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Preclinical Optimization of Antibody-based Radiopharmaceuticals for Cancer Imaging and Radionuclide Therapy – Model, Vector, and Radionuclide Selection

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Abstract

Intact antibodies and their truncated counterparts (e.g. Fab, scFv fragments) are generally exquisitely specific and selective vectors, enabling recognition of individual cancer-associated molecular phenotypes against a complex and dynamic biomolecular background. Complementary alignment of these advantages with unique properties of radionuclides is a defining paradigm in both radioimmunoimaging and radioimmunotherapy, which remain some of the most adept and promising tools for cancer diagnosis and treatment. This review discusses how translational potency can be maximized through rational selection of antibody-nuclide couples for radioimmunoimaging/therapy in preclinical models.

Introduction

The advantages antibodies provide in oncologic nuclear medicine are manifold, allowing precision in non-invasively delivering diagnostic and therapeutic radionuclides to cancer cells based on specific molecular phenotypes. In turn, this has enabled quantitative readout of antigen status via imaging modalities including positron emission tomography, as well as novel treatment options for therapeutic intervention. However, the path to these clinical ideals winds through multiple stages of design, benchtop analysis and preclinical development. Nuclear medicine at its core remains inextricably linked by the vastly different disciplines of biology, chemistry, radiochemistry, and nuclear physics. A sound understanding of elements of each of these disciplines is consequently necessary for optimal design and application of radiopharmaceuticals for imaging and therapy. Our aim in this review is to provide a window into the dominating factors to be considered when designing antibody-based strategies for interrogating oncologic unknowns. A spotlight on preclinical aspects such as the choice of the antibody-based targeting vector, mouse models, the

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radioisotope selection for imaging or therapeutic purposes, dosimetric considerations, or the optimization of the biodistribution at the organ and sub-cellular level, are provided. Insights toward ultimate clinical application are also considered. In doing so, we hope to simultaneously aid the understanding of researchers new to the field, researchers currently focused exclusively on either biological or physical aspects, and clinicians, alike.

Selecting Antibody-Based Tumor Targeting Vectors as Radiopharmaceuticals

The high degree of specificity for binding to their target epitopes in a complex biological milieu combined with the advances in the synthesis and biochemical engineering of their structure and affinity have made antibodies one of the most popular class of molecules used in biomedical research and the biopharmaceutical industry (1–5). This is increasingly evident in the oncology space wherein 27 antibodies currently have USFDA approval status as drugs for the treatment of various cancers (<http://www.antibodysociety.org/news/approved-antibodies/>). Indeed, the utility of antibodies to treat cancer is attributed to their primary ability for binding to tumor-associated antigens, which are over- or aberrantly-expressed on the surface of neoplastic cells (6). Arguably, Fab and F(ab')₂ units that form the antigen-binding arms of an antibody as well as single chain variable fragments (scFv) comprised of the variable light (V_L) and heavy chains (V_H) within the Fab are sufficient and capable of binding to target antigens on tumor cells *in vitro* (Figure 1). However, these fragments are rapidly cleared from systemic circulation via renal filtration, thus reducing their *in vivo* residency and tumor availability (7,8). Furthermore, the monovalent binding of scFv and Fab fragments limits their avidity for binding to the target at the tumor site. More recently, engineered bivalent antibody fragments such as diabodies and minibodies have shown better *in vivo* pharmacokinetics and tumor targeting compared to the aforementioned Fab subunits (9,10). On the other hand, full-length antibodies benefit from their molecular weight (~150 kDa) and size (~20 nm) making them ineligible for rapid renal clearance, while being conferred greater tumor bioavailability due to FcRn-mediated recycling in systemic circulation (11). Taken together, full-length antibodies possess features that favour them to achieve higher tumor uptake, albeit with less-than ideal tumor penetration capabilities (7,8,12). Notably, the long serum half-lives of full-length antibodies due to FcRn-mediated recycling delivers unnecessary radiation burden to non-target organs and serves as a disadvantage for targeted radiotherapy strategies with these agents.

A historically common concern pertaining to “immunogenicity” attributed to the use of full-length antibodies obtained from mouse hybridomas has now been significantly resolved by the ability to produce “humanized” and fully human antibodies (14–16). The latter can be produced from chimeric intermediates, or by screening phage libraries of human transcripts obtained from peripheral blood to enable the identification of potential target binding scFv sequences that can be grafted onto a human IgG scaffold through recombinant DNA techniques (17). More recently, the use of transgenic mouse platforms has enabled the production of fully human antibodies (18–20). IgG1, the most abundant immunoglobulin subtype present in human blood, is also the most common scaffold that is used to develop humanized or fully human antibody-based tumor-targeting vectors (21). From a translational

perspective, the use of humanized or fully human antibodies in preclinical nuclear imaging and radioimmunotherapy (RIT) studies can provide a reasonable estimate of the *in vivo* biodistribution and pharmacokinetics of these tumor-targeting agents in human patients. Conversely, murine antibodies work well in preclinical mouse models but are rapidly cleared in human subjects due to reduced binding to human FcRn and human anti-mouse antibody responses, which limit the *in vivo* residency for effective uptake of murine antibodies in tumors (22). More importantly, repeated administration of murine antibodies in patients might provoke adverse immunologic reactions. That being said, even fully human antibodies can be immunogenic, albeit to a lesser extent (23,24).

With much credit attributed to the use of full-length antibodies, it must be noted that their *in vivo* pharmacokinetics may be viewed as “sluggish” and disadvantageous in the context of radioimmunoimaging and RIT. These applications require the targeting vector to be radiolabeled, and a prolonged residence of the radiopharmaceutical in systemic circulation could impart unnecessary radiation dose to non-target organs such as sites of hematopoiesis (bone marrow) and the biological site of clearance (liver) in cancer patients. Additionally, this process can be inconvenient for patients who have to return to the clinic after 5 or 7 days to undergo a positron emission tomography (PET) or single-photon emission computed tomography (SPECT) scan. In the interest of being fair and current, it must be stated that practical solutions to overcome some of the inherent limitations of using full-length antibodies and antibody fragments as tumor-targeting vectors have been sought. For example, pretargeted immunoPET imaging and pretargeted radioimmunotherapy (PRIT) can significantly reduce the off-target radiation dose for antibodies that target non-internalizing tumor-associated antigens(25–28). In pretargeted imaging studies, tumors can be delineated in PET scans at time points as early as 4–24 h after the injection of the small molecule radioligand (29–32). Similarly, preclinical PRIT studies have shown that repeated and/or fractionated delivery of therapeutic radionuclides to deliver higher effective doses to the tumor is possible while improving the therapeutic index compared to directly labeled antibodies used in RIT (33,34). That being said, the application of pre-targeted strategies is limited to non-internalizing or slowly internalizing tumor biomarkers.

Considerations for Selecting Tumor Models and Preclinical Mouse Models

Further to choosing an ideal antibody-based vector, it becomes paramount to consider the tumor model and the biological background of the preclinical mouse model in which the antibody-based radiopharmaceutical will be tested for radioimmunoimaging and radioimmunotherapy (35–37). Indeed, it is important to recognize that preclinical models can only provide a close approximation of the reality and complexity of tumor biology. Owing to several factors including cost, availability, ease of handling, and most importantly their ability to engraft human cancer cell lines, immunodeficient mice tend to be the most ubiquitously employed preclinical animals used to develop tumor models for oncologic research. Various cancer cell lines obtained from centralized resources such as the American Type Cell Culture serve as reliable platforms that are used for the *in vitro* verification of binding of the antibody-based radiopharmaceutical prior to xenografting cells for growth in immunodeficient mice. The most common and easy-to-develop xenograft model involves the subcutaneous injection of tumor cells in the flank or shoulder of mice and allowing tumors

to grow up to a volume of ~120–150 mm³ before the animals can be used for imaging and/or therapy experiments. The use of subcutaneous xenografts allows easy visualization of tumor growth with the naked eye and routine measurement of tumor volumes in order to monitor growth prior to initiation of RIT and thereafter to evaluate response to therapy. Although subcutaneous xenografts are a reliable and reproducible model for purposes of characterizing the *in vivo* targeting capabilities and biodistribution of radiopharmaceuticals used for imaging and therapy, this model has often been criticized for the lack of anatomical context in the natural site of occurrence of the malignancy and a restricted potential for metastases to distant sites.

On the other hand, orthotopic models can provide anatomical context related to aspects of *in vivo* access/delivery of the radiopharmaceutical at the tumor site and dosimetry to adjacent tissues and organs. However, generating these models can be skillfully challenging and may yield unreliable tumor take rates in mice. Furthermore, monitoring orthotopic tumor growth and its response to therapy often necessitates the use of noninvasive longitudinal imaging via ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI). Alternatively, optical imaging can be used to monitor tumor growth if orthotopic models are generated using tumor cells stably transfected with genes encoding fluorescent proteins such as green fluorescent protein (GFP), red fluorescent protein (RFP), mCherry for fluorescence imaging or luciferase for bioluminescence imaging.

Finally, in an effort to imply greater translational relevance and to attribute a higher likelihood for clinical success of the tumor-targeting radiopharmaceutical agents being tested in preclinical imaging and therapy studies, there has been a rise in the use of patient-derived xenograft (PDX) models (38,39). Results obtained from using PDX models could be immensely powerful as these tumors retain human stromal components during early passages in highly immunodeficient mice, they offer the advantage of representing tumor heterogeneity and are often looked upon as ‘avatars’ of the patients they were derived from. Specifically, PDX models can provide clinical context and a history for response versus resistance to prior treatments shown by the patient. This facilitates novel drug discovery while informing investigators to tailor improved therapies while avoiding the use of therapies/approaches that were previously ineffective in the human patient (40,41). More recently, the ability to generate organoid 3D culture models of human tumors has emerged as an excellent platform that efficiently recapitulates disease progression to enable the identification of novel therapeutic targets (42–44). However, the logistic challenges associated with making PDX tumors and organoids more easily available could pose a limitation to the widespread utility of these models (45). Also, access to patient tumor samples and the need for dedicated core units/facilities with skilled personnel to bank, propagate and maintain these models can be an expensive venture that might be possible at limited research centers that are integrated with cancer hospitals.

Having identified an optimal tumor-targeting antibody-based vector and a relevant tumor model to carry out radioimmunotherapy and radioimmunotherapy studies with, an often overlooked factor that might be crucial to the success of the research strategy is the “biological background” of the preclinical mouse model. This becomes paramount when testing immune-oncology drugs and for monitoring immunotherapy via

radioimmunoimaging. Testing antibody-based radiopharmaceuticals in immunocompetent mice would provide a functional immune status akin to that in human patients, however, this would limit one to using mouse tumors propagated in syngeneic inbred mice generated in a C57Bl/6 of BALB/c background. Although a human population would not be represented by inbred strains of mice, the use of the latter seems to be advocated over outbred stocks because they provide a “better-defined” and consistent genetic background with little phenotypic variation – thus serving as an ideal platform to evaluate the efficacy of anti-cancer drugs – especially in the context of mutations (46,47). Alternatively, the use of genetically engineered mouse models offer the possibility to image and treat tumors at various stages of their development as these models are capable of recapitulating tumor growth from initiation through progression while accounting for stromal involvement and a functional immune system. Finally, one could use humanized mice, which are scientifically appealing and near-ideal candidate models for clinical translation, but would come with the burden of a high expense in the preclinical setting (48,49). For more information on preclinical mouse models, readers are directed to excellent review by Day *et al.*, and Zitvogel *et al.* (36,50). Together, these factors leave most preclinical research to be conducted in immunodeficient mice that allow the reliable growth and testing of human tumors in the various formats discussed above.

The most common immunodeficient strain is the athymic nude mouse, which lacks T cells but has functional B cells and NK cells. However, occasionally the need to develop a metastatic model or a PDX model to test the targeting efficacy and translational potential of a humanized antibody-based radiopharmaceutical agent might necessitate the use of highly immunodeficient strains of mice such as NOD/SCID (NOD.CB17-*Prkdc*^{scid}/NcrCrI) and NSG (NOD.Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}/SzJ) (51,52). The highly immunodeficient status of these strains allows metastatic spread of tumors and facilitates a significantly better take rate and growth of PDX tumors (53). However, the NOD SCID and NSG mice lack immunoglobulin-producing B-cells and thus have low endogenous levels of antibodies in their blood(54). While this may not be of any consequence to the *in vivo* pharmacokinetics of small molecule inhibitor drugs, peptides or other such targeted therapies, it seems to hold great relevance when humanized antibody-based therapeutic entities and human IgG1 Fc-fusion proteins are investigated as tumor-targeting vectors. We recently reported that humanized antibody-based imaging or RIT agents when injected into preclinical models developed in highly immunodeficient strains, display an anomalous pattern of *in vivo* biodistribution wherein the vast majority of radioactivity accumulates in non-target organs such as the spleen, bones and liver, while the antigen-positive tumor tissue is deprived of the antibody-based tumor-targeting vector (55) (Figure 2). Notably, the amount (mass) of a full-length antibody-based vector routinely injected for nuclear imaging or RIT studies is significantly lower than that administered for studies involving therapeutic doses of antibody drugs under preclinical investigation. The most striking observation was made in NSG mice injected with imaging doses of high specific activity ⁸⁹Zr-labeled full-length humanized antibody vectors. These mice were moribund with bone marrow toxicity indicated by the ablation of their marrow, marked anemia and severe neutropenia that led to their death within 2 weeks of being injected with the humanized antibody-based radiotracer. It is likely that the bone marrow of highly immunodeficient mice acted as a depot for Fc-mediated

uptake of the humanized antibody-based radiotracer and led to the ablation of their hematopoietic reservoir. Furthermore, the toxicity observed in mice strains derived from the SCID background might also be attributed to their high radiosensitivity, which is attributed to a mutation in the *Prkdc* gene that encodes a catalytic subunit of a protein kinase implicated in DNA repair (54, 56). The limitation posed by the radiosensitivity imparted by the SCID background may be overcome by using immunodeficient mice that are generated as a result of a double knockout of the common gamma chain gene – *IL2rg* and the recombination activating gene – *Rag1* (NRG; NOD Rag gamma – The Jackson Laboratory) (52) or *Rag2* (R2G2 – Envigo; www.envigo.com/r2g2). Absence of the SCID background but presence of the *Rag* mutation, which encodes an enzyme responsible for V(D)J recombination renders these mice deficient in B- and T-cells while imparting them with radioresistance. A table enlisting the various immunodeficient strains of mice and their reasons for immunodeficiency is presented at the end of this section to enable the reader to pick and choose per their experimental needs (Table 1).

Although non-specific in the context of tumor targeting, the uptake of the full-length antibody and associated radioactivity in non-target organs appears to be mediated via interaction between the Fc portion of the antibody vector and Fc receptors expressed on various myeloid cell populations in non-target tissues of the highly immunodeficient mice (58). This conclusion was drawn from experiments wherein the anomalous biodistribution of the tumor-targeting antibody could be resolved by transiently reconstituting the blood pool of highly immunodeficient mice by pre- or co-injecting them with IgG isolated from mouse serum or human isotype IgG in levels at par with the physiological titer of immunoglobulins in mice. By doing so, the Fc receptors on myeloid cells within the spleen and bones of highly immunodeficient mice could be occupied by the Fc of a non-specific IgG, while allowing the antibody-based radiotracer to accrue in the antigen sink provided by the tumor. Alternatively, the use of deglycosylated or Fc-silent tumor-targeting antibodies was able to successfully avoid the non-specific uptake by virtue of their inability to interact with the FcR on myeloid cells in non-target organs despite the low endogenous levels of immunoglobulins in highly immunodeficient mice.

In conclusion, it is becoming increasingly evident that considering the biological background of the preclinical mouse model could be vital to the success of the *in vivo* tumor-targeting capability of an antibody-based targeting vector for the purposes of radioimmunoimaging and radioimmunotherapy. Additionally, when these strategies are experimented in preclinical models developed using highly immunodeficient strains of mice, additional measures such as reconstitution of the *in vivo* immunoglobulin titers or use of deglycosylated/Fc-silent humanized antibody vectors should be considered in order to achieve optimal tumor targeting while avoiding off-target toxicities.

Radionuclide Selection for Radioimmunoimaging

Concurrent development in radionuclide production and radiochemistry have graced the field of nuclear medicine with a diverse and expanding palette of applicable radionuclides, each with a unique spectrum of characteristics for emitted radiation as a function of its underlying nuclear structure. The parallel increase in biomarker identification and

development of new highly specific and selective antibody vectors targeting these, has immense implications for personalized nuclear medicine: given the array of suitable combinations of nuclide/antibody, how can the physical decay characteristics of the nuclide be optimally matched to the biological behavior of the antibody and the physiology of the target (e.g. heterogeneity, tissue density, volume) in order to maximize diagnostic accuracy and therapeutic potency? A brief review of the relevant techniques and considerations in radioimmunoimaging/therapy provides an obvious starting point to begin to answer this question.

PET

PET surveys the biodistribution of a radiolabeled probe based on coincidence detection of roughly antiparallel 511 keV photons produced co-locally following positron decay, attenuation and subsequent annihilation of the emitted positron. A rigorous discussion of the underlying physics is outside the scope of this review, but has been well-undertaken elsewhere.(59,60) Following list-mode acquisition, the data are typically histogrammed (via e.g. Fourier rebinning) and tomographically reconstructed to obtain a 3D representation of the whole-body biodistribution of the tracer. PET has long been considered as, and remains, the gold-standard for quantitative molecular imaging in both clinical and preclinical oncology. Technical advances in PET instrumentation and image processing have increased the sensitivity, resolution, and quantitative accuracy by large extents. Though ultimately the technique remains limited by the physical properties of the radionuclide used, in addition to some radionuclide-independent aspects (e.g. annihilation photon acollinearity, scatter, detector geometry), we focus our attention here on the radionuclide dependent performance aspects in PET, as optimization is enabled by rational radionuclide selection dependent on thorough consideration of these factors.

Sensitivity in PET is determined by multiple factors. The rate of radiotracer decay together with the positron branching ratio govern the rate of positron decay; the system sensitivity is then a function of these factors, in addition to the system resolution (*vide infra*) and field of view. In part due to photon attenuation, detector geometrical considerations and photon scattering, PET detectors generally utilize a relatively wide photopeak energy window (~350–750 keV); energy deposit in the detector crystals within this range and within a defined coincidence timing window are “counted” as annihilation events. However, positron emission from certain radionuclides is accompanied by a gamma ray(s) also originating or downscattering to within this energy range, termed an “interfering” gamma; Table 2 indicates specific gamma emissions for each relevant radionuclide which are likely to degrade imaging performance in this manner. Such a gamma may be detected simultaneously with one annihilation photon or a second interfering gamma resulting in a false line of response assignment (i.e. mispositioning of the annihilation site), or simultaneously with both annihilation photons resulting in a triple coincidence event. These anomalies lead to image degradation through inaccuracy, information loss or increased noise, and therefore, energy windows may be narrowed in an effort to exclude competing gammas on the basis of energy, at the expense of counting statistics.

It is important to highlight that PET images positron annihilation events; the distribution thereof is distinct from that of the source radiotracer itself, and this has consequent implications with respect to spatial resolution (Figure 3). Positrons are subject to collisional and radiative mechanisms of energy loss following emission as outlined in the Bethe theory (61), and thus travel a finite, energetically and statistically regulated distance from their source before annihilation. The degree of uncertainty in the parent radionuclide's position is directly related to the positron range. The effects of positron transport phenomena vis-à-vis imaging are most profound in the preclinical arena, where positron range kernel of the system point spread function (PSF) generally dominates the contributions from photon acollinearity and detector geometry to the overall system PSF. Therefore, special care (e.g. partial volume correction, PSF modeling (62)) must be taken when quantifying uptake of high-energy positron emitters in small-scale imaging applications (i.e. when the diameter of the region of interest is approximately less 2–3 times the system full-width half-maximal resolution): for example in mice and rats, in clinical investigations of small tumors or other small anatomical structures, and in assessment of small-scale spatial heterogeneity of uptake in larger organs. Likewise, research design should include a preliminary assessment as to whether the nuclide in question is likely to enable the salient features the physiology to be resolved.

SPECT

SPECT, similar to PET, examines the biodistribution of a radiotracer through tomographic reconstruction of decay events based on photon detection. The difference lies primarily in how photon directionality is assessed: while lines of response (LOR) are established in PET via coincidence detection, SPECT relies on collimation for LOR assignment. Current state-of-the-art preclinical SPECT scanners generally utilize multi-pinhole collimator arrays with magnification to obtain sub-millimeter resolution capability. The gain in resolving power comes at the price of decreased sensitivity, as the uncertainty in photon directionality is inversely related to the number of photons rejected via collimation, and is thus nuclide dependent due to differential photon penetration at the pinhole edges as a function of photon energy (i.e. optimal resolution is achieved with relatively low energy gamma emitters, e.g. ^{99m}Tc). ^{131}I presents an interesting example due to equipment challenges relating to its energetic photon emissions as reviewed by Dewaraja et al. (65), and also due to former widespread use in radioimmunotherapy with [^{131}I]I-tositumomab (Bexxar®).

SPECT provides a distinct advantage in its direct capability of multiplexed data acquisition through simultaneous imaging of multiple tracers, which is possible based on identification of photon emissions from different radionuclides *via* energy discrimination. Such acquisitions reinforce antibody-based imaging approaches by enabling simultaneous interrogation of multiple antigens, as well as the combined interrogation of antigen status together with assessment of anatomic, metabolic or microenvironmental signatures (66–68). In a classic example of simultaneous dual-isotope SPECT, Kelly et al. demonstrated potential enhancement of metastatic lesion detectability in prostate cancer with [^{111}In]In-capromab pendetide (ProstaScint®), when augmented with an anatomic (vascular) signature *via* ^{99m}Tc -labeled RBCs in a 40-patient clinical trial (69). Currently the clinical relevance of

SPECT remains upheld do to its pivotal use as a theranostic tool, especially in dosimetric monitoring in radioimmunotherapy.

CLI

In addition to particle and high-energy photon (γ , X-rays) most medically relevant beta emitters (β^+ and β^-) produce intrinsic optical contrast *via* the Cerenkov effect, wherein blue-UV frequency photon radiation is produced during the ephemeral relaxation of the beta particle from super-relativistic phase velocities (i.e. Cerenkov luminescence; CL). This secondary CL is detectable with modern optical imaging instrumentation, and holds promise in clinical settings for intraoperative delineation of tumor margins (i.e. CLI-guided surgery). Furthermore, CLI enables non-invasive preclinical assessment of uptake of diagnostic and therapeutic nuclides that are difficult or impossible to assess using PET/SPECT, including alpha (e.g. actinium-225 (70)) and pure β^- emitters (e.g. yttrium-90 (71)). Further still, CLI makes use of relatively inexpensive and widely distributed small animal optical imaging systems, thus greatly broadening the capability for nuclear imaging research at institutions with less available funding support.

In contrast to PET, CLI favors high-energy beta-emitting radionuclides, e.g. ^{18}F < ^{89}Zr < ^{124}I < ^{68}Ga < ^{90}Y (72, 73). Namely, the advantages afforded by high energy beta emitters stems primarily from two factors: 1) a greater energy differential with respect to the Cerenkov emission threshold produces more photons along the beta trajectory (i.e. higher photon intensity), and 2) increased range of the beta particle results in a greater fraction of imagable photons created near the tissue surface where photon attenuation is decreased. While CLI remains largely a semi-quantitative planar imaging modality due to the non-penetrating nature of blue-UV frequency photons, tomographic techniques are currently being developed for small-scale applications including preclinical imaging and endoscopic techniques (Cerenkov Luminescence Tomography; CLT (74)). Several groups have already evaluated CLI in preclinical radioimmuno applications including tumor localization and intraoperative surgical guidance with promising results (71,75), as expected. CLI procedures implementing agents already approved for other procedures, will likely gain accelerated FDA approval.

Radionuclide and Chelator Selection and Relevant Effects on Biodistribution and Overall Imaging Performance

As a general guideline, the pharmacokinetics of the targeting vector should be well matched with the physical half-life of the radionuclide; the relatively long circulation times necessary for tumoral uptake of intact IgGs dictates the use of radionuclides with multi-hour physical half-lives (e.g. zirconium-89, copper-64; Table 2). In contrast, novel pretargeting strategies utilizing bioorthogonal *in vivo* click chemistry (e.g. inverse electron demand Diels-Alder; IEDDA) have enabled the use of rapidly clearing small molecule conjugates with corresponding short half-life tracers (e.g. fluorine-18, gallium-68 for immunoPET; Table 2), offering preferentially reduced patient radiation burden and shorter imaging timeframes (28,32,76–79). In preclinical imaging, these aspirations can become of secondary importance, affording considerable freedom to align imaging performance toward a specific scientific output using longer half-life nuclides where desired. As an example, indium-111

possesses a longer half-life of 2.8 days that is not optimal for pretargeted clinical imaging. Yet, its use in addressing a preclinical unknown related to the time-dependent on-target stability of CC49 mAb (and associated radioactivity) as-demonstrated by Rossin et al. (80), is entirely justifiable, considering future application of pretargeted CC49 radiotherapy utilizing a similarly long-lived therapeutic radionuclide analog.

With the exception of direct-labeling approaches utilized with e.g. radiohalogens, a chelating moiety is required to confine the radionuclide to the targeting vector – thus, the choice of radionuclide goes hand-in-hand with selection of an appropriate bifunctional chelator. A horde of such ligands is seen given a cursory historical glimpse at previous literature, and excellent comprehensive reviews outlining the selection of suitable chelators for radiometals and their conjugation to relevant targeting vectors are given elsewhere (79,81). For the sake of brevity and scope, here, we distill down the considerations presented in these perspectives to those most relevant to optimization for immuno-applications.

The chemistry required to bind the radiolabel to its respective vector must be time-prudent and suitably mild to avoid degradation of the vector, while also giving a reasonable radiochemical yield. Short half-life radionuclides necessarily require prompt reaction times but it is to note that the small-molecule (pre)targeting ligands they are utilized with in pretargeted immunoimaging/therapy, are often forgiving of higher temperature conditions for accelerated radiolabeling. On the other hand, intact antibodies and larger fragments are quite sensitive in this regard, and generally demand thermal conditions at or below the normal body temperature threshold. In case an intact IgG is required and high-temperature chemistry is unavoidable, a bifunctional ligand may be pre-labeled with the radioisotope before bioconjugation with the vector, or utilized in a pretargeting strategy to a similar end. Furthermore, the radiolabel-chelate complex must exhibit suitable kinetic stability over the application time course in order to maximize on-target signal and minimize potential dosimetric consequences of unbound activity (e.g. increased marrow dose from bone-seeking nuclides such as zirconium-89 or radiolanthanides). As such, intact IgGs have more stringent requirements for kinetic inertness due to long circulation times required for optimal targeting. Even still, some degree of decomplexation is inevitable. Optimal molecular imaging typically favors increased target-to-background contrast over absolute uptake; therefore, an imperfectly stable radionuclide-chelate pair may still be quite suitable if the accumulation site of the unbound radionuclide does not overlap with the tumor.

Assuming the radiolabel is stably retained, a particular advantage of imaging strategies implementing intact antibodies is their relative pharmacokinetic insensitivity toward radiolabel substitutions, and thus nuclide selection can often be conducted on the basis of resolution, dosimetric profile, chelation chemistry, etc. (*vide infra*) without fear of greatly perturbing the targeting efficiency. On the other hand, small molecule conjugates used in immunopretargeting, as well as derived IgG fragments (e.g. F(ab'), F(ab')₂, or scFv fragments), are more sensitive to the radiolabel/chelating moiety – in particular its overall charge – requiring additional consideration regarding changes in target-to-background uptake ratios and dosimetric implications when using non-isotopologs in a combined therapeutic/companion diagnostic role. While occasionally this manifests itself as a limitation, it should be noted that in many cases, a radiolabel-dependent biodistribution is

favorable when attempting to optimize target-to-background contrast. For example, hepatobiliary lesion identification may be improved by using a radionuclide/chelate complex bearing a non-neutral charge, which typically shifts clearance pathways toward renal excretion. Pharmacokinetic optimization with respect to charge dependence is facilitated by the broad spectrum of applicable radiometals (with multiple charge states to choose from) as well as their applicable bifunctional chelators. For instance, radiometals stably chelated by C-functionalized NOTA derivatives (e.g. maleimide-NOTA, *CheMaTech*) are generally similarly stably chelated by its N-functionalized analog NO2A (e.g. maleimide-NODA-GA, *CheMaTech*), but with the latter complexes exhibiting an additional unit of positive charge. An additional advantage provided by radiometals (over non-metallic radiolabels; e.g. radiohalogens) is their propensity to residualize in organelles following cellular internalization and lysosomal degradation, which in turn enhances retention at the target. In contrast, non-metallic radionuclides are typically not retained intracellularly and are more rapidly cleared from the target, leading to decreased target-to background activity ratios. Residualizing labels are therefore preferable when internalizing antibodies are utilized (82,83).

Dosimetric Considerations in Imaging

Radiation dose and exposure in all nuclear medical procedures is radionuclide dependent from macro- and microdosimetric standpoints due to differences in emission type (beta, photon, alpha, electron) and respective energy, half-life, range and linear energy transfer (LET), as well as biological factors including clearance kinetics and tissue radiosensitivity (84). An exhaustive discussion of radionuclide-dependent dosimetric implications is outside the scope of this work, and has been reviewed well elsewhere, particularly in the Medical Internal Radiation Dose Committee (MIRD; www.snm.org) and International Commission on Radiological Protection (ICRP; www.icrp.org) literature.

Although radiation exposure limits set by the US Nuclear Regulatory Commission for clinical diagnostic imaging are unequivocally safe, it is essential to ensure future imaging agents do not exceed these thresholds, and it remains desirable to minimize exposures to the largest extent possible, especially to enable repeated scanning. The average organ-level absorbed dose, $D(r_T, T_D)$, for a particular target organ, r_T , is expressed within the time-independent framework of the MIRD formalism (85) as:

$$D(r_T, T_D) = \sum_{r_s} \tilde{A}(r_s, T_D) S(r_T \leftarrow r_s)$$

where:

$$\tilde{A}(r_s, T_D) = \text{cumulated activity in source } r_s \text{ over time } T_D (\text{Bq} \cdot \text{s})$$

$$S(r_T \leftarrow r_s) = \text{Absorbed dose rate to target } r_T \text{ from sources } r_s, \text{ per unit activity } \left(\frac{\text{Gy}}{\text{Bq} \cdot \text{s}} \right)$$

This equation provides adequate context for discussion of the vector- and radionuclide-dependent aspects amenable to optimization. All variables related to uptake and clearance of the vector, as well as radionuclide decay and administered activity, are contained within the terms $\tilde{A}(r_s, T_D)$; $\tilde{A}(r_s, T_D)$ may be reduced through increasing the rate of clearance (e.g. using a truncated IgG fragment or pretargeting approach, as opposed to an intact IgG), utilizing a shorter half-life radionuclide, or using a nuclide with a high probability of emission of the desired radiation (e.g. high positron intensity for PET). On the other hand, the factors $S(r_T, \leftarrow r_s)$ are dependent on the temporally invariant aspects of radionuclide decay (types, intensities and energies of nuclear transitions) and the anatomy of the subject (organ dimensions/composition and their 3D spatial relationship). At the organ level, the self dose $S(r_T, \leftarrow r_T)$ is generally amplified when the energy of non-penetrating radiations (i.e. alpha, beta, electron) are increased, and also rises with increasing intensity of all nuclear transitions. The cross dose $S(r_T, \leftarrow r_s)$ for r_T, r_s is amplified by increasing beta energies/intensity, and photon intensity; the dependence of photon energy on S is complicated and less relevant for the present discussion. As a case in point, cobalt-55 and terbium-152 have equivalent half-lives and similar photon intensities. However, the comparatively high energy of terbium-152's positron emission (1.14 vs. 0.57 MeV) and lower positron intensity (which translates to an increased administered activity required for similar image quality) effectuate a less favorable dosimetric profile for terbium-152 vs. cobalt-55. Particularly when multiple decay aspects differ, comparison of dosimetric favorability by inspection is obfuscated; in that case, dosimetric analysis utilities such as OLINDA/EXM (Organ Level Internal Dose Assessment/Exponential Modeling; Hermes Medical Solutions) or phantom-based Monte Carlo calculations facilitate assessment of the dosimetric implications of radionuclide selection.

With regard to dose, understanding the spatial heterogeneity of tumors is valuable, particularly in certain applications of imaging in therapy planning (e.g. target definition). Imaging at low resolution can provide inaccurate assessment of the small-scale distribution of disease, and is confounded by differential accessibility of the tracer to the target cells or differential expression of the antigen, leading to unpredicted highly non-uniform tumor cell radiation exposures in a subsequent or concurrent therapeutic scenario. In particular, this becomes problematic in image-based dose assessment/planning for therapeutic nuclides with sub-millimeter particle ranges when the FWHM resolution of the imaging modality is large in comparison. On the other hand, therapy with energetic beta emitters (e.g. yttrium-90) is more forgiving in this respect, but at the cost of increased collateral damage to non-target tissues due to the long particle range (Figure 5). Ideally, a meaningful nuclear imaging-based assessment of heterogeneity, if desired, would be obtained with a nuclide that provides a system FWHM resolution much less than the mean particle range of the therapeutic nuclide to be utilized. While this is not feasible with short range radiation (e.g. alpha and Auger) and rarely possible in clinical practice with PET and SPECT due to current scanner limitations, small animal PET and SPECT can provide this level of resolution for a number of nuclides, maximizing translatable scientific output from preclinical studies. *Ex vivo* autoradiography can be utilized similarly.

The aim to optimize dosimetric aspects in preclinical models must continue to be an ideal; however, it also must be kept in mind that dosimetric observables in preclinical models may

mispredict, for better or for worse, clinical observables. For instance, the minor kinetic instability of [^{89}Zr]Zr-DFO-immunoconjugates is shown to result in elevated bone marrow dose in preclinical models; yet, immunoPET in clinical trials utilizing DFO chelation strategies for zirconium-89 have shown drastically reduced bone uptake, implying increased metabolism as a culprit of *in vivo* zirconium-89 release in murine models (86).

Radionuclides for Radioimmunotherapy – Particle Selection

The arsenal of available therapeutic radioisotopes includes alpha- (α), beta- (β^-) and Auger electron-emitting radionuclides, and encompasses a terrific expanse of decay pathways, effective range and relative biological effectiveness for implementation in cancer treatment (Table 4) (87). It is therefore critical to properly associate the physical properties of these therapeutic radionuclides to the tumor burden, radiosensitivity or homogeneity/heterogeneity in order to optimize the RIT efficacy.

With typical maximum kinetic energies spanning 0.3 and 2.3 MeV, and corresponding particle soft tissue ranges of 0.5 to 12 mm, β^- -emitting radionuclides have the lowest linear energy transfer (LET) ($\sim 0.2 \text{ keV}/\mu\text{m}$) of available therapeutic radioisotopes (Table 4; Figure 4). With such long reach, β^- -emitting radioisotopes can be a precious resource to counterbalance tumor heterogeneity but can result in concurrent toxicity to nontargeted tissues. On the opposite side of the spectrum, Auger electrons, which are emitted in cascade in response to electronic rearrangement in the daughter atom following e.g. electron capture or internal conversion, have very low energy ($< 25 \text{ keV}$) per particle. The correspondingly limited path length (2–500 nm) and high local density of Auger emission imparts a high LET (4–26 $\text{keV}/\mu\text{m}$) but restricts efficacy to single cells (Table 4; Figure 4). Finally, α -particles, naked ^4He nuclei with a +2 charge, are monoenergetically emitted with high initial kinetic energy (5–9 MeV) and corresponding particle range of a few cell diameters (50–100 μm) (Table 4; Figure 4). Due to their high mass and charge, alpha-emitters have therefore the highest LET ($\sim 80 \text{ keV}/\mu\text{m}$) and find particular application for the treatment of small clusters (e.g. micrometastases) or isolated single cancer cells (e.g. lymphoma).

Cytotoxic potency of these therapeutic particles relies on their ability to generate irreparable DNA breaks (whether via direct ionization or indirect effects, e.g. radical induced damage), ultimately leading to cell-cycle arrest, apoptosis, autophagy or necrosis (88–90). High LET particles, with dense ionization events along the particle track, have higher probability of causing irreparable DNA double-strand breaks as compared to low LET particles (Figure 4) (91). The relative biological effectiveness of α -particles with a deterministic endpoint (e.g., therapeutic efficacy or toxicity) lies between 4 and 7.5 and should be considered to predict their therapeutic efficacy or biological effects following accidental or environmental exposure (92). To fully benefit from their high LET, Auger electron emission must occur in close proximity of the nucleus/DNA due to their miniscule range; decay of Auger electron emitters in the cytoplasm, cell membrane or extracellular milieu has been shown to result in no enhancement of the toxicity as compared to low LET radiation (93). In the case of low LET β -particles and extranuclear Auger emission, probability of ionization of DNA is therefore low and cell death mechanisms mainly rely on indirect effects: in particular, formation of free radical oxygen species ($\bullet\text{OH}$) by β - or Auger electron radiations can result

in indirect single or double DNA strand breaks, both to target and surrounding nontarget cells (94,95). In the latter case, this effect, commonly referred as the “crossfire effect”, is suppressed in hypoxic environments due to lowered oxygen density, and thereby manifests itself as a mechanism of radioresistance in hypoxic tissues such as tumors (96). On the other hand, the escalated probability of direct DNA interaction with α -therapy confers a major advantage in this regard (97), and has been previously utilized to overcome radioresistance observed in analogous beta therapy(98,99). In the case of β -particle radioimmunotherapy, the crossfire effect, together with the long particle range of β -emission and antibodies’ long blood circulation time leads to increased non-specific irradiation of, e.g., hematopoietic stem cells in the bone marrow (100). Such toxicity constitutes a severe limitation to the administered activity to patients and thus to radioimmunotherapeutic potential. Other indirect cell killing mechanisms have been demonstrated. *In vitro* evidence of radiation-induced biological bystander effect has been reported with low and high LET radiation (101–103). In this process, unirradiated cells in the neighborhood of irradiated cells have been shown to die from DNA damage due to oxidative mechanisms and stress-inducible proteins, yet the full mechanistic details remain unclear. Finally, the abscopal effect, hypothesized to arise from an upregulated systemic immune response to radiotherapy, results in an antitumor effect in remote lesions and has been reported *in vitro* after alpha irradiation (104,105). Recent observation of such effects opens the way toward combination therapies aligning radioimmunotherapy with targeted immunomodulators and immune checkpoint blockade.

Selection of the ideal therapeutic radionuclide should not only take into account the radionuclide decay mode and its cytotoxic potential, but should also consider radionuclide physical half-life and the coemission of imageable γ -photons, characteristic X-rays or bremsstrahlung radiation that could be used for the quantification of target uptake, dosimetry or for the monitoring therapeutic response (Table 4). Radionuclides simultaneously possessing suitable imaging and therapeutic emissions (e.g. iodine-131) have been historically used for RIT purposes; however, the γ -rays co-emitted together with β -particle constitute a radiation risk and sometimes requires patient hospitalization/isolation. Radionuclide availability and production mode must necessarily be considered: albeit very attractive, due to their high cytotoxic potencies, many α -emitters suffer from severe shortage due to exotic methodology required for production (e.g., ^{211}At [28 MeV α -particle beam] (106)) or special restrictions stemming from non-proliferation mandates relating to legacy nuclear material (^{225}Ac , ^{213}Bi [^{229}Th generator] (107)). Radiolabeling method and coordination chemistry can also restrict the choice of the radionuclide (81). Limitations intrinsic to the use of α -emitting radionuclides should also be considered: many such α -emitters decay through the emission of multiple α - or β -emitting radioactive daughters (e.g. ^{225}Ac , ^{213}Bi , ^{211}At , ^{212}Bi , ^{227}Th) (Table 4) (108). While this property can result in increased delivered cytotoxicity (“nanogenerator” or “in vivo generator” effect), the recoil energy (>100 keV) that accompanies the α -emission can, if not controlled, result in the consistent release of the decay daughters from the coordination complex, breaking any coordination or chemical bonds between the daughter and the radioimmunoconjugate chelating agent. The release of decay daughters ultimately leads to their redistribution from the targeted site and depends mainly on the intrinsic affinity and excretion pathway of the

particular daughter. Accumulation in the bone or kidneys has been observed and can result in undesired dose to these radiosensitive tissues (109,110). Strategies to limit daughters' redistribution are being explored; however, application to RIT remains limited and internalization of the radioimmunoconjugates or nanocarrier sequestration remain the only currently effective options to deal with the problem at the source (111,112). It has to be mentioned that the use of diuretic has been shown to accelerate the excretion of free daughters in the kidneys (e.g., ^{221}Fr , ^{213}Bi) (113). Furosemide and spironolactone are currently used in clinical trials together with α -RIT (^{225}Ac -Lintuzumab [NCT02575963] and ^{212}Pb -TCMC-Trastuzumab [NCT01384253]) (114,115).

Optimization of Biodistribution at the Organ Level

In order to maximize the uptake at the targeted site, the choice of the antigen-antibody is of primary concern. Ideally, the antigen should be uniformly and highly expressed ($>100,000$ copies/cell) among the cell population comprising the tumor, and not shed but easily accessible. However, uniform cellular distribution of the antigen is not a requirement for molecular imaging or therapeutic purposes. The release of membrane-associated proteins into soluble effectors referred as antigen shedding or ectodomain shedding is a major obstacle for radioimmuno-imaging/therapy (116). Solutions to overcome antigen sinks include methods such as the "preloading" with unlabeled antibody (117) or the pretargeting method (26) and render radioimmuno-imaging/therapy possible with circulating antigen. The majority of relevant antigenic targets are therefore surface-expressed and easily accessible from the blood and extracellular fluid. Additionally, the antibody should have a high binding affinity ($K_d \sim 1$ nM) and the immunoreactivity of the radiolabeled immunoconjugates should remain as high as possible ($> 90\%$). Depending on the radionuclide of interest and its potential accumulation sites, the fate of the antigen-antibody complex should be taken into account. If internalized, radioiodinated antibody conjugates are known to be intralysosomally degraded, resulting in the exocytosis of radioiodinated tyrosine or free radioiodine in the blood stream (118). In that case, a non-internalizing antigen-antibody complex should be considered to reduce non-specific and undesired toxicity from free radioiodine. In contrast, the previously mentioned sequestration of residualizing radiometals within cellular organelles, act in favor of internalizing antigen-antibody complexes.

Historically targeted antigens include haematopoietic clusters of differentiation (CD), CD20 and CD22 overexpressed in B cell non-Hodgkin lymphomas or CD33 and CD45 for acute myeloid leukaemia (119). Anti-CD20 antibodies were conjugated to the β -emitters ^{90}Y and ^{131}I . Encouraging clinical results (e.g., higher overall response rate, higher rate of complete response as compared to control) lead to the FDA approval of [^{90}Y]Y-ibritumomab (Zevalin®) in 2002 for the treatment of relapsed or refractory low-grade, follicular B-cell non-Hodgkin lymphoma (120). Similarly, FDA approved a second RIT, [^{131}I]I-tositumomab (Bexxar®) for the treatment of relapsed, refractory and transformed indolent lymphomas (121). For still uncertain reasons, these two approved RITs did not find broad clinical application, resulting in poor sales and discontinuation of Bexxar® in 2014. Anti-CD33 antibody (HuM195; Lintuzumab) was conjugated to both α - and β -emitters (^{213}Bi , ^{225}Ac and ^{131}I) and studied in phase I clinical trials for the treatment of primary refractory or

relapsed advanced myeloid leukemia with or without combination with cytarabine (122–125). Reduced myelosuppression together with improved absorbed dose ratios between the leukemic sites and whole body were strong arguments in favor of the use of the α -emitter conjugates (123). Actinium-225 was considered due to its longer half-life and increased lethal potency as compared to ^{213}Bi . The maximum tolerated dose was determined to be 111 kBq/kg in a phase I clinical trial in relapsed or refractory advanced myeloid leukemia (myelosuppression as a dose-limiting toxicity) and no evidence of radiation-induced nephrotoxicity due to ^{225}Ac -daughters redistribution was observed (124). Other antigenic targets include glycoproteins such as A33; enzymes such as prostate specific membrane antigen (PSMA); signal transduction cell membrane receptors such as the human epidermal growth factor receptors (HER family); tumor vasculature components such as vascular endothelial growth factor receptor (VEGFR) or stromal cells (CD25+ T cells) (119, 126).

Tumor response will be highly dependent on the *in vivo* biodistribution of the radioimmunoconjugates. Radiation doses to the tumor and therapeutic indices (i.e., ratios of absorbed dose to the tumor as compared to radiosensitive tissues) should be optimized. Radiosensitive tumors, such as hematologic cancers (e.g., leukemias, lymphomas) should receive low-LET radiation doses between 1,500 and 2,000 cGy to expect durable response (119,127). Solid tumors, in contrast, generally exhibit high levels of radioresistance (e.g., brain, thyroid tumors) and therefore require escalated doses of ~3,500 up to 10,000 cGy (119,127). Normal tissue radiosensitivity must be mutually considered and doses should ideally remain below the tolerance doses leading to a 5% complication rate at 5 years ($\text{TD}_{5/5}$): dose to the kidneys should not exceed 1,500–2,000 cGy and bone marrow dose should remain below 150 cGy (119, 127,128). Consequently, therapeutic indexes should be at least >10 for the kidneys and >50 for the bone marrow.

Modulation of the radioimmunoconjugates *in vivo* biodistribution can be performed using different antibody fragments (e.g., F(ab)_2 , scFv-Fc, Fab, scFv) (Figure 1). Blood dose has been shown to be significant lower with antibody fragments, mostly due the lack of Fc binding region responsible for prolonged blood retention (13,129,130). Even though higher at early time points, dose rate to the tumor of anti-CEA F(ab')_2 has been shown to fall off rapidly as the clearance of these fragments is faster compared to the intact antibody (130). Fractionation with anti-CEA F(ab')_2 also show a significant reduction of the treatment therapeutic efficacy. The use of serum albumin binding moieties to increase the blood half-life of small molecules or affibodies found promising applications in radiotherapy and can be similarly applied to antibody fragments (131,132). Davé *et al.* recently designed an antibody format referred as Fab-dsFv, composed of the variable fragment of a humanized albumin-binding antibody fused to the C-termini of Fab constant domain (Figure 7A) (133). The anti-albumin variable fragment resulted in an increase serum half-life in both mice ($t_{1/2} = 2.9$ days) and cynomolgus monkeys ($t_{1/2} = 7.9$ days) (133). A sequential delivery approach, referred as pretargeting, has also been developed in order to improve therapeutic indices (Figure 7B) (25). The separation of the antibody administration from the radionuclide has been explored clinically using a non-radiolabelled bispecific monoclonal antibody (TF2) and a radiolabelled (^{111}In for imaging and ^{177}Lu for therapy) hapten peptide (IMP288) (134). This approach resulted in higher tumor-to-tissue ratios (> 20 at 24 h). High doses of ^{177}Lu (2.5–7.4 GBq) were well tolerated with transient grades 3–4

thrombocytopenia in 10 % of the patients (134). On a preclinical level, the use of a tetravalent bispecific antibody with dual specificity for the GPA33 antigen and ^{177}Lu -DOTA-Bn complex or the use of the inverse electron-demand Diels-Alder reaction between a transcyclooctene conjugate and a radiolabelled (^{177}Lu) Tetrazine-DOTA derivative, both resulted in significant therapeutic response in human xenografts with dosimetry suggesting amenability to clinical translation (34,135). Finally, when possible, compartmental-RIT can be considered to deliver high dose to the tumor and reduce non-specific toxicity. As opposed to systemic administration, compartmental administration consists in the specific delivery of the therapy into a specific body cavity such as the peritoneum, pleura or intrathecal space. Compartmental-RIT was demonstrated to be especially successful in the case of recurrent metastatic central nervous system neuroblastoma (Figure 7C) (119,136). Median survival after compartmental ^{131}I -monoclonal antibody therapy targeting GD2 or B7H3 was 33 months as compared to 6 months with non-compartmental RIT (136). Intraperitoneal cavity injection is also preferred for the treatment of progressive ovarian carcinoma with [^{212}Pb]Pb-TCMC-trastuzumab as minimal radioimmunoconjugate redistribution was observed outside of the peritoneal cavity (115).

Optimization of the Sub-Cellular Biodistribution

Optimization of the radionuclide distribution at the cellular level is particularly important in the case of Auger electron RIT as the benefits of this high LET particle are only observed if the radionuclide is localized in intimate proximity to the cell nucleus and DNA. Few options exist to drive the distribution at a cellular level; one such strategy utilizes rapidly internalized antigens such as CD5, CD22 or PSMA. This approach demonstrated promise in both preclinic and a clinical trial using J591, an IgG1 monoclonal antibody targeting the PSMA external domain and ^{111}In (γ - and Auger electron-emitter) (137). Favorable tumor uptake was observed as the antigen-antibody complex internalized and ^{111}In residualized in the cell (137). Fast blood clearance and tumor-to-blood ratios (6.07 ± 1.79 at 6 days post-injection) supported the clinical translation of this RIT (137). A phase I clinical trial involving patients with progressive metastatic prostate cancer despite androgen deprivation was conducted to determine the optimal antibody mass for immunotherapy (unlabeled J591) and for targeted radiation therapy purposes ([^{111}In]In-DOTA-J591) (138). The optimal dose for RIT was determined taking into account risks of hematotoxicity and bone marrow suppression. Hepatic saturation was observed between 10 and 25 mg and this highest value was chosen as optimal dose as it properly balanced hepatic uptake and rapid serum clearance (138). Unfortunately, the antitumor effect of this RIT was limited; only one patient out of 14 benefited from a sustained PSA decline of 90% (138). The choice of the population in this clinical trial is questionable; patients at earlier disease stage with no established metastasis and rising PSA after prostatectomy or radiation would likely constitute a more suitable target population.

Another approach to drive antibody internalization is through appendage of a cell penetrating peptide such as TAT (GRKKRRQRRRPPQGYG). This approach was used in order to target γH2AX , one of the first molecular responses observed after DNA double-strand breaks (139,140). The latter can be driven by radiation therapy, but can also arise from oncogenic stress, genomic instability or chemotherapy. Since γH2AX forms foci of up

to thousands of copies, it appeared as an interesting target for imaging and therapy purposes. Cornelissen *et al.* conjugated an anti- γ H2AX antibody (commercially available), to the aforementioned cell penetrating peptide and further functionalized this antibody-conjugate with a radionuclide or a fluorophore (140). The presence of a TAT was shown to drive the antibody internalization and the conjugation of an Auger electron-emitter (^{111}In) was evaluated. Recently, a mathematical model revealed that double-strand break kinetics remain unaffected by the introduction of an anti- γ H2AX antibody and that the presence of an Auger electron could result in the induction of new DNA damage (141).

Radioimmunoimaging and Radioimmunotherapy in Perspective

The transition of molecular imaging and targeted radiotherapies from a one-size-fits-all approach toward personalized medicine has been empowered through identification of novel cell surface biomarkers and engineered antibodies targeting these, in synchrony with advancements in radionuclide production and chelation chemistry. Accordingly, immunoimaging will continue to provide an enhanced molecular fingerprint of disease to which cognate radioimmunotherapeutics can be precisely and personally tailored; both of which will clearly see expanded clinical roles moving forward. While admittedly, scientific hindrances remain which must be addressed, and the usual regulatory, logistical, and fiscal hurdles must be overcome. It should be mentioned, however, that progress in radioimmunoimaging and RIT directly facilitates progress in related disciplines (e.g. immunotherapy, ADCs) and therefore a wide potential profit margin exists which is increasingly leading pharma to invest in more antibody-related clinical trials. Such investment will ultimately lead to reduction in cost, enabling access to a broader patient population.

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Figure 1.

In vivo pharmacokinetics of tumor-targeted antibody-based vectors as a function of their molecular weight and size. (Left to Right) Full-length (intact) antibodies yield the highest tumor uptake (% ID/g) and tumor-to-blood ratios (image contrast/therapeutic index) at later time points (>72 h post-injection). This is mostly attributed to their high molecular weight (~150 kDa) making them ineligible for renal clearance and the *in vivo* recycling of the antibodies by the neonatal Fc-receptor (FcRn) into the bloodstream – thus increasing their bioavailability to target tumors over multiple passes while within the systemic circulation. Various engineered formats of the antibody such as scFv-Fc-fusion constructs (~100–150 kDa) and minibodies are intermediate antibody fragments that achieve relatively lower uptake in the tumor, but their faster *in vivo* clearance profile enables them to yield high tumor-to-background contrast at early time points. These constructs have been synthesized for the purpose of next day (24 h p.i.) radioimmunoimaging. Finally, a combination of their structural composition (lack of Fc-mediated recycling), extremely rapid renal clearance due to low molecular weight of the smaller tumor-targeting antibody-based constructs (diabodies and scFv(s)) limits their *in vivo* bioavailability and uptake in the tumor. However, diabodies (~50 kDa) can be used for same-day imaging and yield high contrast images as early as 2–4 h p.i. Adapted with permission from Knowles and Wu (13).

Figure 2.

The influence of immunodeficiency status on tumor uptake of the targeting vector. (A) Volume-rendered PET-CT images of nude (Nu/Nu) – less immunodeficient mice versus NOD-SCID gamma (NSG) – highly immunodeficient mice bearing subcutaneously xenografted small cell lung cancer (SCLC) tumors on the right flank. Each animal was injected with the same amount [7.4–9.3 MBq; (200–250 μ Ci), 35–44 μ g] of a ^{89}Zr -labeled humanized monoclonal antibody targeting delta-like protein3 (DLL3) in SCLC (57). However, the remarkably different uptake patterns of the same radiotracer in two different biological backgrounds are evident from the higher uptake (indicated by easier delineation of the organs and a relatively higher intensity of signal) of the antibody-based tracer in the spleen, bones and the liver of the highly immunodeficient NSG mouse strain. Images were scaled individually - though not comprehensible from these images, the tumor uptake in NSG mice is significantly lower than in the nude mice. (B) A graph depicting the inverse correlation between immunodeficiency status of the preclinical mouse model background and tumor uptake versus a progressively increasing non-target accumulation of humanized (non-Fc-silent) antibody-based targeting vectors in mice with highly immunodeficient status.

Figure 3.

Decay properties and imaging characteristics of common positron emitters for traditional and pre-targeted immunoPET. (A) Positron energy spectra of selected PET nuclides outlined in Table 2 (data obtained from the RADAR database). (B) Continuous slowing down approximation (CDSA) range of beta particles in selected ICRP reference tissues (data obtained from the NIST database). (C) Comparison of image quality and resolution with six clinically relevant positron emitters in a preclinical Derenzo phantom (63) with rod diameters of 0.8, 0.9, 1.0, 1.1, 1.2, and 1.3 mm (counter-clockwise from bottom-right sector). (D) ^{124}I -labeled (top, $E_{\beta^+, \text{avg}} = 820 \text{ keV}$) versus ^{89}Zr -labeled (bottom, $E_{\beta^+, \text{avg}} = 396$

keV) α HER2 Fab PET maximum intensity projections (64). Note the variation in clearance route arising from chemical differences imparted to the Fab construct *via* labeling with different radionuclides. Figure (C) adapted from Bunka et al. (63), with permission. Figure (D) adapted from Mendler et al. (64), with permission.

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Figure 4. Relevant considerations for optimization of imaging performance with antibodies and fragments. Adapted from Zhou, et al. (82).

Figure 5. Spatially heterogeneous radiation dose deposition for different therapeutic radionuclides. A) Heterogeneous uptake present in high-resolution preclinical SPECT imaging. B) Segmentation of diseased tissue / source definition for dose calculations (40% max SPECT signal thresholding of tumor-associated activity). C) Radiation dose rate* computed in PHITS v2.88 with CT overlay; note the more uniform dose deposition with ^{90}Y but significant irradiation of surrounding healthy tissue. *Non-penetrating component only; photon components are assumed negligible in calculation; dose rates are individually normalized to the maximum voxel value for each image.

Figure 6.

DNA damage stemming from interaction of alpha, beta, and Auger emissions. Note the high density of ionization along the alpha particle track, and spatially compact region of ionization events produced *via* Auger cascade, reflective of the high LET (and cytotoxic potential) of these radiations.

Figure 7.

Optimization of radioimmunotherapy at the tissue level. A) Schematic representation of an Fab-dsFv (133). An disulfide bonded Fab is conjugated to an anti-HSA variable fragment (Fv) domain containing an incorporated disulfide bond via an amino acid linker. B) Schematic representation of the pretargeting strategy (25). C) Sagittal PET images of [¹²⁴I]I-8H9 antibody following intrathecal injection via an Ommaya reservoir. Images shows good accumulation at the leptomeningeal tumor sites (119).

Table 1

Immunodeficient mouse strains commonly used in oncology research.

Mouse Strain (vendor)	Genotype	Reason for immunodeficiency
Athymic Nude (CRL)	CrI:NU(NCr)- <i>Foxn1</i> ^{nu}	Lacks Thymus: No T-cells
Fox Chase SCID (CRL)	CB17/lcr- <i>Prkdc</i> ^{scid} /lcrIcoCrI	SCID Mutation: Lack of B- cells and T-cells
Fox Chase SCID Beige (CRL)	CB17.Cg- <i>Prkdc</i> ^{scid} <i>Lysf</i> ^{hg-J} /CrI	SCID Mutation: Lack of B- cells and T-cells Beige Mutation: Defective NK Cells
NOD SCID (CRL, JAX)	NOD.CB17- <i>Prkdc</i> ^{scid}	SCID Mutation: Lack of B- cells and T-cells NOD background: Deficient NK Cell Function
BALB/c-Nude (CRL)	CAnN.Cg- <i>Foxn1</i> ^{nu} /CrI	Lacks Thymus: No T-cells
Nude (NU/NU)	CrI:NU- <i>Foxn1</i> ^{nu}	Lacks Thymus: No T-cells
Nude Mice – CD1 (CRL)	CrI:CD1- <i>Foxn1</i> ^{nu}	Transfer of nude gene from CrI:NU- <i>Foxn1</i> ^{nu} to CD-1 mouse through several crosses and back-crosses. Lacks Thymus: No T-cells
NCI SCID/Ncr (CRL)	CB17/lcr- <i>Prkdc</i> ^{scid} /lcrCr	SCID Mutation: Lack of B-cells and T-cells
NCG (CRL)	NOD- <i>Prkdc</i> ^{em26Cd52} <i>Il2rg</i> ^{em26cd22} /NjuCrI	CRISPR/Cas9 edited <i>Prkdc</i> / <i>Ilr2g</i> loci in NOD/Nju mouse carrying SIRP α gene mutation No B-cells and T-cells Decreased NK cells
NSG (JAX)	NOD.Cg- <i>Prkdc</i> ^{scid} <i>Il2rg</i> ^{tm1Wjl} /SzJ	SCID Mutation: Lack of B-cells and T-cells No NK Cells Defective Macrophages and Dendritic Cells No Complement Defective Cytokine Signaling
NRG (JAX)	NOD.Cg- <i>Rag1</i> ^{tm1Mom} <i>Il2rg</i> ^{tm1Wjl} /SzJ	Rag1 Mutation: Lack of Mature B-cells and T-cells No NK Cells Defective Macrophages and Dendritic Cells No Complement Defective Cytokine Signaling
R2G2 (Envigo)	B6;129- <i>Rag2</i> ^{tm1Fwa} <i>Il2rg</i> ^{tm1Rsky} /DwlHsd	Rag2 Mutation: Lack of Mature B-cells and T-cells No NK Cells Defective Macrophages, Neutrophils and Dendritic Cells No Complement Defective Cytokine Signaling
CIEA NOG (Taconic)	NOD.Cg- <i>Prkdc</i> ^{scid} <i>Il2rg</i> ^{tm1Sug} /JicTac	SCID Mutation: Lack of B-cells and T-cells No NK Cells Defective Macrophages and Dendritic Cells Reduced Complement Activity

Mouse Strain (vendor)	Genotype	Reason for immunodeficiency
		Defective Cytokine Signaling

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Table 2

Selected radionuclides for traditional and pre-targeted immunoPET.

Nuclide	Use	$t_{1/2}$	β^+ Intensity	$E_{\beta^+,avg}$ [MeV]	CDSA Range [mm] ^a	Main gamma Emissions [keV] β^+ (intensity)	Selected chelators	Production
⁷² As	PET, CLI	26.0 h	87.8%	1.17	5.16	834 (81.0%), 629 (8.1%) β	Thiol-based	⁷² Se/ ⁷² As generator
⁷⁶ Br	PET, CLI	16.2 h	55.0%	1.18	5.21	559 (74.0%) β , 657 (15.9%) β , 1854 (14.7%), 1216 (8.8%), 2950 (7.4%), 2793 (5.6%)	N/A	Cyclotron: ⁷⁶ Se(p,n) ⁷⁶ Br
⁵⁵ Co	PET, CLI	17.5 h	76.0%	0.570	2.06	931 (75.0%), 477 (20.2%) β , 1408 (16.9%), 1316 (7.1%)	NOTA, DOTA	Cyclotron: ⁵⁸ Ni(p, α) ⁵⁵ Co
⁶⁴ Cu	PET, CLI, beta therapy	12.7 h	17.6%	0.278	0.73	N/A	DiamSar,NOTA, DOTA, TETA	Cyclotron: ⁶⁴ Ni(p,n) ⁶⁴ Cu; Reactor: ⁶⁴ Zn(n,p) ⁶⁴ Cu
¹⁸ F	PET, CLI	110 m	96.7%	0.250	0.62	N/A	NOTA (AIF)	Cyclotron: ¹⁸ O(p,n) ¹⁸ F
⁶⁶ Ga	PET, CLI	9.49 h	57.0%	1.75	8.23	1039 (37.0%), 2752 (22.7%), 834 (5.9%), 2190 (5.3%)	NOTA, DOTA, TRAP	Cyclotron: ⁶³ Cu(α ,n) ⁶⁶ Ga
⁶⁸ Ga	PET, CLI	67.7 m	88.9%	0.830	3.37		NOTA, DOTA, TRAP	⁶⁸ Ge/ ⁶⁸ Ga generator
¹²⁴ I	PET, CLI	4.18 d	22.7%	0.820	3.32	603 (62.9%) β , 1691 (11.2%), 723 (10.4%) β	N/A	Cyclotron: ¹²⁴ Te(p,n) ¹²⁴ I
⁵² Mn	PET, CLI	5.59 d	29.4%	0.242	0.59	1434 (100%), 935 (94.5%), 744 (90%) β , 1334 (5.07%)	DOTA	Cyclotron: ⁵² Cr(p,n) ⁵² Mn
⁹⁰ Nb	PET, CLI	14.6 h	51.2%	0.660	2.50	1129 (92.7%), 2319 (82.0%), 141 (66.8%), 2186 (18.0%)	DFO	Cyclotron: ⁹⁰ Zr(p,n) ⁹⁰ Nb
⁴⁴ Sc	PET, CLI	3.97 h	94.3%	0.632	2.36	1157 (99.9%)	DOTA	⁴⁴ Ti/ ⁴⁴ Sc generator
¹⁵² Tb	PET, CLI	17.5 h	20.3%	1.14	5.00	344 (63.5%), 271 (9.53%), 586 β (9.21) β , 779 (5.54%)	DOTA	Accelerator: Tantalum spallation (proton/heavy ion)(142)
⁸⁶ Y	PET, CLI	14.7 h	31.9%	0.660	2.50	1077 (82.5%), 628 (32.6%) β , 1153 (30.5%), 777 (22.4%), 1921 (20.8%), 1854 (17.2%), 443 (16.9%) β , 703 (15.4%)	DOTA, DTPA	Cyclotron: ⁸⁶ Str(p,n) ⁸⁶ Y
⁸⁹ Zr	PET, CLI	78.4 h	22.7%	0.396	1.23	909 (99.0%)	DFO, HOPO	Cyclotron: ⁸⁹ Y(p,n) ⁸⁹ Zr

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DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; NOTA, 1,4,7-triazacyclononane-1,4,7-triacetic acid; DTPA diethylenetriaminepentaacetic acid; DiamSar, 1,8-Diamino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]-eicosane; TRAP, 1,4,7-triazacyclononane-1,4,7-tris[methyl(2-carboxyethyl)phosphinic acid]; DFO, N'-[5-(Acetyl-hydroxy-amino)pentyl]-N-[5-[3-(5-aminopentyl)-hydroxy-carbamoyl) propanoylamino]pentyl]-N-hydroxy-butane diamide; HOPO, 1,5,10,14-tetra (1-hydroxy-2-pyridone-6-yl)-1,5,10,14-tetraazatetradecane

λ_p Particle range in the continuous slowing down approximation, for the average energy emission

γ_q Denotes potentially interfering gamma

$\gamma_{\geq 5\%}$ Denotes gammas with intensity > 5%; excludes annihilation photons (511 keV)

Table 3

Desirable radionuclide decay characteristics for high-performance immunoimaging.

Imaging Modality	Performance Criteria	Advantages Provided
PET	High positron intensity	High sensitivity, decreased image degradation from interfering gammas
	Low positron energy	High resolution, decreased patient radiation burden
	No or low-abundance accompanying decay modes	Decreased detection of false coincidences, decreased radiation burden to patient and personnel
SPECT	High gamma intensity	High sensitivity
	Low gamma energy (<~300 keV)	High resolution, high quantitative accuracy
CLI	High beta energy (positron or negatron)	High sensitivity, enhanced detectability at sub-surface depth
All modalities	Generator-based production	Decouples radiopharmacy from the need for on-site cyclotron and otherwise required shipment logistics
	Theranostic isotope pair	Identical pharmacokinetics for therapeutic and diagnostic components; faster FDA approval
	High kinetic stability	Increased diagnostic and therapeutic sensitivity, decreased non-target radiation exposure
	Swift radiolabeling kinetics	Minimal thermal or chemical degradation of the targeting vector, minimal tracer decay pre-administration

Table 4

Selected radionuclides for radioimmunotherapy.

Nuclide	Use	$t_{1/2}$	Primary therapeutic emissions			Main Gamma Emissions [keV] ^b (intensity)	Recommended chelators	Production
			Emission	Intensity/Net Yield	Energy [MeV] _{avg}	CDSA Range ^a		
²²⁵ Ac	Alpha therapy, CLI	9.92 d	α	400%	5.8–8.4	47–86 μ m	DOTA	²²⁹ Th Generator;
			β	300%	0.198–0.660	0.43–2.5 mm		Accelerator: ²³² Th proton spallation; Cyclotron: ²²⁶ Ra(p,2n) ²²⁵ Ac
⁷⁷ As	Beta therapy, CLI	38.8 h	β^-	100%	0.226	0.53 mm	Thiol-based	⁷⁷ Ge/ ⁷⁷ As Generator
²¹¹ At	Alpha therapy	7.21 h	α	100%	5.9–7.5	49–71 μ m	N/A	Cyclotron: ²⁰⁹ Bi(α ,2n) ²¹¹ At
²¹² Bi	Alpha therapy, CLI	60.6 min	α	100%	6.05	51 μ m		²²⁴ Ra Generator
			β^-	100%	0.56–0.77	2.0–3.1 mm	727 (6.67%)	
²¹³ Bi	Alpha therapy, CLI	45.6 min	β^-	300%	0.198–0.660	0.43–2.5 mm	DOTA, NETA, DTPA	²²⁹ Th Generator
			α	100%	5.9–8.4	49–86 μ m		
⁶⁴ Cu	PET, CLI, beta therapy	12.7 h	β^+	17.6%	0.278	0.73 mm	Diamsar, NOTA, DOTA, TETA	Cyclotron: ⁶⁴ Ni(p,n) ⁶⁴ Cu;
			β^-	38.5%	0.191	0.41 mm		Reactor: ⁶⁴ Zn(n,p) ⁶⁴ Cu
⁶⁷ Cu	Beta therapy, SPECT, CLI	61.8 h	β^-	100%	0.141	0.25 mm	See above	Reactor: ⁶⁷ Zn(n,p) ⁶⁷ Cu;
								Accelerator: ⁶⁸ Zn(p,2p) ⁶⁷ Cu
⁶⁷ Ga	SPECT, Auger therapy	3.26 d	Auger e ⁻	229%	>0.0075	>5 μ m	NOTA, DOTA, TRAP	Cyclotron: ⁶⁸ Zn(p,2n) ⁶⁷ Ga
			CE	33%	~0.08–0.09	0.10–0.12 mm		(21.4%), 300 (16.6%)
¹²³ I	SPECT, Auger therapy	13.2 h	Auger e ⁻	108%	>0.023	>11 μ m	N/A	Cyclotron: ¹²⁴ Xe(p,2n) ¹²³ I
¹³¹ I	Beta therapy, SPECT, CLI	8.04 d	β^-	100%	0.182	0.38 mm	N/A	Reactor: ¹³⁰ Te(n, γ) ¹³¹ I
								(7.16%), 284 (6.12%)
¹¹¹ In	SPECT, Auger therapy	2.80 d	Auger e ⁻	116%	>0.019	>7 μ m	DOTA, DTPA, H ₃ Octapa	Cyclotron: ¹¹¹ Cd(p,n) ¹¹¹ In
			CE	14%	~0.14–0.22	0.24–0.51 mm		(94.1%)

Nuclide	Use	$t_{1/2}$	Primary therapeutic emissions			Main Gamma Emissions [keV] ^b (intensity)	Recommended chelators	Production
			Emission	Intensity/Net Yield	Energy [MeV] _{avg}	CDSA Range ^a		
¹⁷⁷Lu	Beta therapy, SPECT, CLI	6.65 d	β−	100%	0.134	0.23 mm	113 (6.17%), 208 (10.4%)	DOTA, DTPA, H ₄ Octapa Reactor: ¹⁷⁶ Lu(n,γ) ¹⁷⁷ Lu
²¹²Pb	Alpha therapy, CLI	10.6 h	α	100%	6.05	51 μm	239 (43.6%), 727 (6.67%)	²²⁴ Ra Generator
¹⁸⁶Re	Beta therapy, SPECT, CLI	3.72 d	β−	100%	0.347	1.0 mm	137 (9.47%)	Reactor: ¹⁸⁵ Re(n,γ) ¹⁸⁶ Re
¹⁸⁸Re	Beta therapy, SPECT, CLI	17.0 h	β−	100%	0.763	3.0 mm	155 (15.6%)	Reactor: ¹⁸⁷ Re(n,γ) ¹⁸⁸ Re
⁴⁷Sc	Beta therapy, SPECT, CLI	3.35 d	β−	100%	0.162	0.31 mm	159 (68.3%)	⁴⁷ Ca/ ⁴⁷ Sc Generator; Reactor: ⁴⁷ Ti(n,p) ⁴⁷ Sc
¹⁴⁹Tb	Alpha therapy	4.1 h	α	16.7%	3.97	27 μm	352 (29.4%), 165 (26.4%), 389 (18.4%), 652 (16.2%), 853 (15.5%), 511 (14.2% ; pos. annihilation)	Accelerator: Tantalum spallation (proton/heavy ion) (142)
⁹⁰Y	Beta therapy	64.0 h	β−	100%	0.934	3.9 mm	N/A	Reactor: ⁹⁰ Zr(n,p) ⁹⁰ Y

NETA, [4-[2-(bis-carboxymethylamino)-ethyl]-7-carboxymethyl]-[1,4,7-triazonan-1-yl]-acetic acid; DOTAM/TCMC, 1,4,7,10-tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane; H₄Octapa N,N'-bis(6-carboxy-2-pyridylmethyl)-ethylenediamine-N,N'-diacetic acid; MAG3, N-[N-[(benzoylthio)acetyl]glycyl]glycylglycine.

^aParticle range in the continuous slowing down approximation, for the average energy emission (beta) or most probable energy emission (alpha) in soft tissue

^cDenotes gammas with intensity > 5%