Expert Opinion

Tailoring antibodies for radionuclide delivery

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Therapeutic antibodies are well established as an important class of drugs in modern medicine. The exquisite specificity and affinity for a specific target offered by antibodies has also encouraged their development as delivery vehicles for agents such as radionuclides to target tissues, for radioimmunoimaging and radioimmunotherapy. Specifically, in nuclear medicine, radionuclide-conjugated antibody molecules make it possible to image diseased loci with greater sensitivity than other imaging modalities such as magnetic resonance imaging. Furthermore, two radionuclide-conjugated antibodies have recently been approved for the therapy of non-Hodgkin's lymphoma. However, optimal implementation of antibodies has been limited by the extended circulation persistence that is characteristic of native antibodies, which is responsible for increased background activity in radioimmunoimaging applications and dose-related normal organ toxicities in radioimmunotherapy. In this article the current status of radiolabelled intact antibodies is reviewed, focusing on strategies to improve their pharmacokinetic properties to suit a desired application. Examples from the literature that represent different approaches to accomplishing this task in terms of their successes as well as limitations, and perspectives for the future are discussed.

Keywords: pharmacokinetics, pretargeting, radioimmunoimaging, radioimmunotherapy, radiolabelled antibodies and antibody fragments

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1. Introduction

At present, modern medicine is at a state where a new class of diagnostics and therapeutics capable of differentiating between diseased and normal tissue with exquisite specificity, are within reach. These entities are termed molecular targeting agents and are extensively reviewed by Britz-Cunningham and Adelstein [1]. Antibodies and antibody fragments are key examples of molecular targeting agents, in addition to other classes of molecules including kinase inhibitors, small antisense molecules, aptamers, peptides and chimaeric fusion molecules. In contrast to physiological tracers, molecular targeting agents accumulate in target tissues *in vivo* by interacting specifically with a molecular species that is abundant in a disease state compared with the normal tissue. In the case of antibodies and antibody fragments, the molecular targets are generally cell surface molecules. These can include mutated gene products as well as proteins with normal structure, which are aberrantly expressed in the diseased tissue. Critical for the accumulation of antibodies within cells or tissues where the target antigen is expressed, are the specificity and affinity of antibodies for the target molecule, and the density of antigen epitopes. In addition, because antibodies are bivalent molecules, bivalent binding can lead to increased functional affinity if the density and geometry of available epitopes are favourable.

A variety of cargoes can be attached to molecular targeting agents for tissue-specific delivery *in vivo*, including radionuclides, paramagnetic particles, fluorescent dyes and particles, for detection by imaging modes such as γ-camera, single-photon

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emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging and optical imaging. Molecular targeting can also be utilised for delivery of therapeutic payloads, including therapeutic radionuclides, photodynamic therapy agents, and liposome or nanoparticle encapsulated drugs [2,3]. Radioisotopes are especially attractive for attachment to molecular targeting agents such as antibodies, and are the focus of this review. Radionuclides that emit single photons, detectable by γ-cameras or SPECT, are widely used in medical imaging. Radioisotopes such as ^{99m}Tc, ^{123}I and ^{111}In are essentially pure γ-emitters with half-lifes of 6, 13.2 and 68 h, respectively, and are energies readily detected by the cameras used in hospital nuclear medicine departments. Thus, it is no surprise that these are the radionuclides that have commonly been conjugated to antibodies for radioimmunoimaging (RII). More recently, imaging of isotopes that emit positrons during decay, such as ^{18}F, ^{64}Cu and ^{124}I (half-lifes 109 min, 12.7 h and 4.2 days, respectively), has garnered increasing interest as the availability of clinical PET scanners has improved. The development of dedicated small animal scanners (such as micro-SPECT and micro-PET imagers) has accelerated preclinical investigations. Radionuclides that emit hard β-particles capable of killing tumour cells, including ^{131}I and ^{90}Y (half-lifes 8 days and 64 h, respectively), remain the mainstay of investigations of radioimmunotherapy (RIT). The emission path length of ^{90}Y is relatively long (mean range 275 µm, maximum range of 500 – 600 µm) and its best application may be the treatment of bulky lesions. ^{131}I together with other β-emitters, such as ^{67}Cu and ^{177}Lu (half-lifes 61 h and 6.8 days, respectively), are characterised by a shorter distance of penetration (< 200 µm) and are suitable for treatment of small-volume and disseminated disease. In addition to β-emitters, therapeutic nuclides producing α-particles, such as ^{212}Bi, ^{213}Bi and ^{211}At (half-lifes 1 h, 45.7 min and 7.2 h, respectively), have also shown promise in RIT. Their high-energy particles (4 – 9 MeV) and short-distance penetration (40 – 100 µm) are more appropriate for addressing single-cell metastatic disease, leukaemias and highly vascularised tumours [4,5]. Auger emitters, such as ^{67}Ga, ^{195m}Pt, ^{123}I and ^{125}I (half-lifes, 3.3 days, 4.0 days, 13.2 h and 60.1 days, respectively), cause potent cytotoxicity and are mostly limited to the eradication of microscopic residual disease [6,7].

The use of intact antibodies in RII and RIT poses several challenges, which need to be addressed. As large globular proteins (molecular weight 150 kDa), antibodies demonstrate poor tumour penetration, especially in the case of solid tumours with high interstitial pressure or those protected by the blood–brain barrier [8]. In addition, the slow kinetics of antibody uptake and blood clearance results in the generation of images obscured by high background, and/or increased bone marrow and normal organ toxicity. The question of how one can exploit the benefits of antibodies and avoid the limitations associated with their use comes to mind. In this article, the use of antibodies and antibody fragments as molecular targeting agents in RII and RIT is reviewed: what is their current clinical implementation; what modifications and strategies are now used for tailoring antibody targeting, pharmacokinetics and biodistribution; what obstacles of antibodies and antibody fragments in disease targeting are being encountered; and finally, what lies in the future?

2. Clinically available radiolabelled antibodies

2.1 Radioimmunotherapy

Significant effort has been applied to the development of antitumour antibodies for RIT [9-11]. Beginning in the 1980s, mAbs began to establish themselves as an important class of therapeutic drugs, and progress and registrations have accelerated in recent years. At present, there are 17 antibodies approved by the FDA, 6 of them indicated for malignant disease, including two conjugated to radionuclides [201]. Conjugation of a mAb to a radionuclide can overcome some of the limitations inherent to unconjugated or 'cold' antibodies. In order for cold antibodies to produce a cytotoxic effect, they need to either interact directly with target cells and induce a biological response, or effectively engage with a functional host immune system. Patients treated with mAbs are often immune compromised either due to their current disease or as a side effect of previous therapy, thus limiting the ability to recruit host immune responses. Biologically active antibodies must directly contact every target cell, a process that can be restricted by their large size and limited ability to penetrate tumour tissue. In contrast, radioactivity, specifically delivered to the tumour site by the antibody, can kill cells deeper within the tumour, which are otherwise not accessible to cold antibodies due to their poor tissue penetration. This 'field' effect of regional killing induced by the local delivery of radiation is also critical in cases where antigen expression is heterogeneous; a common feature in cancer. Other cargoes attached to antibodies include drugs (e.g., doxorubicin, calicheamicin, DM1) and toxins (e.g., ricin A). Gemtuzumab ozogamicin (Mylotarg®, Wyeth/Celltech Group) is a representative of the drug immunoconjugate group, which has been approved by the FDA for the treatment of acute myeloid leukaemia. However, the effectiveness of immunoconjugates is generally limited by labile linkers, low drug potency and normal tissue crossreactivity of the mAb in some cases. Although progress has been made with the introduction of peptide conditionally stable linkers instead of chemically labile functional groups, further improvement of drug immunoconjugates is required for future clinical success [2]. Importantly, the advantage of RIT over conventional radiotherapy, chemotherapy and immunotherapy is the potential to control the delivery of a patient-specific dose or radiation to the tumour with minimal toxicity to normal organs. Thus, RIT has been extensively investigated and in some clinical settings has demonstrated higher response rates than those achieved with unconjugated antibodies [12-15].

An antibody radioconjugate recently approved by the FDA for the treatment of relapsed/refractory B-cell non-Hodgkin's lymphoma (NHL) is ^{90}Y-labelled ibritumomab-tiuxetan

(Zevalin®, IDEC Pharmaceuticals Corp.). It is a combination of the CD20-antibody ibritumomab (the parental murine antibody of rituximab [Rituxan®, Genentech, Inc.]) linked to tiuxetan, a chelating agent used for labelling with ^{90}Y. The therapeutic ^{90}Y radioisotope is a β-emitter with a half-life of 64 h and penetration in soft tissue ranging 1 – 5 mm [16]. In a clinical trial, in which patients were treated with ibritumomab, data analysis showed an overall response rate of 80%. Moreover, ibritumomab treatment of rituximab-refractory disease gave an overall response rate of 46% [17].

Another FDA-approved RIT agent is ^{131}I-labelled tositumomab (Bexxar®, Corixa Corp.), which also targets CD20-positive cells (B-cell NHL). The high-energy β-particles emitted by the ^{131}I radioisotope with an 8-day half-life are cytotoxic over distances of ~ 1 – 2 mm [16,18]. In clinical trials of tositumomab, objective response rates ranged 54 – 71% in heavily pretreated patients, and 97% with 63% achieving complete response in newly diagnosed patients [19].

Although ibritumomab or tositumomab treatment of B-cell lymphomas is effective in inducing cell death, the acute as well as potential long-term side effects of the treatment must also be weighed. The main toxicity and dose-limiting side effect associated with RIT is myelosuppression caused by the radiation from the antibody, which can be directly bound to lymphocytes and tumour cells in the bone marrow, and also represents a circulating source of radiation in the blood. Late effects include reports of therapy-related myelodysplastic syndrome and the restriction of future therapeutic administration due to irreversible stem cell and stromal damage caused by radiation [19,20]. One strategy to reduce radiation exposure was the development of both ibritumomab and tositumomab as murine (as opposed to chimaeric or humanised) antibodies. Increased immunogenicity was projected to promote rapid clearance from the circulation, thus reducing radiation side effects. The drawback of this approach is that > 50% of patients develop human anti-mouse antibody (HAMA) response, which limits therapy to a single administration [21-23]. Another strategy was to increase the administered activity and tumour dose, which leads to a myeloablative effect that can be subsequently corrected by autologous stem cell rescue [24,25].

RIT treatment of solid tumours, which are radioresistant in comparison to lymphomas, presents additional challenges because higher tumour doses are needed to achieve objective responses [26,27]. However, administration of higher amounts of activity will also result in increased red marrow toxicity, which further limits the efficacy of the administered RIT. Thus, the lack of an FDA-approved radioimmunotherapeutic agent for solid tumours is not a surprise. Some Phase I and II clinical studies have shown promising results of applying RIT to small-volume disease and in an adjuvant setting [28,29]. A Phase II clinical trial of RIT using the anti-carcinoembryonic antigen (CEA) ^{131}I-labelled labetuzumab, after salvage resection of colorectal metastases to the liver, has demonstrated a median overall survival of 5 years and 8 months, and a median disease-free survival of 1 year and 6 months [29]; a significant improvement over historical and contemporaneous controls.

It is clear that new approaches to increase the clinical efficacy of radiolabelled anticancer mAbs are needed. A promising possibility lies in engineering antibodies with controlled targeting, distribution, pharmacokinetic and clearance properties to optimise their use. Several recent excellent reviews have focused on the evaluation of engineered antibody fragments [30-32] in both preclinical and clinical settings.

2.2 Radioimmunoimaging

As the most sensitive noninvasive modality presently available, imaging using radioactively labelled probes plays an important role in the detection and analysis of disease. As is the case in other molecular imaging applications, RII requires concentration of the radiation-emitting antibody at the targeted cells or tissue site. Antibodies are particularly good for specific delivery of radioisotopes to the target because of their superior antigen specificity and affinity. There are several examples of antitumour antibodies labelled with single-photon emitting isotopes for clinical γ-camera or SPECT imaging. These include [^{111}In]satumomab pendetide (Oncoscint®, Cytogen Corp.), which is specific for the mucin-like surface glycoprotein TAG-72; [^{111}In]capromab pendetide (Prostascint®, Cytogen Corp.), which targets the prostate-specific membrane antigen; and [^{99m}Tc]arcitumomab (CEA-Scan®, Immunomedics, Inc.), which binds cell membrane-bound CEA.

To be clinically useful, an imaging application needs to produce high-contrast images within a reasonable amount of time following administration of the tracer. Herein lies the major technical problem with imaging using intact, native antibodies. Specifically, the inherently slow kinetics of tumour uptake and blood clearance leads to high background activity and requires a long interval (hours to days) before clear images can be obtained. In addition, most radionuclides, commonly used in clinical imaging, have relatively short half-lifes (for patient safety and practical issues), which limits the imaging strategies that can be employed. Other concerns include HAMA response [33] (which can be reduced or eliminated by the use of chimaeric human/mouse, humanised or fully human antibodies), crossreactivity with normal tissue or nonspecific uptake, hepatic and/or renal uptake, and metabolism of the radiolabelled protein with loss of radiolabel at the target site. Some of these shortcomings can be modified through engineering antibody fragments with faster target uptake kinetics and blood pool clearance while retaining their favourable target binding and avidity.

3. Strategies for improving antibody radionuclide delivery

3.1 Engineered antibody fragments for direct labelling approaches

The generation of mAbs and fragments with specificities for a variety of tumour-associated antigens has been accelerated

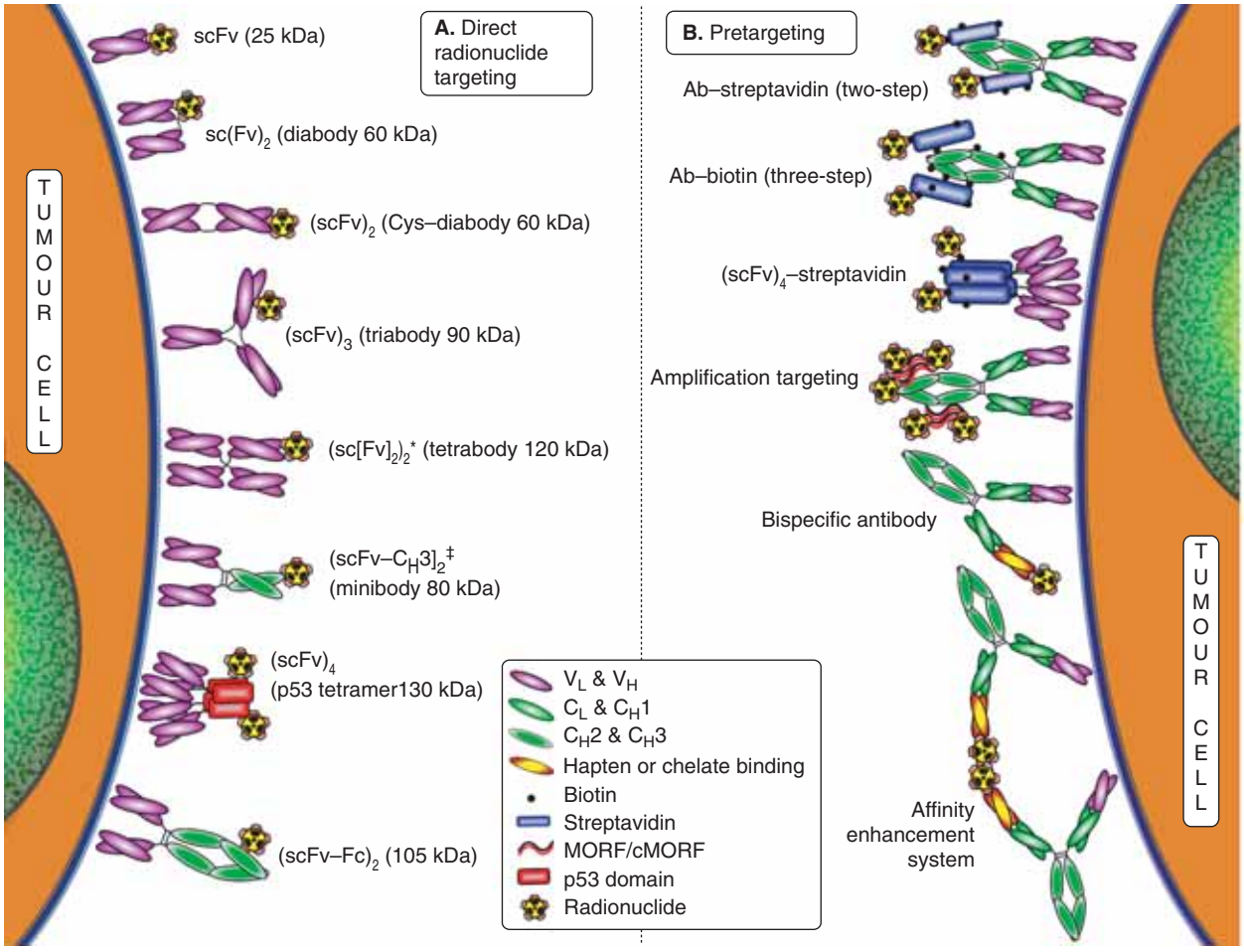

Figure 1. Strategies for improving the efficacy of radiolabelled antitumour antibodies. A. Direct radionuclide targeting. Direct radiolabelling of antibodies entails their covalent linkage to cytotoxic or imaging radionuclides prior to administration. Engineered mono-, di- and multivalent antibody fragments include scFv, diabodies (assembles both covalently and noncovalently), tria- and tetrabodies, minibodies with variable dimerisation domains, tetramers and scFv fused to the entire antibody Fc region. **B. Pretargeting.** Pretargeting strategies aim for the selective delivery of radionuclides to tumours, thereby diminishing the systemic toxicities of these cytotoxic agents. For radionuclide pretargeting, an Ab–streptavidin conjugate is allowed to accumulate within a tumour and is then used to capture a biotin–chelator–radionuclide complex (two-step). In three-step pretargeting, an Ab–biotin conjugate binds to the tumour and is then followed by streptavidin and radiolabelled biotin. Two-step pretargeting can be optimised by the use of engineered tetravalent single chain variable fragment fused to streptavidin ([scFv]$_4$–streptavidin). Amplification pretargeting uses the concept of complementarity of oligos (MORF and cMORF) to enhance the radionuclide signal from the tumour site. Bispecific antibodies that bind to two different antigens (tumour and hapten) can be pretargeted to the tumour before delivery of the cytotoxic hapten-captured radionuclide. An improved version of the latter is the affinity enhancement system, where a bivalent hapten is used to connect two bispecific antibodies on the tumour surface, thereby improving their functional affinity.

*Both covalently and noncovalently dimerised tetrabodies have been developed. ‡Variations to IgG1 C_H3 domain (minibody) include IgE C_H4 (SIP) and helix-loop-helix (miniantibodies).

Ab: Antibody; C_H: Constant heavy chain; C_L: Constant light chain; cMORF: Complementary morpholino oligonucleotide; Fc: Crystallisable fragment; scFv: Single-chain variable fragment; MORF: Morpholino oligonucleotide; V_H: Variable heavy chain; V_L: Variable light chain.

by progress in recombinant DNA technology. The traditional method for isolating target-specific antibody variable (V) genes is from murine hybridomas producing tumour-specific mAbs. Alternatively, V genes can be selected from large natural or synthetic libraries using phage [34,35], bacterial [36] or yeast [37] display as well as *in vitro* display on ribosomes [38]. Once specific V genes are cloned, they can be expressed in the context of different antibody formats, depending on the properties desired (e.g., affinity, specificity, stability, half-life). Smaller antibody fragments, such as Fab′ (produced by enzymatic cleavage from an intact antibody) and single-chain variable fragments (scFv; variable heavy [V_H] and variable light [V_L] chains, genetically connected through a flexible linker; Figure 1A) have been shown

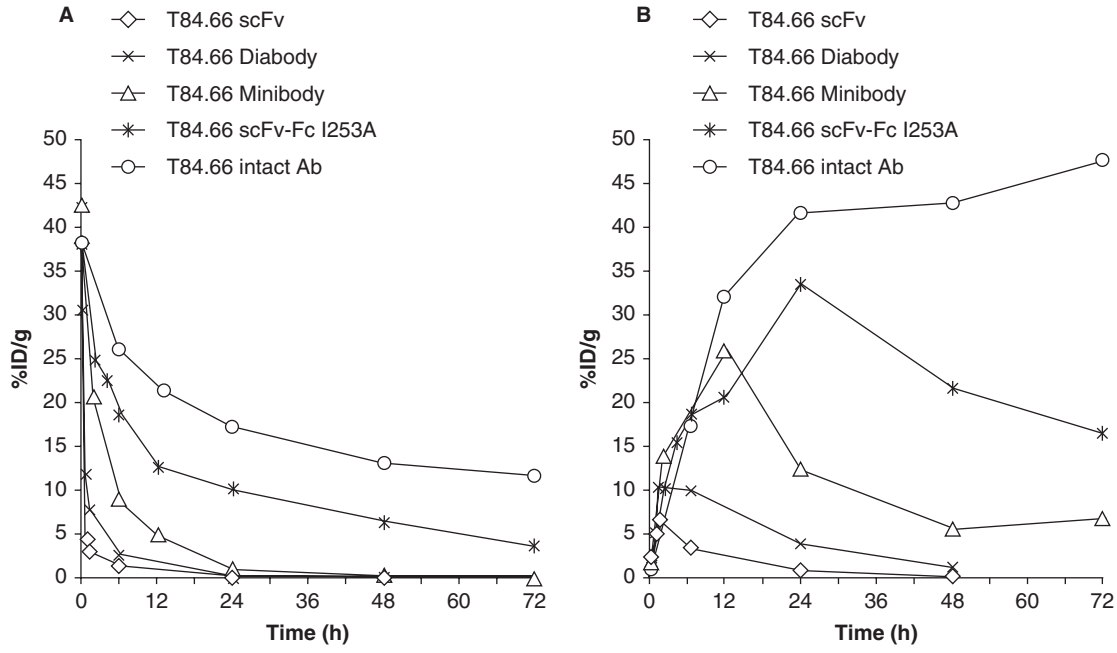

Figure 2. Effect of antibody format on blood and tumour kinetics of various engineered anti-CEA T84.66 antibody fragments in athymic nude mice bearing LS174T human colon adenocarcinoma xenografts. A. Blood clearance curves represented as normalised radioiodine activity in the serum as a function of time. **B.** Tumour uptake, expressed as percentage injected dose per gram of radioiodine-labelled antibody fragments. Data used for the generation of each curve was gathered from the works of Wu *et al.* [63], Yazaki *et al.* [76], Williams *et al.* [133] and Kenanova *et al.* (V Kenanova, unpublished data).
Ab: Antibody; CEA: Carcinoembryonic antigen; Fc: Crystallisable fragment; ID: Injected dose; scFv: Single-chain variable fragment.

to be superior in their ability to extravasate and penetrate solid tumours *in vivo*, when compared with intact antibodies [39,40]. However, because these fragments are monovalent they have lower avidity and overall affinity for the antigen than intact antibodies and multivalent antibody fragments. In addition, due to their small size (~ 27 kDa for scFv) and rapid circulation clearance, the time of exposure to the tumour is diminished. Renal uptake further limits these forms of mAbs. To enhance the desired affinity and stability of scFv molecules, methods for introducing greater diversity into the scFv selection libraries have been further developed. These include random mutagenesis using the error-prone polymerase chain reaction and/or chain shuffling [41], homology modelling, sequence analysis and statistical approaches [42-45], which all aim to enhance the scFv affinity and stability.

Beyond enhancing the scFv properties, targeting efficacy of antibody fragments can be improved by the use of multimeric formats with higher avidity and affinity for the antigenic target (Figure 1A). Efficient multimerisation can be accomplished by shortening or deleting the flexible interdomain peptide linker in scFv fragments. These engineered alterations result in dimeric, trimeric and tetrameric scFv molecules [31]. The dimeric molecules, also known as 'diabodies', have demonstrated stability under *in vivo* conditions and improved the enrichment of tumour xenografts when compared with their scFv counterparts [46,47]. Both the trimers [48-51] and tetramers [52-54] still require additional *in vivo* characterisation to confirm their feasibility for tumour imaging or therapy applications. Examples of generating higher molecular weight species are many, and include the fusion of scFvs to various protein domains capable of multimerisation; for example, amphipathic helices [55], leucine zippers [56], light-chain κ-constant domains [57] and helical linkers [58]. It is important to realise that because parameters such as serum clearance, targeting, tumour retention and elimination are interrelated and dependent on antibody valency, structure and molecular size, there is always a compromise when antibody fragments are engineered for particular *in vivo* applications.

3.1.1 Preclinical studies

3.1.1.1 Single-chain antigen-binding proteins (scFv)

Numerous animal studies using radiolabelled scFvs (Figure 1A) against various targets have demonstrated the ability of these molecules to target the tumour and rapidly clear from the circulation (Figures 2A and 3A), thus producing high tumour:blood and tumour:normal tissue ratios [39,54,59-62]. Disappointingly, the tumour uptake and retention achieved is uniformly quite low (Figures 2B and 3B). Recombinant scFv fragments made from the anti-p185^{HER2} C6.5 and 4D5

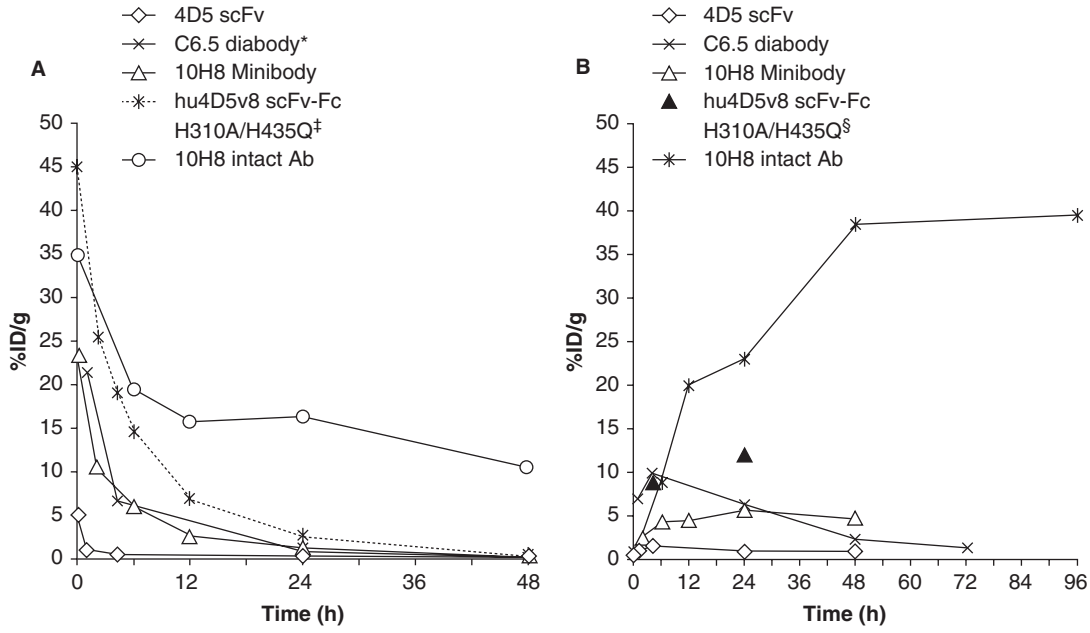

Figure 3. Comparison of the relationship of antibody format to blood and tumour kinetics of various engineered anti-HER2 recombinant antibody fragments in mice bearing HER2-positive xenografts. A. Blood clearance curves. **B.** Tumour uptake. Curve data were extracted from the works of Willuda *et al.* [54], Adams *et al.* [46], Olafsen *et al.* [84], Kenanova *et al.* (V Kenanova, unpublished data), and Olafsen *at al*. [85].

*The C6.5 diabody blood curve overlap with the minibody could be due to the lack of more intermediate time points between 4 and 24 h. ‡The blood curve for the scFv-Fc fragment is hypothetical and uses the T84.66 scFv-Fc fragment clearance kinetics data. This assumption was based on the closely overlapping 10H8 and T84.66 minibody as well as the 10H8, hu4D5v8 and T84.66 intact antibody clearance curves. Hence, the same antibody format possibly results in similar blood clearance profile. §Only two tumour uptake values were determined from a ^{64}Cu imaging study with the hu4D5v8 scFv-Fc fragment.
Ab: Antibody; Fc: Crystallisable fragment; ID: Injected dose; scFv: Single-chain variable fragment.

(Figure 3) mAbs demonstrated similar pharmacokinetics and tumour retention (~ 1% injected dose per gram [%ID/g]) in mice bearing p185^{HER2}-positive tumour xenografts [54,62]. Other scFvs, derived from B6.2, CC49, 741F8 and T84.66 (Figure 2) mAbs, and the MFE-23 and L19 scFvs, with specificities for human breast tumour metastases [59], pancarcinoma antigen TAG 72 [39], p185^{HER2} [60], CEA [61,63] and fibronectin ED-B domain [47], respectively, demonstrated slightly higher tumour uptake, ranging 3 – 5 %ID/g at 1 – 4 h postinjection. Comparative pharmacokinetic studies in athymic mice revealed more rapid plasma clearance of scFv than of the corresponding Fab′ fragments, as well as an extremely rapid whole-body clearance. Despite their rapid clearance, the scFvs show tumour uptake comparable to that of the Fab′ fragment, resulting in tumour to normal tissue ratios equal to or greater than those obtained with the Fab′ fragment. In addition, the clearance of radiolabelled scFvs occurs through the kidneys. This observation is consistent with the established renal filtration molecular weight cutoff of ~ 60 kDa, below which proteins are filtered by the glomerular basement membrane, and lysosomal degradation occurs after tubular reabsorption [64]. This raises concerns regarding nephrotoxicity if radiometals are used for scFv labelling, as the radiometal-labelled metabolites remain trapped in the lysosomal compartment for extended periods of time, thus irradiating the kidney tissue [65]. Taken together, these preclinical animal studies suggest that although scFvs are able to localise at the tumour site effectively, there is generally low tumour uptake and retention, accompanied by high kidney activity, which limits potential clinical use of scFvs. Instead, scFvs have found use as building blocks for the generation of multivalent molecules, which are characterised by increased molecular weight, longer circulating half-lifes and reduced renal uptake. These characteristics should contribute to improved tumour accumulation and, together with multivalent binding to the antigen, could prove more effective in RIT and RII.

3.1.1.2 Multivalent scFv constructs

Increasing valency is an effective way of improving the functional affinity of an antibody to its antigen. Multivalent scFvs (Figure 1A) exhibit increased affinity compared with monovalent forms [55]. They are also generally characterised by longer elimination half-lifes when compared with scFv fragments [32,60]. These molecules also offer an improvement over both intact IgGs and scFvs in tumour *in vivo* targeting, thus allowing for fast blood clearance, rapid uptake and retention in the tumour. Thus, radiolabelled multivalent scFvs are expected to show enhanced performance as imaging and therapeutic agents. Different strategies for scFv

multimerisation have and are being explored, all of them aiming to optimise tumour uptake and clearance profiles. The most straightforward approach for the production of multimeric scFvs is based on the spontaneous formation of noncovalently associated dimers, such as the 50-kDa diabodies ($[scFv]_2$) [66], trimers (triabodies) or tetramers (tetrabodies) with shortened or no additional linker peptide between the V_L and V_H chains (Figure 1A) [31,48,49]. The addition of a cysteine at the C terminus of the scFv results in the production of two covalently linked scFvs via a site-specific dimerisation ($sc[Fv]_2$) [60]. Other strategies for the promotion of multimer formation include fusion of the homodimerisation human constant immunoglobulin-γ1 C_H3 constant domain, the C_H4 domain of human IgE or even the entire Fc (γ1 C_H2 and C_H3 constant domains) antibody region to the C terminus of the scFv polypeptide chain to produce a minibody, 'small immunoprotein' or scFv-Fc molecules, respectively. By using this strategy, very promising tumour targeting data have been reported [67-71].

3.1.1.3 Diabodies

Diabodies (Figure 1A) are the most extensively characterised group and have demonstrated high antigen affinity, increased tumour retention and blood clearance slower than that of their scFv counterparts [31]. Biodistribution studies using a cysteine-linked divalent anti-$p185^{ERB-2}$ 741F8 $sc(Fv)_2$ showed a twofold improvement of *in vivo* tumour localisation compared with the corresponding monomeric scFv [60,72]. The importance of high-avidity target binding for improved tumour uptake was demonstrated by the radioiodinated scFv(L19) dimer. This fragment exhibited fourfold (20.23 %ID/g) and sevenfold (11.78 %ID/g) higher tumour uptake at 4 and 24 h postadministration, respectively, in F9 murine teratocarcinoma grafted nude mice, when compared with the monomeric L19 scFv [47]. In a different study, the CC49 covalent $sc(Fv)_2$ dimer showed tumour uptake > 6 %ID/g, 30 min after administration, which remained at this level for 6 h [58]. High tumour uptakes were reported in animal studies where CC49 noncovalently linked $(scFv)_2$ generated in yeast and bacteria showed equivalent tumour targeting and achieved 12.5 and 11.4 %ID/g, respectively, at 6 h postinjection [73]. Impressive results were also demonstrated by three diabodies based on affinity mutants of the human anti-$p185^{ERB-2}$ scFv molecule C6.5 [46] (Figure 3). Biodistribution studies, performed in severe combined immunodeficient mice bearing SKOV-3 tumours, showed 3- to 37-fold more diabody retained in the tumour at 24 h compared with the parental scFv monomers [74]. Wu and colleagues demonstrated excellent targeting by both radioiodinated (Figure 2) and radiometal-conjugated $(scFv)_2$ forms of the anti-CEA T84.66 scFv, with tumour uptakes ranging 5 – 15 %ID/g compared with the monomeric form (1 – 5 %ID/g) [75,76]. A covalently disulfide-linked version of the same diabody, designed to allow site-specific conjugation and radiolabelling for tumour targeting applications, showed similar behaviour *in vivo* [77].

In addition to the numerous biodistribution studies of various diabodies demonstrating improved pharmacokinetic properties compared with scFv fragments, an animal therapy study investigating the potential of C6.5 diabody in RIT of breast carcinoma was undertaken by Adams *et al.* [78]. A single dose of 150 µCi of ^{90}Y-labelled C6.5 diabody resulted in significant inhibition of the established MDA-361/DYT2 tumours, whereas a higher dose of 200 µCi produced complete responses lasting > 1 year in 25% of the treated mice. Thus, the diabody molecules can be used as targeting vehicles for the RIT of solid tumours. However, the radiometal conjugated diabody preparations also exhibited high renal retention, and long-term follow up in three non-tumour-bearing mice indicated a spectrum of kidney damage including increased serum creatinine levels, early-stage renal disease in one mouse, and severe renal damage in a second mouse. This observation will complicate further RIT investigations with diabodies, which, like scFvs, are below the cutoff molecular size for renal clearance.

Because of their improved tumour residence time, compared with scFvs, and rapid clearance kinetics, compared with intact antibodies and larger fragments, antitumour diabodies are favourable agents for RII [77,79,80]. Small animal PET imaging experiments using T84.66 ^{124}I-labelled diabody $(scFv)_2$ [79] showed specific localisation to CEA-positive LS174T xenografts as early as 4 h after tracer injection with relatively low activity elsewhere in the animals (Figure 4D). Impressively, CEA-positive tumours, as small as 11 mg, were visualised with high sensitivity and clarity. Similar tumour-targeting kinetics was observed in micro-PET studies with the Cys-diabody $sc(Fv)_2$, conjugated with the macrocyclic chelate 1, 4, 7, 10-tetraazacyclododecane-*N*, *N′*, *N″*, *N‴*-tetraacetic acid (DOTA) and labelled with ^{64}Cu [77]. However, this particular protein–chelate–radionuclide combination resulted in elevated liver and kidney activities. In a different setting using a clinical PET scanner, the ^{124}I-labelled anti-HER2/neu C6.5 diabody was used to image and quantitate uptake effectively in tumour xenografts as small as 0.1 g (SKOV-3) or 0.3 g (MDA-361/DYT2) [80]. Similar to the anti-CEA T84.66 diabody [79], clear images of the HER2/neu-positive tumour masses were generated at 4 h post-^{124}I-labelled C6.5 diabody administration, with tumour to background ratios comparable with those of the anti-CEA T84.66 diabody. In addition, active uptake by normal tissues was diminished by altering the labelling strategy, leading to lower background and improved PET/computed tomography (CT) imaging. These examples suggest that diabodies represent a promising new class of tumour-specific probes for PET imaging (currently the most sensitive modality using radionuclide-labelled probes) of both tumour masses and metastases.

3.1.1.4 Triabodies and tetrabodies

Trimers and tetramers (Figure 1A), composed of scFvs in tandem, are multimeric species expected to have pharmacokinetic properties that are more desirable than diabodies. Specifically, they should bind their antigens and remain at the target site

longer, due to their increased avidity. Their serum clearance kinetics are also expected to be relatively fast due to the lack of Fc region. In addition, the triabody and tetrabody molecular weights of ~ 90 and 120 kDa, respectively, are favourable for hepatic clearance, thus reducing the likelihood of inducing renal toxicity when in a radiometal-conjugated form.

There are very few reports describing the *in vitro* and *in vivo* properties of triabodies, mainly due to production and purification hurdles. Two studies report BIAcore® (Biacore AB) biosensor binding experiments, which confirm that trimeric scFvs showed increased antigen binding affinities due to their enhanced avidity of binding [48,49]. *In vivo* analysis after radiolabelling was performed to evaluate the pharmacokinetics, biodistribution and tumour-imaging properties of TNT-3 triabody in solid tumour-bearing mice [51]. The terminal serum half-life ($T_{½}\beta$) of the ^{125}I-labelled TNT-3 triabody was estimated to be 7.9 h with a tumour %ID/g and tumour:blood ratio exceeding that of the corresponding diabody and scFv. Immunoscintigraphy using ^{131}I-triabody preparations revealed that at 24-h postinjection, the acquired images were distinct and showed uptake in LS174T tumours with minimal activity in normal tissues compared with the parental [^{131}I]TNT-3 intact antibody. Further studies are required to confirm the potential of triabodies for the diagnosis and monitoring of solid tumours using noninvasive imaging techniques.

Superior tumour and serum pharmacokinetics of tetravalent over divalent forms of scFvs was also observed in a study in which both the *in vitro* and *in vivo* properties of a tetravalent scFv [53] of the murine mAb CC49 recognising TAG-72 were evaluated [52]. The binding affinities for the tetramer and CC49 IgG were similar and fourfold higher than the bivalent $sc(Fv)_2$ molecule. Biodistribution studies in LS174T tumour-bearing mice demonstrated that at 6-h postadministration, the tumour %ID/g achieved by the radioiodinated tetramer was 21.3 compared with 9.8 and 17.3 for the radioiodinated $sc(Fv)_2$ and IgG, respectively. The elimination half-life ($T_{½}\beta$)of the tetramer was 2.8 h, compared with 1.3 and 5.5 h for the $sc(Fv)_2$ and IgG, respectively. Finally, in an animal therapy study, the therapeutic potential of the same CC49 antibody tetravalent and bivalent $sc(Fv)_2$ multimers was investigated [53]. Mice received either a single intravenous injection (1000 µCi) or four injections (4 × 250 µCi) of ^{131}I-labelled $(sc[Fv]_2)_2$ or ^{131}I-labelled $sc(Fv)_2$. No ^{131}I-label systemic toxicity was observed in any of the treatment groups. The results showed that RIT delivery for $sc(Fv)_2$ and $(sc[Fv]_2)_2$ in a fractionated schedule clearly presented a therapeutic advantage over a single administration in this model. The treatment group receiving tetravalent scFv revealed a statistically significant prolonged survival with both single and fractionated administrations, thus suggesting promising prospects for this reagent in cancer therapy and diagnosis.

3.1.1.5 *Higher molecular weight multivalent fragments*

In addition to multimerisation relying on self-association, multimeric antibody fragments can be generated by the use of immunoglobulin constant domains, homomultimeric molecules, helix bundles and coiled-coil structures [32,81] (Figure 1A). One promising intermediate molecular weight fragment is the anti-CEA minibody ($[scFv\text{-}C_H3]_2$ 80 kDa), which is produced through the use of the IgG1 C_H3 constant domain as a homodimerisation domain. The minibody, derived from the anti-CEA antibody T84.66, showed high, specific uptake in LS174T tumours (21.4 – 32.9 %ID/g) (Figure 2B), relatively rapid blood (Figure 2A) and normal tissue kinetics, and hepatic clearance in preclinical studies [32,67,76]. In comparison with the corresponding radiolabelled T84.66 diabody, the minibody achieved significantly higher tumour uptake and longer tumour retention in the same tumour system [76]. Anti-CEA minibodies have also been successfully implemented in high-resolution small animal PET imaging of CEA-positive tumours in live mice using both ^{64}Cu [82] and ^{124}I [79] (Figure 4C) radionuclide labels. Tumour masses were visible as early as 4 – 5 h after tracer injection. More recent work with the radioiodinated 10H8 [83] minibody recognising the p185^{HER2} receptor tyrosine kinase showed modest tumour uptake (5.6 %ID/g at 12 h) [84] (Figure 3B) compared with the anti-CEA minibody (Figure 2B). This resulted in lower tumour:blood ratios for the anti-p185^{HER2} minibody (3:1 at 48 h) compared with the anti-CEA minibody (65:1 at 48 h), as the serum clearance kinetics of the two minibodies was very similar (Figures 2A and 3A). Possible explanations of the observed low tumour activity include rapid dehalogenation and/or metabolism, active internalisation and proteolytic degradation, or antigen shedding with rapid clearance of antigen–antibody complexes. The tumour uptake of the radiometal-labelled form of the same minibody was similar to the radioiodine-labelled 10H8 minibody; however, in addition to the expected liver clearance, the kidneys showed unexpectedly high activity (34 %ID/g) [85]. A minibody derived from the anti-p185^{HER2} hu4D5v8 (trastuzumab) antibody also exhibited unimpressive tumour uptake and elevated kidney uptake, which can be explained by the presence of a crossreacting antigen in the proximal tubules of the mouse kidney. The inconsistency in the behaviour of the anti-CEA and anti-p185^{HER2} minibodies support the conclusion that although antibody fragments of the same format can demonstrate similar serum persistence, their tumour uptake may vary depending on the specific tumour antigen system.

Similar to the minibody, a dimeric molecule termed small immunoprotein (SIP) was generated via the use of the homodimerising C_H4 domain of the human IgE secretory isoform (IgE-S2) and a short linker attaching the L19 scFv region [70]. The human recombinant scFv, L19, recognises the extradomain B (ED-B) of fibronectin (a marker of angiogenesis) and specifically targets tumour neovasculature. Although the maximum tumour uptake of the SIP molecule was about fourfold less than that of the anti-CEA minibody, it achieved two- to five-times higher tumour uptake than that of the corresponding L19 $(scFv)_2$ with a tumour:blood ratio of 70:1 at 144 h.

Apart from antibody-derived dimerisation domains, regions of other endogenous proteins, responsible for the formation of multimers, can also be used in multivalent antibody fragment design and engineering. The pharmacokinetics and biodistribution of tetravalent and divalent forms of scFvs were also investigated in a study where anti-p185^{HER2} dimeric and tetrameric 4D5 antibody scFv molecules (Figure 1A) were generated by the use of a synthetic helix-turn-helix domain and the multimerisation domain of p53 tumour suppressor protein, respectively [54]. The dimer, termed miniantibody, was able to successfully target and localise at the tumour site *in vivo* when labelled with ^{99m}Tc; however, no significant increase in tumour uptake was noted when compared with the monomeric scFv. Tetramerisation, on the other hand, resulted in increased serum persistence and significantly reduced tumour target off-rate, due to the avidity effect. The ^{99m}Tc-labelled tetrameric 4D5-p53 molecule localised at about threefold higher levels on the tumour than the miniantibody and remained stably bound for ≥ 72 h. The overall cumulative tumour uptake, however, was modest (4.32 %ID/g) and was explained by the dissociation of the multimerised peptides in serum. More stable derivatives of these formats are under investigation for improved tumour uptake [86].

3.1.1.6 *Single-chain Fv-Fc antibody fragments*

One approach for tailoring antibody serum persistence is the reduction of antibody size either through proteolytic cleavage or protein engineering techniques. However, there is a delicate balance between half-life and tumour uptake; therefore, it is not always possible to produce antibody fragments with both optimal tumour uptake and clearance kinetics. Manipulation of the biology responsible for the extended circulation half-life of intact antibodies offers an alternative approach in tailoring their pharmacokinetics for radionuclide delivery. The protective neonatal Fc receptor (FcRn) is responsible for maintaining the levels of IgGs in the circulation by favouring antibody recycling rather than lysosomal degradation [87-89]. Specific antibody amino acids essential for FcRn interaction have been identified [90-93]), and mutation of these amino acid residues correlates with reduced antibody circulation half-life [91,94,95]. To improve the tumour residence and uptake achieved by the T84.66 minibody further, Wu and colleagues designed an even larger anti-CEA fragment; assembled V_L-linker-V_H-hinge-C_H2-C_H3 ([scFv-Fc]$_2$ 105 kDa; Figure 1A), which showed pharmacokinetic behaviour similar to intact antibodies [68] (V Kenanova, unpublished data) (Figure 4A), along with five variants carrying specific mutations in their FcRn binding site [71]. Biodistribution studies in Balb/c mice demonstrated that it is possible to produce molecules with a spectrum of terminal half-lifes ranging from 12 days to 8 h, yet retain the same overall structure. *In vivo* serial imaging with ^{124}I-labelled scFv-Fc recombinant variants, using a small animal PET scanner, revealed that the mutant with the most rapid clearance kinetics was the fastest to localise at the tumour site and disappear from

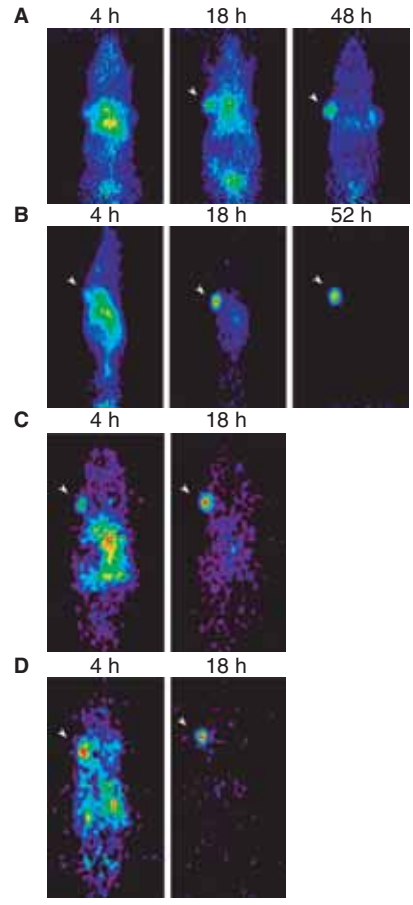

Figure 4. Serial *in vivo* imaging by micro-PET. Engineered anti-CEA antibody fragments, radiolabelled with ^{124}I were followed by micro-PET imaging over time, in mice xenografted with LS174T tumours on the left shoulder. All images are showing a coronal section of the mouse. **A.** scFv-Fc fragment with intact human γ1 C_H2 and C_H3 domains (Fc region). This fragment was shown to exhibit slow blood clearance and tumour localisation properties similar to those of intact antibodies. **B.** scFv-Fc fragment with two mutations in its Fc region (His310Ala/His435Gln) shown to have accelerated serum and tumour kinetics due to compromised interaction with the protective FcRn receptor. **C.** Minibody, a dimer of scFv fused to the C_H3 dimerisation domain ([scFv-C_H3]$_2$), exhibiting serum characteristics similar to the scFv-Fc His310Ala/His435Gln variant. **D.** Diabody, a noncovalent dimer of two scFv (sc[Fv]$_2$), is the fastest clearing divalent fragment. The difference in clearance rates is easily visualised from the serial images. The images were compiled from the work of Kenanova *et al.* [71] and Sundaresan *et al.* [79]. Reproduced with permission from KENANOVA V, OLAFSEN T, CROW DM *et al.*: Tailoring the pharmacokinetics and positron emission tomography imaging properties of anticarcinoembryonic antigen single-chain Fv-Fc antibody fragments. *Cancer Res.* (2005) **65**(2):622-631.
CEA: Carcinoembryonic antigen; C_H: Constant heavy chain; Fc: Crystallisable fragment; FcRn: Neonatal Fc receptor; PET: Positron emission tomography; scFv: Single-chain variable fragment.

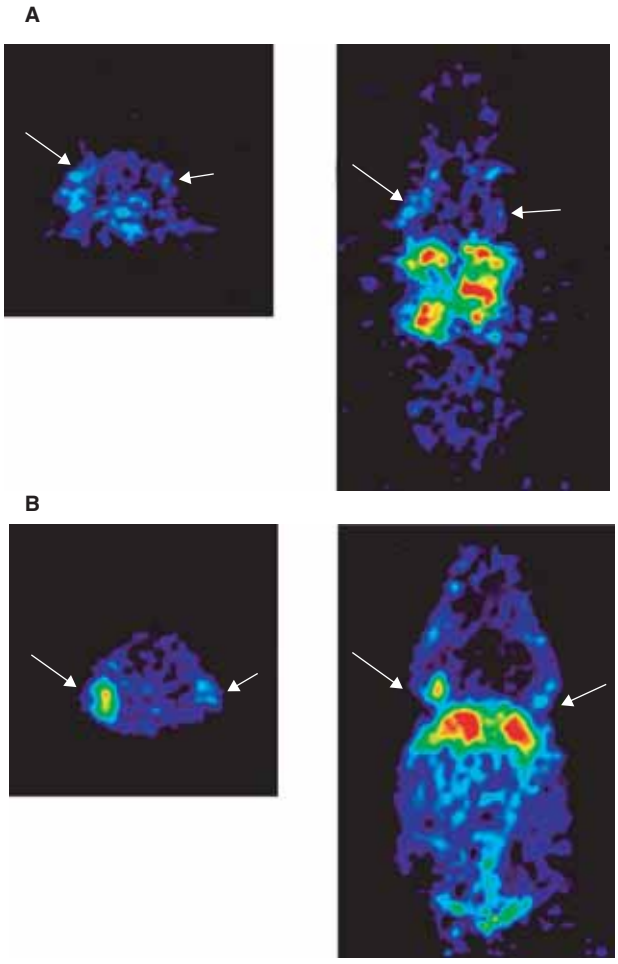

Figure 5. *In vivo* imaging by micro-PET. Engineered anti-HER2 antibody fragments, radiolabelled with ^{64}Cu and imaged by micro-PET in mice xenografted with HER2-expressing MCF7/HER2 tumours on the left shoulder. The panels on the left represent a transverse view, whereas the panels on the right show a coronal section. **A.** Minibody, showing reduced tumour uptake and high activity in the liver and kidneys 18 h after tracer administration. **B.** scFv-Fc His310Ala/His435Gln mutant revealing improved tumour localisation and reduced kidney uptake 18-h postinjection. Micro-PET images reproduced with permsission from OLAFSEN T, KENANOVA VE, SUNDARESAN G *et al.*: Optimizing radiolabelled engineered antip185HER2 antibody fragments for *in vivo* imaging. *Cancer Res.* (2005) **65**(13):5907-5916 [85].
Fc: Crystallisable fragment; PET: Positron emission tomography; scFv: Single-chain variable fragment.

the circulation, resulting in high-contrast images at ~ 18 h postinjection (Figure 4B). Although not as suitable for tumour imaging, due to higher background, the radioiodinated fragments with intermediate circulation kinetics ($T_{½}\beta$ 21.2 – 28.4 h) (Figure 2A) achieved higher maximum tumour uptake (18 – 33.6 %ID/g), retained over a longer period of time (> 72 h) (Figure 2B) in biodistribution studies (V Kenanova, unpublished data). The advantage of attenuating receptor interactions is that one can generate several antibody variants of the same format, with a variety of blood and tumour kinetics, providing greater choice in selecting the agent that would best suit a specific *in vivo* application.

This approach of trimming circulation half-life while retaining larger molecular size was also implemented in the p185^{ERB-2} system [85]. Similar to the fastest clearing anti-CEA scFv-Fc variant from the previous study, an anti-p185^{ERB-2} $(scFv\text{-}Fc)_2$ containing the same amino acid mutations was generated. This ^{64}Cu-conjugated fragment, when evaluated by micro-PET, exhibited improved tumour targeting (12.2 %ID/g) (Figures 3B and 5B) and reduced kidney uptake (13.1 %ID/g), when compared with both radiometal-conjugated anti-p185^{ERB-2} 10H8 and hu4D5v8 minibodies [84,85] (Figures 3B and 5A). Thus, by manipulating both the fragment size and biology of receptor interactions, the authors managed to improve the tumour uptake and reduce the kidney activity exhibited by the p185^{ERB-2} antibody fragment in p185^{ERB-2} overexpressing xenografts. This approach can also be translated to engineering intact antibodies [93] or fusion proteins containing complete antibody Fc regions with a spectrum of circulating half-lifes.

3.1.2 *Clinical studies*

Analogous to the preclinical animal studies, several clinical studies have confirmed the ability of radiolabelled scFvs to target and rapidly accumulate at tumour masses in patients [96-98]. In clinical imaging studies of the anti-CEA scFv MFE-23 using SPECT and radioimmunoscintigraphy [96] or a handheld γ-detecting probe for radioimmunoguided surgery [98], CEA-expressing tumour masses were imaged soon after tracer administration. Clearance of background radioactivity allowed for imaging with the highest median ratios of tumour:normal tissue and tumour:blood at 22-h postadministration for SPECT or between 72 and 96 h for radioimmunoguided surgery. As observed in preclinical investigations, these patient studies confirmed elevated kidney uptake of the radioiodinated scFv and faster serum clearance compared with the corresponding intact parental antibody (A5B7) [99]. A ^{123}I-labelled scFv of the anti-TAG-72 monoclonal antibody CC49 was also used in SPECT and whole-body imaging of patients with colorectal carcinomas [97]. Both primary and metastatic tumour sites in all patients were visualised 18 h after tracer administration but the image contrast was less than optimal with clear visualisation of normal organs (i.e., spleen and testicles). The authors suggested that optimal tumour imaging doses could be determined through escalation of the mass dose of scFv protein. The image findings reconfirmed very fast scFv serum clearance due to rapid equilibrium with the extracellular fluid and soft tissues, with elimination from the body almost exclusively through the kidneys. Although scFv RIT applicability is rather limited and renal parenchyma retention of radioiodinated fragments or metabolites is undesirable, scFv RII has its advantages. Patients with colorectal as well as many other carcinomas are found to develop liver

metastases. Thus, it is important to note that because radiolabelled scFv fragments exhibit exclusive renal clearance and are able to better penetrate tissues, they can be used for imaging liver metastases. Alternative approaches to decrease renal activity are also available and reviewed by Behr *et al.* [100].

The genetically engineered fully human diabody L19 $(scFv)_2$, with specificity for the ED-B domain of fibronectin [101], has also been the subject of clinical evaluation. As this marker is derived from the vasculature, it serves as a pan-carcinoma marker for malignancy. The smallest lesion detected though the use of anti-ED-B ^{123}I-labelled L19$(scFv)_2$ by immunoscintigraphy was a 4 – 6 mm liver metastasis. The imaging results demonstrated the ability of L19 $(scFv)_2$ to localise efficiently in aggressive primary tumours as well as metastases, and to clear rapidly through the renal route. Well-defined images were acquired at 6 and 21 h. The variation in antibody uptake correlated with the differential antigen expression found in tumours examined by immunohistochemistry. This study demonstrated the potential use of the L19 diabody for obtaining information on the growth potential of aggressive versus benign lesions as well as in the follow up of patient response to therapy through the use of immunoscintigraphy. The short half-life, the ability of L19 $(scFv)_2$ construct to extravasate and localise rapidly at both the primary tumour as well as metastases, and its rapid renal excretion make this fragment an especially valuable imaging agent for the production of high-quality, low background images within a short time after molecular tracer administration.

Lower molecular weight antibody fragments, such as scFvs and diabodies, possess faster clearance rates, resulting in increased tumour:blood ratios and reduced background activity. However, maximum uptake and retention in the tumour are often quite low. Thus, candidate fragments characterised by tumour uptake closer to that of intact antibodies combined with clearance that is more rapid should provide improved imaging capabilities, and would probably have intermediate molecular mass. In a pilot clinical study, ^{123}I-labelled chimaeric T84.66 anti-CEA minibody (80 kDa) was used and its tumour targeting ability, as well as biodistribution, pharmacokinetics and immunogenicity were characterised [102]. SPECT images obtained at 18 – 24 h clearly demonstrated the ability of the anti-CEA minibody to detect lesions ≥ 1 cm. In addition, tumour imaging with the radioiodinated minibody revealed three infiltrative lesions undetected by computed tomography. The minibody was well tolerated, with no significant human antiminibody responses. Although a radionuclide with a longer half-life, such as ^{111}In, may be better suited to evaluate the pharmacokinetic properties of the minibody, the initial results are encouraging and further clinical evaluation of the imaging and therapeutic potential of this construct are worth pursuing.

3.2 Pretargeting

3.2.1 Animal studies

3.2.1.1 Streptavidin–biotin-based pretargeting system

In nuclear medicine, pretargeting offers the potential to greatly reduce the systemic toxicity associated with conventional RIT. The concept of pretargeting is based on a multistep mechanism, in which the signal molecule or toxic agent does not react directly with the target. Instead, a facilitating intermediary (usually antibody based) is allowed to bind its target directly. Subsequently, the radionuclide is introduced systemically in the form of a low molecular weight agent that binds rapidly to the intermediary. The outcome of this sequence is essentially fixing of the signal or toxic molecule on the target. Several pretargeting strategies have been based on the use of the high-affinity interaction between streptavidin (StAv) and biotin (dissociation constant $[K_d] \sim 10^{-15}$ M) [103-105] (Figure 1B). A common two-step scenario (Figure 1B) includes infusion of the intermediary StAv-conjugated antibody first. After allowing sufficient time for target localisation (48 – 72 h), radionuclide-labelled biotin is administered. The radionuclide–biotin complex is either captured by the antibody–StAv prelocalised to the tumour cells or cleared rapidly through the kidneys due to its low molecular mass. Another variant of the StAv–biotin pretargeting system, termed a three-step pretargeting (Figure 1B), includes infusion of a biotinylated antitumour antibody. StAv is then administered to bind the tumour-localised IgG–biotin. The final step is the administration of radiolabelled biotin [106].

The production of StAv–antibody complexes is generally accomplished by chemical conjugation of StAv with mAbs. These complexes are not easily produced and are quite immunogenic, which limits their commercialisation and widespread availability. In addition, more uniform and predictable orientation of the produced conjugates is required for clinical applications. For these reasons, recombinant DNA and protein engineering techniques, offering significant advantages over chemical conjugation, have been used to provide a variety of antibody-based agents specifically for pretargeting.

StAv is a homotetrameric protein and when genetically linked with a scFv results in fusion proteins (scFv–StAv), which bind antigen tetravalently (Figure 1B). In addition, these fusions are characterised by improved pharmacokinetic properties with faster tumour localisation and serum clearance when compared with IgG–StAv complexes. Preclinical biodistribution studies using tetravalent scFv–StAv molecules revealed improved tumour:normal tissue ratios resulting in enhanced therapeutic index [107]. A preclinical therapy study using scFv antibody fragments from the CD20-specific antibody B9E9 genetically engineered as StAv fusions followed by radiolabelled biotin, demonstrated increased therapeutic efficacy in Ramos B cell lymphoma xenografted mice [108,109]. Similar results in lymphoma and leukaemia xenografted mice were also achieved by the use of anti-IL-2 receptor α-antibody scFv–StAv fusion protein followed by [^{90}Y]- or [^{213}Bi]biotin, respectively [110]. Even with more challenging solid tumours, a similarly formatted construct using humanised scFvs specific to the EGP40 antigen was used in a therapy study with nude mice bearing SW1222 human colon carcinoma xenografts. A single dose of [^{90}Y]DOTA-biotin (800 µCi) produced cures in these mice [111]. The therapeutic potential of three radiolanthanides was also evaluated in a study comparing the

tumour targeting and biodistrubution properties of ^{149}Pm-, ^{166}Ho- and ^{177}Lu-labelled DOTA-biotin in nude mice bearing human colorectal carcinoma xenografts, after pretargeting with the CC49 scFv–StAv fusion protein [112]. Here, the authors used the existent pretargeting system as a tool for evaluation of novel radionuclides. Taken together, these collective data show promise for pretargeted RIT using antibody fragments in cancer, although the immunogenicity of the components, especially avidin/StAv remains a major obstacle. Engineering efforts are not only directed towards improvement of the *in vivo* properties of the mAb fragment but also towards masking the immunogenic StAv epitopes (e.g., deletion of T- and B-cell epitopes, pegylation), thus allowing for multiple administrations of the antibody fragment–StAv fusion proteins.

3.2.1.2 Amplification pretargeting

The administration of antibody fragments with fast distribution kinetics in pretargeting strategies obviates the requirement for using clearing agents. Unfortunately, smaller fragments, such as scFvs and Fabs, exhibit reduced tumour uptake, which requires a strategy for amplifying the signal at the target site. Such an approach, termed amplification targeting, was investigated by Hnatowich and colleagues [113]. This nucleic acid-based pretargeting strategy was designed to show that amplification targeting leads to increased accumulation of radioactivity in the tumour target. In the experiment, human colorectal carcinoma xenografted mice received the anti-CEA antibody MN14 conjugated with phosphordiamidate morpholino oligonucleotides (MORFs), followed by a polymer linked to multiple copies of the complementary MORF (cMORF), and finally [^{99m}Tc]MORF (Figure 1B). The results demonstrated that the hybridisation in tumour of both polymeric cMORF to the antibody MORF and the radiolabelled MORF to the polymeric cMORF was very efficient. Tumour accumulation of radiolabelled MORFs was more than triple for study animals receiving all three components compared with control animals. Furthermore, antibody–MORFs and polymer–cMORFs were rapidly cleared from normal organs such as liver, spleen and kidneys, but remained in the tumour, thus improving the target:nontarget ratios. Although the experimental layout requires extensive optimisation of the oligomers and peptides employed, the concept is sound and should be applicable with smaller antibody fragments. In addition, the potential low immunogenicity of MORFs provides them with a significant advantage over StAv in therapeutic studies.

3.2.1.3 Bispecific antibody pretargeting

Another pretargeting approach relies on the use of bispecific mAbs (bsMAbs) (Figure 1B). The principle behind this pretargeting strategy is that antibodies could be constructed with dual binding specificity: one to a particular target and the other capable of binding a radiolabelled chelate or hapten. The accumulation of an antichelate antibody at the target site could be used subsequently to capture a radiolabelled chelate. Bispecific antibodies can be produced by chemically combining Fab′ fragments of one antibody with specificity to a tumour antigen with another antibody specific for the effector molecule. More recently, divalent haptens have been used as effector molecules [114] (Figure 1B). This method, termed the affinity enhancement system, greatly increases the residence time of the effector molecule at the target site and is explained by the ability of the bivalent effector molecule to connect two bispecific antibodies on the tumour surface, thereby increasing their functional affinity [115].

In an attempt to improve the affinity enhancement system pretargeting scheme further, Goldenberg and colleagues designed a pretargeting system consisting of bispecific antibodies to CEA and diethylenetriaminepentaacetic acid (DTPA), together with a divalent indium-loaded DTPA ([In]DTPA) peptide capable of binding ^{99m}Tc [116]. Bispecific antibodies were prepared by chemically combining the IgG, $F(ab')_2$ and Fab′ components of a humanised antibody against CEA with the Fab′ fragment of a chimeric anti-(In)DTPA antibody. A ^{99m}Tc-labelled divalent (In)DTPA peptide was used as the effector molecule. The results demonstrated that IgG × Fab′ conjugate had the highest tumour uptake along with the longest retention time, followed by the $F(ab')_2$ × Fab′ and the fastest clearing Fab′ × Fab′ conjugate. The $F(ab')_2$ × Fab was selected as the best candidate out of the three for pretargeting RIT. The study suggests that increasing the valency of a bsMAb to the tumour antigen as well as the use of smaller antibody fragments could improve pretargeting.

As in antibody–StAv-based pretargeting, there is a need to develop more uniform bsMAb products with predictable orientation. This can be achieved by substituting chemical conjugation with genetically engineered bsMAbs or fragments with bivalent specificity for the tumour target. Recombinant bsMAbs and fragments would not only simplify production but their reduced molecular size compared with the conventionally prepared Fab′ chemical conjugates could lead to improved pharmacokinetic and tissue penetration properties for both RIT and RII [117]. Preclinical examples supporting the improved therapeutic efficacy of recombinant bsMAbs-based systems are reviewed by Sharkey *et al.* [118]. In one study, the trivalent, recombinant fusion protein, hBS14, which binds bispecifically to CEA and the hapten histamine-succinyl-glycine, was evaluated for its potential in radionuclide delivery to tumours in mice [119]. The 80-kDa fusion protein cleared rapidly from the circulation, similar to Fab′ × Fab′ bsMAb [120], but with tumour uptake two- to threefold higher. Tumour localisation was shown to occur within 3 h of the radiolabelled hapten injection. Based on these preliminary studies, hBS14 is a promising candidate for use in pretargeted imaging and therapy of CEA-expressing tumours. Although difficulties in obtaining the appropriate combination of dual specificity while retaining immunoreactivity is a challenge, overall, the preclinical results are encouraging and support further optimisation of recombinant bsMAb preparations.

3.2.2 Clinical studies

The StAv–biotin-based pretargeting strategy has proven quite effective in animal studies, in which tumours were cured with virtually no toxicity [121]. These promising results have encouraged the initiation of several Phase I and II clinical trials [122-124]. Although these trials produced objective therapy responses with substantial tumour:whole body ratios, significant organ toxicities (gastrointestinal tract, kidneys) were also noted. Clinical studies using the three-step StAv method (Figure 1B) have not resulted in similar toxicities, but this is most likely due to target selection, lower radioactivity administered, and the specific tumour/therapy system, notably the regional treatment of brain or ovarian cancers [125,126]. Phase I clinical studies with bsMAb pretargeting using a ^{131}I-labelled peptide hapten were carried out in lung, colorectal and medullary thyroid cancer patients. Although some responses were observed, dose escalation has been limited by haematological toxicity [127-129].

Based on the success of numerous therapy studies in animal models discussed above, the anti-CD20 B9E9 scFv–StAv tetrameric construct was translated to a clinical setting [130]. The outcome of the Phase I pilot trial in 15 patients with B-cell NHL included two complete remissions and one partial response. Low whole-body, marrow, and liver and relatively modest kidney and bladder exposure were estimated. Immune responses to the fusion protein were found in three patients, which could be partially ameliorated by pegylation of the StAv [131]. The tumour dose of 26 ± 4 cGy/mCi resulted in 49:1 average tumour:whole body radiation dose. Importantly, when extrapolated to a patient with a body surface of 1.8 m^2, the mean tumour dose at the low 15-mCi/m^2 ^{90}Y dose would be 600 – 800 cGy. These values are similar to the ones achieved by ibritumomab and tositumomab at their maximum tolerated doses [13,132]. Furthermore, the authors estimated that ^{90}Y-DOTA-biotin doses of ~ 100 mCi ^{90} should produce a myelotoxic effect similar to that of a typical ibritumomab or tositumomab dose. Thus, the pretargeting scheme using the engineered antibody B9E9 fusion protein carries the potential to specifically deliver substantially higher radiation doses to tumours compared with currently used clinical RIT strategies.

4. Expert opinion and conclusion

The implementation of antibody molecules for radionuclide delivery has advanced, as evidenced by the FDA approval and recent marketing of two RIT agents (ibritumomab and tositumomab) for the treatment of NHL. These join the previously approved RII agents (Oncoscint, Prostascint and CEA-Scan) for cancer detection. Several additional radiolabelled antibody products are in clinical evaluation for RIT of lymphoma, colon cancer and prostate cancer. Most encouraging is the intense level of research at the preclinical stage, which is enhanced by advances in library screening approaches for generating lead antibodies, antibody engineering and sophisticated instrumentation for preclinical small animal imaging. Direct targeting as well as pretargeting approaches to radionuclide delivery have both been exhaustively explored in murine models, and a solid understanding of the requirements for effective imaging and RIT is emerging.

However, progress in the clinic remains slow, at least for radioimmunoconjugates. Even as we continue to optimise the antibody agents and the radioimmunoconjugates, many challenges remain, relating to fundamental issues of the biology of disease in humans. Biological factors include target expression and density, normal tissue expression and/or crossreactivity; shedding of antigen into the circulation, heterogeneity of antigen expression, size of the target lesion (e.g., micrometastases versus primary tumour), internalisation of target-associated antibody, metabolism and subsequent trapping/elimination of the radioactive moiety, and vascularisation of the target tissue.

Further challenges arise due to the sheer complexity of not only producing and delivering a biological product but one that is radiolabelled in addition for clinical use. Institutional, regulatory and economic obstacles must be overcome, even to institute pilot imaging studies or Phase I RIT studies: a forbidding task for most academic and nonprofit organisations. Even when FDA approval is achieved, commercial success relies on widespread adoption and implementation of a new modality, which is more complex for a radiolabelled biological product compared with conventional drugs currently in use. For example, routine delivery of RIT for cancer treatment may require the cooperation of oncologists, nuclear medicine personnel, radiation oncology, and/or many other specialties within a hospital. Shipment, storage and waste disposal of hazardous, time-dependent reagents also need accommodation.

Nonetheless, there is cause for optimism. As reviewed in this paper, investigators have extensive control over the design and properties of antibodies and can optimise them for delivery of radionuclides for imaging or therapy. The broad utility of antibodies as a platform for developing molecularly targeted agents is well recognised. As information continues to flow from the maturing fields of genomics and proteomics of disease, the molecular entities that are characteristic of disease are being identified, and development of antibodies is a facile approach for generating agents that target new markers, for disease detection and intervention. These forces will motivate the field to overcome the remaining barriers and enable wider implementation of radiolabelled antibody-based agents for imaging and therapy.

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