5.1 Reducing renal uptake

It has been demonstrated that sdAbs bind their target rapidly and excess material is cleared very fast from blood, making imaging possible shortly after probe administration [26,42,50,54,55]. The rapid blood clearance of sdAbs through the kidneys is a double-edged sword resulting in kidney uptake values ranging between 100 and 300%IA/g a few hours after injection [54,59]. This high kidney retention puts limits on the detection of targets in organs such as pancreas that are located close to kidneys. Also, high radioactive exposure should be avoided when considering clinical applications. Apart from kidneys, the uptake of sdAb probes in other off-site organs such as liver or spleen is generally low unless the target is expressed in these organs [21,59].

Kidney uptake is a common problem for probes with a molecular weight below the renal threshold of glomerular filtration (60 kDa) [66]. Megalin was identified as an important receptor for sdAbs that are filtered in the glomeruli. Renal uptake in megalin-deficient mice was reduced by > 40%. The high renal uptake of radiometal-labeled sdAbs is partially blocked by a co-infusion of gelofusin and lysine, leading to improved tumor targeting of the probe [53].

The kidney uptake of sdAbs is dependent on the sequence of the sdAb, the linker or chelator used for radio-coupling, the type of radionuclide, and the injected mass [26,55,67]. When taking an sdAb tracer to the clinic, various format adjustments of the selected lead compound are required, as described by Xavier *et al.* [26]. First, the hexahistidine tag used for purification and labeling the different sdAb candidates should be removed because it is redundant for tracer functionality and adversely affects biodistribution [26]. Secondly, like all products for clinical administration, the sdAb should be produced and purified under Good Manufacturing Practices (GMP)-grade conditions. The availability of a hexahistidine tag-free, GMP-produced, and purified sdAb requires also the development of a PET-labeled analog for clinical PET imaging. Preferably, the positron-emitting radionuclide should have a short half-life to allow rapid sdAb imaging after tracer injection and to maximally limit radiation exposure [68]. Ga is a good option as it has the advantage of being available via a generator system without the need of a cyclotron and it can be used in combination with a clinical-grade NOTA chelator for protein coupling [68]. All these clinical adjustments have an implication on the renal clearance, resulting in kidney uptake values of 30 – 40%IA/g (instead of 100 – 300%IA/g) [26]. ^{18}F-labeled sdAbs reach even lower kidney uptake values [36].

2.5.2 sdAbs crossing the BBB

The small size of sdAbs, together with other favorable properties (e.g., stability and protease resistance), makes them conceivable as compounds crossing the BBB. Moreover, the absence of an Fc part predicts that they cannot be exported from the brain via the Fc-receptor-mediated efflux system like full-length Abs [69]. Drugs with a molecular weight exceeding the limit of free diffusion across the BBB (400 Da) need to be engineered to effectively pass the BBB by active transport [70]. Obviously, sdAbs (15 kDa) are too

large to cross the intact BBB freely without such modifications or specific selection procedures. Muruganandam *et al.* were the first to report a selection procedure for sdAbs crossing the BBB and also the putative transport mechanism was subsequently published [69,71]. Recently, other groups also reported sdAbs crossing the BBB *in vitro* and *in vivo,* which is a token for the increasing interest for such sdAbs [72-76]. Most of these studies are investigating these sdAbs for a therapeutic purpose. However, when considering *in vivo* brain imaging with sdAbs crossing an intact BBB, some conditions and limitations should be taken into account: i) administration of several milligrams of tracer by perfusion via the carotid of a mouse, as reported in a recent study [74], is not common for *in vivo* imaging studies. In most sdAb-imaging studies the tracer amount is about thousand times lower and administered by tail vein injections [26]. ii) Caljon *et al.* quantified the uptake of a radiolabeled sdAb in the brain using a intracerebral microdialysis method and the detected amounts were only about 0.0005%IA [72]. Nabuurs *et al.* confirmed that the sdAb uptake levels in the brain are too low for *in vivo* SPECT imaging [73]. iii) Several investigators use parasite infections or osmotic stress as models to detect sdAb probes in the brain [72-74]. These disease models disrupt the BBB which in turn increases uptake of the tracers. Iqbal *et al.* [77,78] used sdAb-based probes to successfully image brain tumors in mice, which probably also had an impaired BBB.

Multiple investigators propose to increase the blood circulation time to improve BBB penetration [72,73]. Possible approaches include adjustment of the molecular size of the probe above the threshold of glomerular filtration (60 kDa), incorporation of an sdAb binding to albumin, or PEGylation of the sdAb (**Figure 1**). These are already well-known methods for therapeutic applications in general [78-81], but future will show whether they have an added value for brain imaging. Increasing the blood retention time will probably improve the absolute uptake in the brain, but it will also increase the background signal in blood.

To conclude, limited amounts of sdAbs might indeed cross the BBB and dedicated selection methods can be implemented to obtain better-performing sdAbs [71-74]. However, so far the sdAb amounts diffusing in the brain are too low to allow reliable non-invasive *in vivo* imaging [72,73]

3. Expert opinion: the future of sdAbs in clinical molecular imaging applications

Camelid sdAbs are readily obtained as monomeric target-binding units against a wide variety of biomarkers (**Table 2**). These stable and soluble sdAb probes bind their antigen with high affinities and specificities. The molecular weight of sdAbs is well below the renal cutoff of glomerular filtration allowing imaging within hours after injection. Various labeling methods can be employed, according to the preferred imaging application. All these properties make camelid sdAbs ideally suited as *in vivo* molecular imaging probes for both preclinical and clinical applications. The first clinical trial with a camelid sdAb as a molecular imaging tracer targeting the breast cancer marker HER2 is currently ongoing (EudraCT 2012-001135-31). Additional sdAbs are in the pipeline, and these will be investigated in the near future with diverse objectives toward molecular imaging applications, both in preclinical and clinical settings:

Preclinical in animal models:

1) *The sdAb as scientific research tool* to understand pathogenic mechanisms and molecular pathways involved in animal disease models: it may provide insights in the role of a protein or cell type in particular diseases by locating and tracking the biomarker or cell in the body of a living animal model.
2) *The sdAb as an early decision-making tool* for disease biomarkers as well as its targeting compounds in both imaging and therapy applications: the *in vivo* expression of the target protein both at the target sites and at the non-targeting sites can be evaluated using molecular imaging. The presence of the target in non-targeted sites could have implications for the possible toxicity of the compound. The specificity of the compound binding to the target can also be investigated *in vivo*.

In the clinic:

1) *The sdAb as a whole-body distribution tool* for a targeted therapeutic in clinical trials. The *in vivo* expression pattern of the target or the biodistribution of the therapeutic compound itself can provide valuable information regarding possible adverse events.
2) *The sdAb as a patient stratification tool*. Some patients are more likely to respond to a particular therapy than others, according to the presence of biological markers throughout the body. Pharmaceutical companies are faced with huge costs and increasing bureaucracy for clinical studies. Most of the drugs entering clinical trials do not get market approval. It is becoming increasingly important to identify upfront, patient populations for these trials into possible responders and non-responders. Screening for the presence or absence of particular biomarkers in patients will select the appropriate patient population for which the drug could then be approved more easily.
3) *The sdAb as a companion diagnostic of a therapeutic* helping to make a therapeutic decision in the clinic:
   a) *Diagnosis/prognosis*. Early diagnosis of a particular molecular event or biomarker can help to select patients eligible to a therapeutic regimen targeting this event or biomarker. In case the molecular event or biomarker is associated with a prognostic value,

imaging might also determine the optimal treatment procedure even if it does not target the molecular event itself.

b) *Treatment evaluation*. The imaging diagnostic can be used to follow up patients who are under treatment to evaluate the response to therapy. In this case a molecular probe should be used that does not compete with the binding of the actual drug so that the therapeutic compound does not hinder binding of the probe.

4) *The sdAb as a radio-immunotherapy assistance tool for*:
   a) *Dose determination*. Whole-body imaging can help to find the balance between high radioactive uptake in the tumor while keeping the radioactive exposure to the rest of the body as low as possible.
   b) *Image-guided radiotherapy*. To delineate tumor zones that need different radiation exposure. Imaging hypoxic parts that are more resistant to radiotherapy could define regions where a higher radiation dose is needed, while limiting the exposure to the surrounding tissues.
5) *The sdAb as an image-guided surgery tool:* A typical application for optical molecular imaging has been proposed whereby a fluorescently labeled probe can visualize tissues that express a particular marker. This is for instance very helpful to ensure that the entire tumor tissue expressing a particular marker is removed during surgery.

Whole-body, non-invasive molecular imaging is complementary to possible alternative detection methods like immunodetection on biopt samples. This standard but invasive method does not provide any information about other sites in the body where no sample was taken. The genetic screening is another diagnostic method; however, that is not available for all diseases and does not provide local information such as the location of tumor lesions throughout the body.

A cost/benefit analysis should be performed before taking a new molecular imaging tracer to the clinic. The cost of the tracer and the imaging procedure is one driver; however, the expenses related to hospitalization and drugs are another, especially in fields where molecular imaging can be of great help, such as (e.g.) oncology and cardiology. Hence, molecular imaging could reduce part of these costs through early, whole-body imaging of the disease. Molecular imaging could also be helpful in reducing costs during therapeutic drug development, mainly by increasing the success rate of approval. It can assist in the selection of new drug candidates and in better screening and follow-up of patient groups in clinical trials.

In comparison with therapy, non-invasive, *in vivo* imaging can be performed using a small tracer dose (microdosing). As a result the authorities require less stringent toxicity studies. Since there is a need for specific imaging tracers, but few are on the market yet, relatively fast progression toward clinical translation would be possible.

Declaration of interest

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