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Understanding the In Vivo Fate of Radioimmunoconjugates for PET and SPECT

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Abstract

Over the past 25 years, antibodies have emerged as extraordinarily promising vectors for the delivery of radionuclides to tumors for nuclear imaging. While radioimmunoconjugates often produce very high activity concentrations in target tissues, they also are frequently characterized by elevated activity concentrations in healthy organs as well. The root of this background uptake lies in the complex network of biological interactions between the radioimmunoconjugate and the subject. In this review, we seek to provide an overview of these interactions and thus paint a general picture of the *in vivo* fate of radioimmunoconjugates. In order to cover the entire story, we have divided our discussion into two parts. First, we will address the path of the entire radioimmunoconjugate as it travels through the body. And second, we will cover the fate of the radionuclide itself, as its course can diverge from the antibody under certain circumstances. Ultimately, our goal is to provide the nuclear imaging field with a resource covering these important — yet often underestimated — pathways.

Keywords

Monoclonal antibodies; antibodies; radioimmunoconjugates; pharmacokinetics; pharmacodynamics; FeRn; radioiodine; radiometals; copper-64; zirconium-89; indium-111; positron emission tomography; PET; immunoPET; single photon emission tomography; SPECT; immunoSPECT

INTRODUCTION

The advent of monoclonal antibodies as medical tools unquestionably represents one of the most important developments in healthcare over the last quarter century. Indeed, over this period, monoclonal antibodies (mAbs) have emerged as a leading class of biological drugs.^{1–2} If we include the 10 antibodies that received USFDA approval in 2017, a total of 63 monoclonal antibodies are now approved and marketed in the United States alone.

Moreover, a growing pipeline of monoclonal antibodies in clinical trials suggests that several antibodies will garner regulatory approval per year in the near future.³ This growth has been fueled in part by several biotechnological advances, including improvements in (a) hybridoma and phage display technology, (b) recombinant DNA technology, (c) transgenic mice, and (d) industrial bioreactors.

Like many other fields, the nuclear imaging community — and specifically those interested in the imaging of cancer — has been quick to adopt mAbs. Indeed, the high affinity and specificity of mAbs for their *in vivo* targets have made antibodies extremely promising vectors for the delivery of radionuclides to tumor tissue for positron emission tomography (PET) and single photon emission computed tomography (SPECT). Over the past 25 years, a wide variety of radioimmunoconjugates labeled with nuclides ranging from ¹¹¹In to ⁸⁹Zr have been developed and translated to the clinic. Yet while radioimmunoconjugates often produce very high activity concentrations in target tissues, they also are frequently characterized by elevated activity concentrations in healthy organs such as the liver and spleen. The root of this background uptake lies in the complex network of interactions between the radioimmunoconjugate and the biochemical milieu of the subject.

In this review, we seek to provide an overview of these interactions and thus paint a general picture of the *in vivo* fate of radioimmunoconjugates. More specifically, we will seek to answer several questions about radioimmunoconjugates that are often asked by researchers new to the field, including “Why are their serum half-lives so long?”, “Why don’t they penetrate tumors particularly well?”, and “Why do radioimmunoconjugates produce so much background uptake in the liver (or the spleen)?” To provide answers to these queries, we have had to draw from a wide variety of fields, including literature on immunoPET, immunoSPECT, antibody pharmacokinetics, antibody-drug conjugates, immunotherapy, and immunology. In order to cover the entire story, we have divided our discussion into two parts. First — in *The Fate of the Antibody* — we will address the path of the *entire* radioimmunoconjugate as it travels through the body, covering topics such as absorption, distribution, elimination, and interaction with the immune system. And second — in *The Fate of the Radionuclide* — we will cover the journey of the radiolabel *itself*, as its course can diverge from the antibody under certain circumstances. Ultimately, our goal is to provide the nuclear imaging field with a resource covering these important yet often underestimated pathways and thereby facilitate the incorporation of these critical lessons into the creation of the next generation of radioimmunoconjugates.

THE FATE OF THE ANTIBODY

The Biochemical Processes Governing the In Vivo Behavior of Antibodies

Simply put, pharmacokinetics is the study of what the body does to a drug. When considering the pharmacokinetics of radioimmunoconjugates, it is absolutely critical to remember that the body has extensive machinery for the processing of its own endogenous antibodies that are created by B-cells and mass-produced by plasma cells in response to an infection or disease. As a result, any exogenous mAb — such as a radioimmunoconjugate — will be acted upon by the same cellular machinery that has been put in place for the catabolism of endogenous immunoglobulins (Ig).

Administration and Absorption: The first step in the journey of any radioimmunoconjugate is entering the body. Most tumor-targeting mAbs are administered intravenously. This route provides the most direct access to the body's vascular network for the systemic distribution of the immunoglobulin. In recent years, however, cost and logistical factors have led to the approval of the subcutaneous administration of a few antibody formulations.⁴⁻⁵ This approach relies on the diffusion of the mAb into the lymphatic system, which ultimately drains into the blood. While this method produces bioavailabilities between 50–80% over a period of 2–8 days⁴, it is relatively slow and subjects antibodies to pre-systemic elimination by soluble peptidases in the interstitial space of the extracellular matrix surrounding the site of injection. Collectively, these drawbacks as well as other practical concerns make subcutaneous administration unsuitable for radioimmunoconjugates.^{4, 6-7} As a result, radioimmunoconjugates are administered strictly intravenously.

Regardless of the route of administration, circulating antibodies are immediately susceptible to cell uptake via fluid-phase pinocytosis or receptor-mediated endocytosis. Thankfully, antibodies can be rescued from intracellular catabolism through their interaction with the neonatal Fc-receptor (FcRn), which is expressed by a variety of cells in the body, including the endothelial cells lining blood vessels, hepatocytes, macrophages, monocytes, and dendritic cells (Figure 1).^{4, 8} Specifically, FcRn binds to the C_H2-C_H3 domains within the F_c portion of the antibody when the latter is engulfed within the acidified endosome (pH ~ 6.0–6.5) of the cell (Figure 2). The FcRn-bound antibody is protected from lysosomal degradation and is translocated to the apical or basolateral surface of the cell where it dissociates from the FcRn under conditions of neutral pH and is released back to the extracellular space or the blood. The FcRn-mediated recycling of antibodies is a key mechanism for maintaining the homeostasis of endogenous immunoglobulins and an important contributor to the bioavailability and long serum half-lives of exogenously introduced antibodies.⁹ To wit, mice lacking FcRn have demonstrated lower bioavailability of exogenous antibodies.¹⁰

Distribution: The next step along the antibody's journey involves its transport from the blood vessels to the interstitial space of tissues where it can find tumor cells and bind to its intended target. Owing to their high molecular weight and polarity, antibodies diffuse across vascular endothelial cell membranes extremely slowly. As a result, antibodies rely on convective transport to facilitate their extravasation from blood vessels to the interstitial space of tissues.⁴ Convective transport, however, depends on the fluid flow rate from the blood to the tissue as well as the 'sieving effect' exercised by the paracellular pores of the vascular endothelium. The latter often results in higher concentrations of monoclonal antibodies being driven into tissues — such as the spleen, bone marrow, and liver — which have naturally fenestrated capillaries with large paracellular pores and sinusoids with ~100 nm clefts.^{4, 11} On the other hand, the presence of tight junctions in the vascular endothelium of the brain prevents the convective transport of antibodies across the blood brain barrier.⁴

The distribution of antibodies *within* tumor tissue occurs via diffusion and convection. Yet again, however, the macromolecular size of an antibody becomes a limiting factor, and both these locomotion processes proceed extremely slowly. The diffusion coefficient for a 150

kDa monoclonal antibody within solid tumors has been reported to be $\sim 1.3 \times 10^{-8} \text{ cm}^2/\text{s}$, whereas that of a macromolecular protein such as bovine serum albumin is $\sim 1.2 \times 10^{-7} \text{ cm}^2/\text{s}$.^{12–13} The intratumoral movement of an antibody is further hindered by interstitial fluid pressure (IFP) and binding site barriers, both of which act to further complicate the efficient transport of antibodies in solid tumors.^{13–16}

Interstitial fluid pressure presents strong a *physical* resistance to the movement of antibodies within tumors. The binding site barrier, however, represents a *biochemical* resistance to the movement of the antibody that stems from its high affinity for its intended target. The latter phenomenon limits the antibody to the tumor cells that are situated in close proximity to the point of extravasation. This localization of antibodies within the perivascular space along the tumor periphery and gives rise to a heterogeneous pattern of uptake in solid tumors. One can imagine that antibodies that bind to tumor-associated shed antigens face a greater challenge from the binding site barrier due to the densely packed shed antigen present within the extracellular spaces of the tumor interstitium.¹⁷ Preclinical studies by Rudnick *et al.* and Glatt *et al.* have shown that antibodies with moderate binding affinity for the tumor target were able to achieve higher tumor uptake than counterparts with high affinity for the same target.^{18–20} However, since lowering the binding affinity of the antibody can compromise its specificity for binding to the target, it has been proposed that the binding site barrier may also be overcome by increasing the dose of unlabeled antibody. Doing so would facilitate the saturation of the target in the perivascular space of the tumor while allowing the radiolabeled antibody to extravasate further and achieve better tumor penetration.^{21–22}

Biotransformation and Elimination: Of the three modes of drug elimination — excretion, secretion, and biotransformation (via metabolism or catabolism) — the third is known to be the major contributor to the systemic elimination of monoclonal antibodies. Like endogenous immunoglobulins, exogenous antibodies are broken down into peptides within the reticuloendothelial system. The uptake of antibodies for intracellular catabolism occurs via 3 pathways: (i) non-specific fluid phase endocytosis (pinocytosis), which occurs throughout the body in endothelial cells lining the blood vessels; (ii) receptor-mediated endocytosis, which is carried out by Fc-receptors expressed by immune cells; and (iii) target-mediated disposition (TMD), which occurs when the Fab portions of the antibody bind to targets such as antigens expressed on tumor cells.

Once it has distributed inside a tumor, an antibody is predominantly eliminated by intracellular catabolism via target-mediated disposition (TMD). As the name implies, a prerequisite for TMD is the binding of the monoclonal antibody's Fab domains to the target antigen. The finite expression of the target on a tumor makes this a 'saturable' process. The rate of this elimination pathway is governed by five factors: (i) the level of target expression; (ii) the dose of the antibody administered; (iii) the rate of target turnover; (iv) the kinetics of receptor internalization; and (v) the kinetics of intracellular catabolism. The strong dose dependence of this process causes it to follow non-linear kinetics, whereby the clearance of the antibody decreases as a function of dose.⁴ Moving beyond tumor tissue, pharmacokinetic models that account for the FcRn-mediated recycling of antibodies have shown that the liver acts as the principal site of antibody catabolism. However, when antibodies do not undergo receptor-mediated endocytosis and target-mediated disposition, organs such as the skin,

muscle, and gastrointestinal tract contribute significantly to the *in vivo* catabolism of antibodies as well.^{6, 8, 10, 23} Notably, the size of full-length antibodies limits their glomerular filtration and renal excretion. However, full-length antibodies *can* be excreted via the kidneys in humans with pathologies such as multiple myeloma, which is frequently accompanied by renal dysfunction.²⁴ Furthermore, antibody fragments including F(ab')₂ and Fab' are excreted through the kidneys in both mice and humans.^{24–25}

At this point, a brief shift in focus to the Fc region of the antibody can help us understand the role that this domain plays in elimination. Residues close to the hinge region within the distal portion of the C_H2 domain interact with Fc-gamma receptors (FcγRs) expressed by several immune effector cells. This interaction can trigger the phagocytosis and catabolism of antibodies. However, at present, there is no clear consensus on the ability of FcγRs to influence the *in vivo* biodistribution and elimination of antibodies. Arguably, the relatively large pool of endogenous immunoglobulins should outcompete any exogenously injected antibodies for binding to FcγRs. Clinical investigations such as the one performed by Cartron *et al.* have demonstrated the role of FcγRs in the response to therapy with monoclonal antibodies. This work focused on a cohort of non-Hodgkin's lymphoma patients bearing a single nucleotide polymorphism (SNP) in the gene for FcγRIIIa that gives the receptor higher affinity for IgG. It was found that patients with this SNP exhibited improved survival following treatment with rituximab compared to a cohort of patients who were given the same dose of the antibody but had a low affinity polymorph in their genotype.²⁶ Despite these intriguing results, subsequent investigations found no statistically significant differences between the *in vivo* pharmacokinetics of rituximab in patients with high, medium, and low affinity FcγRIIIa polymorphs.^{21, 27} Similarly, preclinical studies using various FcγR knockout mice revealed no change in the disposition of targeted versus non-specific antibodies.^{21, 28–29} Taken together, these results support the role of the IgG-FcγR interaction in mediating immune effector cell functions and suggest that this interaction does not necessarily have any influence on the *in vivo* pharmacokinetics of antibodies. However, the *in vivo* pharmacokinetics could change if the exogenous antibody forms large immunocomplexes *in vivo* or has been specifically engineered to bind FcγR with high affinity.²¹

Finally, as discussed earlier, the expression of FcRn on a variety of cells plays a key role in both the homeostasis of endogenous immunoglobulin levels as well as the long serum half-lives of exogenously administered antibodies. Under physiologic conditions, endogenous immunoglobulins are present at concentrations between 65–130 μM (50–100 g in 5 L of blood in an average healthy adult human) and comprise ~ 20% of the total plasma protein by weight. While FcRn-mediated recycling *is* efficient, the limited expression of the receptor on cells limits the capacity of the process. This is an important consideration for the administration of exogenous antibodies. Notably, exogenous antibodies that are administered at 10 mg/kg do not perturb the *in vivo* homeostasis or efficiency of the FcRn recycling system. However, there *are* two scenarios in which the FcRn system can be saturated, leading to a dramatic systemic elimination of antibodies: (1) multiple myeloma patients have excessively high plasma IgG concentrations (100 mg/mL) and (2) extremely high concentrations (2 g/kg) of intravenous immunoglobulin (IVIG) are often used to treat patients suffering from autoimmune diseases.^{4, 30} Given that radioimmunoconjugates are

typically administered in doses <100 mg, there is very little likelihood that immunoPET or immunoSPECT could interfere with the FcRn-mediated recycling system.

IgG Subclass and In Vivo Behavior

Perhaps not surprisingly, the *in vivo* fate of IgGs also depends upon their subtype. There are four subclasses of IgG antibodies — IgG₁, IgG₂, IgG₃, and IgG₄ — and each has a distinct yet critical role to play in the immune response. IgG₁ antibodies are the most prevalent form and are induced primarily by both soluble protein antigens and membrane proteins. IgG₂ antibodies, in contrast, are almost solely responsible for the response to bacterial capsular polysaccharide antigens. IgG₃ antibodies are often the first to respond to viral infections and are particularly potent inducers of effector functions. And finally, IgG₄ antibodies are often formed in response to long-term exposure to allergens.

While the 4 subclasses share 90% of their amino acid sequence, small differences in their structures can translate into dramatic changes in their *in vivo* behavior. For example, the length of the hinge region — the flexible linker between the Fab arms and the Fc region — varies from 12 amino acids for the IgG₂ and IgG₄ subtypes to 15 amino acids for the IgG₁ subtype and 62 amino acids for the IgG₃ subtype (Figure 3).³¹ The length of this linker influences the affinity of each immunoglobulin for FcγRs receptors. More specifically, because the binding site is located in the lower hinge region, it can be partially or totally shielded by the Fab arms (Figure 2). As a result, a longer and more flexible hinge region facilitates stronger interactions between the immunoglobulin and FcγRs receptors, making IgG₁ and IgG₃ antibodies more potent inducers of effector mechanisms than IgG₂ and IgG₄ antibodies. Variations in the amino acid composition of the hinge regions, specifically the number of cysteine residues, can also result in different numbers of disulfide bonds between the heavy chains, ranging from 2 for the IgG₁ and IgG₄ subclasses to 11 for the IgG₃ subtype (Figure 3).

As we have noted, members of the IgG₂ and IgG₄ subclasses have lower affinity for FcγRs than their IgG₁ and IgG₃ cousins. As a result, the former have often been selected as platforms for the development of therapeutic antibodies in cases in which the recruitment of effector function may not be required. However, both of these subclasses seem to be sensitive to the redox environment in serum, leading to undesirable re-arrangements of their disulfide bonds.³² In the case of IgG₂ antibodies, fully functional covalent dimers can be formed, presumably to improve the avidity of these antibodies to their target.³³ Another phenomenon observed with IgG₂ antibodies is the shuffling of disulfide bonds to generate three different isoforms: IgG₂-A, IgG₂-B, and IgG₂-A/B (Figure 4A). Although the serum half-lives of the isoforms remain identical, the conversion of IgG₂-A to IgG₂-B induces a loss of activity in some cases.³⁴ IgG₄ antibodies present another unique feature: they can exchange Fab arms *in vivo*!^{35–36} More specifically, two different IgG₄s can recombine to form a pair of monovalent-bispecific antibodies (Figure 4B).³⁷

The structural differences between the IgG subclasses can also affect their pharmacokinetics. For instance, as we have explained earlier, the half-life of IgG antibodies is related to the interaction between the FcRn receptor and the C_H2-C_H3 interface of the immunoglobulin. In the case of IgG₃ antibodies, a crucial histidine at position 435 is replaced with arginine. This

lowers the affinity of IgG₃ antibodies for the FcRn receptor and results in a dramatically shorter serum half-life: 7 days compared to 21 days for IgG₁, IgG₂ and IgG₄ antibodies.³¹

Modulating the *In Vivo* Behavior of Antibodies

In the preceding pages, we have addressed the principal biochemical processes that govern the *in vivo* fate and pharmacokinetics of antibodies. In this section, we will ‘zoom in’ and cover several properties of immunoglobulins that have been artificially modulated — with varying degrees of success — to ‘improve’ the *in vivo* behavior of antibodies. Again, as above, much of this information has been gleaned from the antibody and antibody-drug conjugate literature. However, the overarching lessons apply to radioimmunoconjugates as well.

Fc-Mediated Interactions: In an effort to produce therapeutic antibodies with longer-lived serum half-lives, several laboratories have synthesized Fc-mutated variants of antibodies that have stronger binding to FcRn at pH 6 but little to no binding to the receptor at pH 7.4. For example, the M252Y/S254T/T256E — nicknamed YTE — mutant of an antibody targeting respiratory syncytial virus (RSV) has shown an *in vivo* half-life of more than 60 days, 3 times longer than that of its parent antibody.^{5, 38} Conversely, antibodies with shorter *in vivo* half-lives can be created as well. One way to achieve this is by mutating key amino acid residues such as I253, H310, and H435 within the Fc portion of the antibody to lower the affinity of the antibody for FcRn and thus prevent FcRn-mediated recycling.³⁹ Furthermore, the oxidation of the methionine residue at position 252 within the C_H2-C_H3 domain during the production of an antibody can decrease its binding affinity for FcRn. This has also been shown to dramatically reduce an antibody’s *in vivo* serum half-life.^{5, 40}

A different approach to shortening the *in vivo* half-life of antibodies is predicated on the highly pH-driven nature of the Fc-FcRn interaction. It has been reported that antibodies having identical Fc regions but different antigen binding Fab domains can exhibit different binding affinities for FcRn, ultimately resulting in dramatically different *in vivo* serum half-lives. Interestingly, in this work, the serum half-lives of the antibodies correlated strongly with their kinetics of dissociation from FcRn at pH 7.4. In other words, antibodies with slower FcRn dissociation kinetics at pH 7.4 had shorter serum half-lives.^{5, 41} A similar observation was reported for a pair of interleukin-targeting antibodies with different Fab arms but almost identical Fc domains, identical binding affinities to FcRn at pH 6.0, and similar isoelectric points (pI). In this case, the antibody that had a patch of positively charged amino acid residues in the variable (F_v) domains of its Fab region displayed stronger binding to FcRn and only dissociated at pH >7.4. Predictably, the variant bearing a patch of positively charged amino acid residues in its Fab region had a significantly shorter *in vivo* half-life (~9 days) compared to its counterpart (~22 days).^{5, 42}

Glycosylation: Given the dominant role of FcRn as a mediator of the *in vivo* fate of antibodies, it is noteworthy that the FcRn-mediated recycling of antibodies is *independent* of their glycosylation status. Both genetically aglycosylated and chemoenzymatically deglycosylated variants have been reported to have serum half-lives similar to those of their glycosylated counterparts.^{5, 43} Furthermore, while the deglycosylation of antibodies can

abrogate Fc-Fc γ R interactions, it is generally thought that this phenomenon will not influence the *in vivo* pharmacokinetics due to the occupancy of Fc γ R sites by endogenous Ig(s). On the other hand, the rapid clearance of glycosylated antibodies with high mannose glycans has been attributed to the processing of these antibodies by specific receptors, namely the mannose receptor (ManR) and the asialoglycoprotein receptor (ASGPR). Sinusoidal endothelial cells and hepatic parenchymal cells such as hepatocytes express ManR(s), whereas non-parenchymal cells of the liver such as the Kupffer cells are known to express ASGPR(s).⁵

Charge: Because antibodies are glycoproteins with varying compositions of amino acids and sugars, they can have different isoelectric points. In general, antibodies are slightly more basic (pI > 6) than most serum proteins (pI < 5.5).⁴⁴ This facilitates the purification of antibodies and allows them to interact with anionic glycan chains on cell surfaces as well as heparin sulfate proteoglycans present in the extracellular matrix. Antibodies can be ‘cationized’ to confer a more basic pI or ‘anionized’ to provide a more acidic pI. Importantly, ‘cationized’ antibodies are characterized by rapid clearance from circulation, short serum half-lives, and increased binding to tissues. In some cases, this augmented binding can be non-specific due to the electrostatic interactions between the ‘cationized’ antibody and negatively charged cell surfaces *in vivo*. Li *et al.* have recently shown that an antibody with a higher (more basic) pI produces more accumulation in the liver and spleen than an antibody with a lower (more acidic) pI.^{5, 44–45} On the other hand, antibodies modified to have more acidic pIs tend to repel negatively charged cell surfaces, thereby minimizing non-specific binding in both target and non-target tissues while concomitantly increasing serum half-lives. However, work from the groups of Khawli and Igawa has demonstrated that shifts in pI of at least 1–2 units are needed in order to produce meaningful alterations in the *in vivo* behavior of charge-modified antibodies.^{5, 46–47}

In light of this article’s focus on radioimmunoconjugates, it is important to note that the attachment of bifunctional chelators to antibodies can influence the charge and biodistribution of antibodies as well. Despite a growing appreciation for the importance of site-specific modification, the vast majority of radioimmunoconjugates are synthesized via the indiscriminate coupling of amine-reactive bifunctional chelators to lysine residues on the surface of the immunoglobulin.^{48–49} Not surprisingly, this approach can alter the pI of immunoconjugates relative to their parent antibodies.^{50–51} This, in turn, can impact both the physicochemical properties and *in vivo* behavior of the immunoconjugate if the change in pI is >1–2 units. In addition, the conjugation of bifunctional chelators and prosthetic groups to IgGs can also dramatically affect the *in vivo* biodistribution of the resultant radioimmunoconjugate if the cargoes are inadvertently attached within the antibody’s antigen-binding domains, thus impairing the immunoreactivity of the construct. Ultimately, all alterations to the surface of an antibody should be performed cautiously, as these efforts can inadvertently (a) alter the tertiary structure of the antibody, (b) introduce new antigen determinant sites on the antibody, and (c) result in the loss of the antibody’s affinity for its antigen and/or FcRn.

Aggregation: While the inter-chain disulfide bonds of the hinge region are important for the stability of an antibody, much of its macromolecular structure is held together by intra- and inter-domain hydrophobic interactions, hydrogen bonds, and van der Waals forces. These non-covalent interactions are critical for maintaining the tertiary structure of the immunoglobulin. However, non-covalent interactions can also be detrimental to the *in vivo* performance of antibodies. For example, hydrophobic patches of residues in the complementarity determining regions (CDR) and the Fc domain have been implicated in the aggregation of antibodies, though this behavior varies on an antibody-to-antibody basis. The process of modifying antibodies to form radioimmunoconjugates and antibody drug conjugates (ADC) makes them particularly susceptible to aggregation via the unfolding of subdomains and the exposure of residues and regions that are hotspots for aggregation.⁵² This phenomenon has been reported more frequently in the ADC world, as ADCs are often found to be more hydrophobic and prone to aggregation than their parent antibodies, due in part to the hydrophobicity of their cytotoxic payloads.^{52–54} As expected, the *in vivo* pharmacokinetic profiles of ADCs with higher drug-to-antibody ratios (DAR) differ significantly from those of their counterparts with lower DARs. While the former are more cytotoxic *in vitro*, they are cleared much more rapidly than the latter and are less effective *in vivo*.^{55–56}

Unsurprisingly, the same principles extend to radioimmunoconjugates. The conjugation of hydrophobic chelators to antibodies can also perturb the tertiary structure of the immunoglobulins and increase their propensity for aggregation. Lub-de-Hooge *et al.*, for example, have reported this behavior in their investigations using ¹¹¹In-labeled trastuzumab.⁵⁷ Likewise, desferrioxamine — the current ‘gold standard’ chelator for ⁸⁹Zr — is a rather hydrophobic molecule and has been known to create immunoconjugates that are prone to aggregation.^{58–59} Price *et al.* have shown that the over-conjugation of DFO to trastuzumab yields radioimmunoconjugates with high chelator-to-antibody ratios and high specific activities but poor *in vivo* biodistribution profiles due to their increased propensity for aggregation.⁶⁰ Specifically, this study demonstrates that aggregation prompts an increase in the uptake of radioimmunoconjugates in the liver of tumor-bearing mice. Intriguingly, it remains unclear whether hepatocytes in the parenchyma of the liver or non-parenchymal cells such as the Kupffer cells and endothelial cells of the liver are responsible for catabolizing aggregated proteins.^{61–65} It is also important to emphasize that aggregation is not the *only* cause of elevated uptake in the liver. Indeed, the *in vivo* formation of soluble immunocomplexes between radioimmunoconjugates and shed antigen can boost liver uptake as well.^{66–69} This phenomenon is distinct from the hepatic accumulation of protein aggregates, though, as the accumulation of immunocomplexes in the liver occurs soon after the administration of the radioimmunoconjugate and can be alleviated by the co-injection of unlabeled target-specific antibody.

Immunogenicity: Like most xenobiotic drugs, monoclonal antibodies are capable of eliciting anti-drug antibody (ADA) responses.^{4–5} This can impact both the *in vivo* fate of the monoclonal antibody and — far more importantly — the subject to whom the antibody was administered. While many of the problems with ADA responses have been overcome due to the field’s shift to humanized and fully human antibodies, some of these agents may

nonetheless elicit ADA responses (from <1% up to 10%).^{8, 70} An infamous case is that of Humira (adalimumab), a TNF alpha-targeting fully human antibody that provokes an ADA response in 28% of patients.^{5, 71} Recent reports have shown that the co-administration of methotrexate as an immunosuppressant with adalimumab might help overcome the ADA-driven clearance and immunogenicity of the antibody.^{8, 72}

THE FATE OF THE RADIONUCLIDE

Up until this point, we have been drawing upon data from biochemistry, cell biology, physiology, pharmacology, and biophysics to describe the factors that govern the behavior and pharmacokinetics of antibodies. Yet the focus of this review is not antibodies but rather *radioimmunoconjugates*. This is an important distinction. Of course, many of the important lessons that we have learned regarding the *in vivo* fate of antibodies also apply to radioimmunoconjugates. However, our discussion cannot stop there. The very entity that makes radioimmunoconjugates useful for immunoPET and immunoSPECT — the radionuclide — demands further attention. Most of the time, the radionuclide behaves exactly as we would hope: as a simple passenger along for the ride. However, in some circumstances — such as following the catabolism of the immunoglobulin — the paths of the antibody and the radionuclide can diverge. In these cases, it is critical to understand the fate of the radionuclide itself, as it can produce signal (and radiation dose) regardless of whether or not it remains attached to the antibody.

In the second half of this review, we will turn our attention to these radionuclides. We will provide an overview of the different radionuclides that have been used in immunoPET and immunoSPECT probes and discuss what is known regarding the *in vivo* fate of these nuclides when they are part of radioimmunoconjugates. In addition, we will address several other important lessons on the *in vivo* fate of antibodies that have been gleaned from the preclinical and clinical study of radioimmunoconjugates. The following section will be organized by radionuclide, as there are several considerations that are specific to individual isotopes.

Before we begin, a brief introduction to the structure of a radioimmunoconjugate might be helpful. General speaking, a radioimmunoconjugate has three parts: (a) an antibody, (b) a radionuclide, and (c) a linker between the radionuclide and the antibody.

Radioimmunoconjugates labeled with metallic radionuclides have a fourth component: a chelator whose purpose is the stable sequestration of the radiometal during the radioimmunoconjugate's *in vivo* journey. The identity of the antibody itself is, of course, determined by its molecular target. The radionuclide is chosen primarily on the grounds of its physical properties. Specifically, the radioactive emission must be appropriate for the desired imaging modality (either photons for SPECT or positrons for PET), and the radionuclidic half-life must be compatible with the multi-day serum half-life of the antibody. The choice of the linker between the moieties is governed by several factors, including the desired regioselectivity and site-specificity of the bioconjugation reaction. And finally, the chelator is selected in response to the radiometal, as different radiometals can have dramatically different coordination chemistries. With this quick anatomy lesson complete, it is now time to move on to our first radionuclide.

The Isotopes of Iodine

From a nuclear medicine perspective, iodine is a remarkably versatile element. Indeed, there are four different radioisotopes of iodine that can be used for nuclear imaging and therapy. Specifically, ^{123}I and ^{125}I are gamma emitters suitable for SPECT imaging, ^{124}I is a positron-emitting isotope used for PET imaging, and ^{131}I emits both β - and γ - rays and can thus be harnessed for both SPECT and radioimmunotherapy (RIT). Traditionally, the radioiodination of antibodies has been performed via the direct electrophilic radioiodination of tyrosine residues within the antibody to create radioiodotyrosines. Technically speaking, such directly labeled mAbs are not “radioimmunoconjugates” *per se*; however, several approaches for the synthesis of radiolabeled immunoconjugates based on radioiodinated prosthetic groups have gained popularity in recent years (*vide infra*). Interestingly, several factors — including cost, availability, and ease of radiochemistry — led to the widespread study of ^{131}I -labeled constructs for RIT prior to the advent of therapeutic radioimmunoconjugates labeled with β -emitting radiometals such as ^{90}Y , ^{177}Lu , and ^{67}Cu . As a result, many of the lessons learned about the *in vivo* behavior of radioiodine-bearing antibodies stem from preclinical and clinical investigations of ^{131}I -labeled radioimmunoconjugates.

The chief concern surrounding the *in vivo* fate of radioiodinated antibodies is the ease with which the radionuclide can be separated from the antibody itself. Along these lines, several *in vitro* studies have demonstrated that the cellular internalization of radioiodinated antibodies leads to their rapid degradation within lysosomes and the extracellular release of monoiodotyrosine (Figure 5).^{73–75} This metabolite is further broken down by deiodination enzymes, releasing free radioiodide into the bloodstream. This release of radioiodide into the blood stream creates two interrelated problems:

- First, the radioiodine will be taken up by any tissues that express the sodium-iodide (Na^+/I^-) symporter, an integral membrane protein present in the thyroid gland and the stomach among other tissues. This phenomenon has been reported in several preclinical *in vivo* studies.^{76–78} Moving to the clinic, the uptake of radioiodine in the thyroid was also observed by De Nardo *et al.* in ^{131}I -Lym1-treated patients with non-Hodgkin’s lymphoma and chronic lymphocytic leukemia despite attempts to block this phenomenon via the infusion of Lugol’s solution or a saturated solution of potassium iodide.^{79–80} Interestingly, however, no hypothyroidism was detected during follow-up in these patients over several years.
- Second, the lysosomal degradation of radioiodinated antibodies results in the rapid *in vivo* clearance of radioiodine from all tissues except those that metabolize or process iodine. Theoretically, this could be beneficial for nuclear imaging and therapy, as it leads to lower retention of radioactivity in non-target organs. However, this phenomenon translates into far lower activity concentrations in tumor tissue compared to those created by radioimmunoconjugates labeled with residualizing radionuclides such as ^{90}Y , ^{177}Lu , $^{64/67}\text{Cu}$ or ^{89}Zr . A study by De Nardo *et al.* comparing ^{67}Cu - and ^{131}I -labeled Lym-1 antibodies in patients with non-Hodgkin’s lymphoma

demonstrated that the mean biological tumor half-life of ^{67}Cu in the tumor was 8.8 days compared to only 2.3 days for ^{131}I .⁸⁰

In order to circumvent this limitation, several residualizing prosthetic groups have been developed to help trap the nuclide within the lysosomes of target cells. These include non-metabolizable carbohydrates,^{81–85} positively- or negatively-charged aromatic compounds,^{86–89} bulky ionic polyhedral boron clusters,⁷⁶ and short D-amino acid peptides that are resistant to proteolytic degradation (Figure 6).^{90–93} Diethylenetriaminepentaacetic acid (DTPA) has also been added to some short D-amino acid peptides owing to its own residualizing properties.^{94–95} In murine models of cancer, these strategies have been shown to increase the retention of radioiodine in tumor tissue as well as reduce the uptake of radioiodine in the thyroid.^{81–92, 94–96} Unfortunately, however, radiolabeling antibodies using these prosthetic groups is often less efficient than direct radioiodination. In addition, these residualizing prosthetic groups can also slow the clearance of radioactivity from non-target tissues. For example, radioimmunoconjugates bearing carbohydrate-based prosthetic groups have displayed higher uptake in the liver, whereas antibodies conjugated to positively charged residualizing agents have produced higher activity concentrations in the kidneys.^{85,88} To the best of our knowledge, no radioimmunoconjugates bearing any of these residualizing agents have reached clinical trials, leaving room for further improvement prior to clinical implementation.

Radiometals

Over the past two decades, researchers have increasingly turned to the array of metallic radionuclides for immunoPET and immunoSPECT. Radiometals not only have promising physical properties but also can help eschew some of the issues surrounding the non-residualizing isotopes of radioiodine. As we mentioned in our introduction to this section, radiometals — unlike radiohalogens — require a chelator for their stable sequestration *in vivo*. Not surprisingly, different radiometals are compatible with different chelators. As a result, a wide range of bifunctional chelators have been developed and evaluated in both the laboratory and the clinic.⁹⁷

The Isotopes of Copper: Since the late 1990s, several research teams have focused their attention on the application of copper to nuclear medicine. While the metal has several radioisotopes suitable for medical applications, two are particularly compatible with antibodies: ^{67}Cu and ^{64}Cu . ^{67}Cu is a β -emitter with a half-life of 2.6 days. Like ^{131}I , its mean β energy of 141 keV (mean range of 0.2 mm) is suitable for the treatment of small tumors. ^{67}Cu also emits γ -rays but does so in lower abundance and lower energy than ^{131}I , reducing the radiation burden to non-target organs. In addition, its physical half-life of 2.6 days dovetails well with the biological half-life of antibodies. Nevertheless, its high cost and the lack of a reliable supply have limited its widespread use. In contrast, ^{64}Cu is a positron-emitting radiometal that can be produced readily and relatively inexpensively in high specific activities. Unfortunately, its physical half-life of 12.7 hours is somewhat incompatible with the pharmacokinetics of full-length antibodies. That said, this has not stopped several laboratories from bringing ^{64}Cu -labeled immunoPET agents into the clinic.^{98–100}

The *in vivo* metabolism of both ^{64}Cu - and ^{67}Cu -labeled antibodies has been studied in several preclinical mouse models.^{101–103} Yet regardless of the antibody or tumor model, the catabolism of the antibody in the tumor was found to be fast and led to the formation of a final product consisting of the radiocopper-chelator complex attached to a short protein trapped within the lysosome. For example, in a study focusing on the *in vitro* fate of a ^{67}Cu -CTPA-labeled variant of the chCE7 antibody in neuroblastoma cells, Novak-Hofer *et al.* found that the radionuclide was trapped in the lysosomes of tumor cells due to the accumulation of low molecular weight metabolites corresponding to ^{67}Cu -CTPA with and without a lysine residue.¹⁰⁴ Attempts to increase the lysosomal pH up to 6.0 did not result in the release of ^{67}Cu from the cells, suggesting that the positive charge of the chelator was not the only thing responsible for the retention of the radionuclide in this subcellular compartment. These data, of course, stand in stark contrast to that obtained with radioiodine-labeled antibodies. Indeed, when compared to analogous antibodies labeled with isotopes of iodine, copper-based radioimmunoconjugates produce better tumor uptake across the board.

The one caveat of Cu-based radioimmunoconjugates is the uptake of the radionuclide in the liver. Despite the development of several highly stable macrocyclic chelators for ^{64}Cu and ^{67}Cu such as TETA, BAT, DOTA, and CTPA (Figure 7), ^{64}Cu and ^{67}Cu remain susceptible to copper-binding proteins that are abundant in the liver. Indeed, Rodgers *et al.* observed the formation of a 35 kDa metabolite of the ^{67}Cu -CTPA-1A3 antibody that is believed to correspond to a transchelation product in which ^{67}Cu is bound to superoxide dismutase (SOD), an endogenous antioxidant enzyme.¹⁰⁵ Li *et al.* found a similar metabolite using a ^{64}Cu -DOTA-labeled variant of cetuximab as well as a second metabolite with a molecular weight of 11 kDa, consistent with the mass of metallothionein.¹⁰³ In humans, Mirick *et al.* demonstrated that the copper from ^{67}Cu -2IT-BAT-Lym1 was not transferred to SOD but rather to ceruloplasmin, the major copper-carrying protein in the blood.¹⁰⁶

To overcome this issue, several transchelation-resistant cross-bridged chelators have been created.¹⁰⁷ Unfortunately, the harsh conditions required for the metalation of some of these chelators are incompatible with sensitive biomolecules such as immunoglobulins. More recently, a family of sarcophagine-based chelators has been developed by Sageron and coworkers.¹⁰⁸ Voss *et al.* used a member of this family to radiolabel the anti-GD2 monoclonal antibody 14.G2a with ^{64}Cu and subsequently observed significantly reduced uptake and retention in the liver, a result which suggests that the chelator-radiometal complex is stable against *in vivo* transchelation.¹⁰⁹

Due to their smaller size, the metabolism and excretion of antibody fragments differs from that of full-length antibodies and thus lie outside of the scope of this review. A few words, however, might be appropriate given that ^{64}Cu is commonly used as a radiolabel for antibody fragments. Generally speaking, preclinical imaging and biodistribution studies reveal high non-target accumulation of radiolabeled antibody fragments in the kidneys regardless of the radionuclide employed, as the renal system is the typical route of elimination and clearance of these constructs. In the case of ^{64}Cu -labeled antibody fragments, the formation of a small molecular weight metabolite — most likely a radiocopper complex attached to a lysine residue — that is retained in the cells of the proximal tubules of the kidney further exacerbates this issue.¹⁰¹ Rogers *et al.* demonstrated

that the anomalous non-tumor-specific concentration of radioactivity in the kidneys can be circumvented by modulating the properties of the chelator.¹⁰⁵ In this work, a comparative analysis of four different copper chelators — BAT, TETA, CTPA, and PCBA — revealed that more lipophilic and positively charged metabolites were more likely to accumulate in the kidneys. Zimmerman *et al.* have developed an alternative strategy by introducing a cleavable triglycine linker between the antibody fragment and the chelator in ⁶⁷Cu-chCE7 F(ab')₂ to prevent the formation of metabolites bearing positively charged lysine residues. Interestingly, the efficacy of this approach proved to be dependent on the identity of the chelator. A construct bearing this linker and the cationic CTPA chelator produced significantly lower kidney retention than an analogous 'traditional' conjugate. However, a ⁶⁷Cu-labeled fragment combining this linker with the anionic DO3A chelator yielded slower clearance from the blood and increased activity concentration in the tumor.¹¹⁰

Indium-111: Unlike copper or iodine — each of which have several different medically useful radioisotopes — indium has only one radioisotope that can be used in nuclear medicine: ¹¹¹In. ¹¹¹In is a gamma-emitting radiometal with a half-life of 2.8 days and thus is very well suited for immunoSPECT imaging. *In vitro* studies have clearly illustrated that ¹¹¹In possesses residualizing properties that can contribute to the improved uptake and retention of ¹¹¹In-labeled radioimmunoconjugates in tumors compared to radioiodinated antibodies.^{74–75} Unfortunately, however, the residualizing nature of ¹¹¹In also increases its retention in non-target organs. Moreover, the release of ¹¹¹In from its chelator can create other issues, as it has been shown that the radionuclide can transchelate to iron carrying proteins such as transferrin and ferritin in the blood.¹¹¹

In light of these concerns, the metabolism of ¹¹¹In-labeled radioimmunoconjugates has been studied to better understand their uptake and retention in non-target tissues. Indeed, several articles explore the *in vivo* fate of ¹¹¹In-labeled antibodies in healthy mice¹¹² and rats.^{113–114} In these studies, it has been found that the intact ¹¹¹In-labeled antibody is the only radioactive species present in the blood. However, up to four ¹¹¹In-containing species can be — but are not always — detected in the liver and kidneys: the intact radioimmunoconjugate itself, a low molecular weight metabolite (likely the ¹¹¹In-chelator attached to a small peptide), an ¹¹¹In-labeled fragment of the original antibody, and an ¹¹¹In-transferrin moiety. Rogers *et al.*¹¹⁴ have also shown that the low molecular weight metabolite — in their case positively identified as ¹¹¹In-DTPA-ε-lysine — was also present alongside ¹¹¹In-DTPA in the feces and urine as well.

Several of these observations were reinforced by the work of Motta-Hennessy *et al.* who studied the catabolism of a CEA-targeting ¹¹¹In-SCN-Bz-DTPA-NP-4 antibody in athymic nude mice bearing GW-32 xenografts.¹¹⁵ Radioactivity from the tumor was recovered in 3 fractions corresponding to radioimmunoconjugate-antigen complexes, the native radioimmunoconjugate, and a low molecular weight fraction (LMWF). Further analyses of the LMWF via SDS-PAGE and instant thin-layer chromatography (iTLC) identified ¹¹¹In-SCN-Bz-DTPA and ¹¹¹In-SCN-Bz-DTPA-peptide fragments as the prime metabolites. In the same study, the authors confirmed the presence of the intact antibody and/or the low molecular weight fraction in other organs and biological fluids. Those findings were consistent with the metabolites found in healthy mice and rats.^{112,113–114} Furthermore,

DeNardo *et al.* reported the presence of radiometabolites in the plasma of non-Hodgkin's lymphoma patients injected with the ^{111}In -labeled mouse IgG_{2a} ^{111}In -2IT-BAD-Lym that targets the (HLA)-DR10 antigen on the surface of malignant B lymphocytes.¹¹⁶ Interestingly, radiometabolites have not been described in the plasma of humans administered with ^{131}I - or ^{67}Cu -labeled variants of the same antibody.⁸⁰ This example clearly underscores the influence of the nuclide itself on the *in vivo* processing of radiolabeled antibodies.

Interestingly, the identity of the chelator can also influence the number of metabolites. This was demonstrated effectively by Kinuya *et al.*, who studied the effect of chelator structure on the metabolic fate of ^{111}In -labeled radioimmunoconjugates of the monoclonal antibody T101 in normal mice. To this end, the antibody was conjugated with three different chelators — B4M-DTPA, CHX-B-DTPA and cDTPA (Figure 8) — and then radiolabeled with ^{111}In . The authors found that the three radioimmunoconjugates cleared from the whole body with similar rates. However, the two constructs bearing thiourea-linked variants of DTPA (1B4M-DTPA and CHX-B-DTPA) more quickly from the liver but more slowly from the blood than the cDTPA-bearing conjugate. This phenomenon was attributed to the greater stability of these two conjugates as well as the higher lipophilicity of the 1B4M-DTPA and CHX-B-DTPA chelators. In addition, differences were observed in the metabolites present. Two species were found in the liver after the administration of all three constructs: the intact radioimmunoconjugate itself and an ^{111}In -labeled small molecular weight metabolite. However, another, minor metabolite was observed in the case of ^{111}In -cDTPA-T101: ^{111}In -labeled transferrin. The latter was also exclusively found in the serum of mice injected with the ^{111}In -cDTPA-T101, although the rate of transchelation to transferrin was low (2% in serum after 5 days). These results suggest that all 5 carboxymethyl arms of the 1B4M-DTPA and CHX-B-DTPA chelators (compared to the 4 available for conjugated cDTPA) are necessary to stably coordinate ^{111}In . Interestingly, however, a clinical study using the anti-CEA C110 antibody radiolabeled with ^{111}In *via* a benzylisothiocyanate derivative of DTPA (*p*-SCN-Bz-DTPA) showed that the transchelation of ^{111}In to circulating transferrin can occur at a modest level in patients.¹¹⁷

Several strategies have been employed to reduce the uptake of radioactivity in the liver following the administration of ^{111}In -labeled radioimmunoconjugates. One of the most common preclinical approaches is predicated on pre-dosing animals with unlabeled antibody prior to the administration of the radioimmunoconjugate in order to reduce the formation of radiolabeled immunocomplexes.¹¹⁸ An attempt at translating this strategy to the clinic seems to have had only limited success in decreasing the levels of shed antigen in the blood and thus signal in the liver.¹¹⁹ However, the pre- or co-injection of unlabeled antibodies alongside ^{111}In -labeled mAbs has proven beneficial in other cases in which the target antigen is *not* shed.^{120–123} In a different approach, Studer *et al.* introduced a tetrapeptide linker (Ala-Leu-Ala-Leu) between the antibody and the chelator to promote the specific cleavage of the ^{111}In -chelator complex in the liver.¹²⁴ However, this approach led to very limited — if any — improvements in the activity concentrations retained in the liver of mice. The authors suggest that the ^{111}In -chelate complexes *were* successfully cleaved from the antibody but were themselves subsequently (and unfortunately) trapped in the liver.

Zirconium-89: Over the last decade, ^{89}Zr has garnered a great deal of attention as an emergent radiometal for immunoPET imaging, primarily due to its emission of low energy positrons (~ 395.5 keV) and the advantageous match between its physical half-life ($t_{1/2} \sim 3.3$ d) and the *in vivo* pharmacokinetic profiles of antibodies. A wealth of ^{89}Zr -labeled antibodies have been developed, evaluated in murine models of disease, and, in some cases, translated to the clinic over the past few years.^{58, 125} Both preclinical and clinical studies with ^{89}Zr -labeled radioimmunoconjugates have shown the liver to be the non-target organ with the highest uptake and retention of radioactivity.¹²⁶ This is most likely due to a number of factors, including the liver's role as the primary site of the catabolism of antibodies as well as the residualizing nature of the radionuclide itself. Unfortunately, to the best of our knowledge, no extensive metabolic studies have yet been conducted using ^{89}Zr -labeled antibodies.

While ^{89}Zr 's multiday half-life allows for the collection of imaging data as late as ten days after the administration of the radioimmunoconjugate, preclinical images taken at later time points often reveal relatively high activity concentrations in the bones of mice. It is well-known that Zr^{4+} is an osteophilic (bone-seeking) cation, as illustrated in several prior reports that employed ^{93}Zr and ^{95}Zr .^{127–128} This accumulation in bone suggests that desferrioxamine (DFO; Figure 9) — the most widely used chelator for ^{89}Zr — may allow for the release of the radiometal into the bloodstream or its transchelation to plasma proteins. Abou *et al.* studied the biological fate of ^{89}Zr by directly injecting the radiometal chelated by oxalate, citrate, and DFO.¹²⁹ In this work, the authors confirmed that weakly-chelated ^{89}Zr (*i.e.* ^{89}Zr bound by bidentate oxalate or tridentate citrate ligands) was readily taken up by the bone. More specifically, ^{89}Zr seems to accumulate in the non-soft tissue, mineralized constituents of the bone: most of the radioactivity was found in the epiphysis, whereas the cells of the marrow did not accumulate significant radioactivity. As a result, the authors suggest that ^{89}Zr is most likely chelated by hydroxyapatite. In contrast, the administration of DFO-bound ^{89}Zr produced no uptake in the bone. These data prompted the authors to suggest that in the case of ^{89}Zr -DFO-labeled antibodies, the elevated activity concentrations in the bone arise not from the cleavage of ^{89}Zr -DFO or the release of free $^{89}\text{Zr}^{4+}$ but rather the rapid metabolism of the antibody and the ^{89}Zr -DFO complex. Several groups have tried to develop more suitable chelators for ^{89}Zr , specifically through the use of octadenate coordination environments. Readers interested in these efforts are directed to a comprehensive review by Heskamp *et al.*¹³⁰

Though little clinical data is available on the metabolism of ^{89}Zr -labeled antibodies, it is interesting to note that the bone uptake patterns observed in mice are typically not seen in humans, leading many to believe that DFO is indeed a suitable chelator for ^{89}Zr in the clinic.^{96, 131–135} Studies using ^{89}Zr -DFO-labeled trastuzumab in HER2-positive esophagogastric adenocarcinoma¹³⁵ and ^{89}Zr -labeled cmAb-U36 antibody in head and neck cancer¹³¹ reported relatively low activity accumulation and retention in the liver and spleen. In contrast, higher activity concentrations were observed in both organs during trials with ^{89}Zr -huJ591,⁹⁶ ^{89}Zr -cetuximab,¹³² or ^{89}Zr -rituximab.¹³³ These data suggest that the identity of the antibody may be the driving force behind the ultimate distribution of ^{89}Zr in healthy tissues. That said, these differences may also be due to differences in the mass of antibody

tracer that was used in each case and might therefore be resolved by the pre-injection of unlabeled antibody.

Notably, the growing popularity and clinical utility of ^{89}Zr -labeled antibodies as immunoPET imaging agents for cancer has benefited greatly from our better understanding of the *in vivo* fate of antibodies. In a seminal feasibility study in patients with Her2-positive metastatic breast cancer, Dijkers *et al.* demonstrated the dramatic influence that the dose of antibody can have on the *in vivo* biodistribution of ^{89}Zr -DFO-trastuzumab in patients who had been treated with therapeutic doses of trastuzumab versus those who had not.¹³⁶ Their findings suggested that the optimal delineation of tumor lesions in treatment-naïve patients was achieved by co-dosing 1.5 mg of the ^{89}Zr -DFO-trastuzumab admixed with 48.5 mg of un-labeled trastuzumab (Figure 10). Furthermore, the addition of significantly higher amounts of unlabeled trastuzumab — *i.e.* 48.5 mg unlabeled antibody in a 50 mg total dose vs. 8.5 mg unlabeled antibody in a 10 mg total dose — yielded a better *in vivo* biodistribution profile of the radiotracer. This has been attributed to the ability of a higher dose of unlabeled antibody to prevent the elimination of the tracer and its *in vivo* clearance due to binding the shed ectodomain of Her2 in treatment-naïve patients.

CONCLUDING REMARKS

In the preceding pages, we have tried to paint as clear a picture as possible of the *in vivo* journey of both the antibody and radionuclide components of radioimmunoconjugates. It is our intention that this work will be useful to both experienced researchers and those new to the field. For the latter, we hope this review provides a compelling introduction to the *in vivo* interactions that dictate the *in vivo* behavior of radioimmunoconjugates. For the more veteran researcher, we hope that this work reinforces the key features that govern the pharmacokinetics of antibodies and revisits how these might be leveraged to improve the performance of radioimmunoconjugates.

In the end, one thing became abundantly clear to us as we wrote this review: we need more data. The solution — more studies — is simple, but things may not be as straightforward as they seem. We work in a difficult funding climate, and metabolic studies aren't exactly the 'sexiest' of investigations. Thus, we will end with the earnest hope that both funding agencies and grant reviewers remain mindful of the paramount importance of these fundamental investigations. They may not necessarily end up in the highest tier of journals, but they are nonetheless absolutely critical to the future success of the field.

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Figure 1.

FcRn recycles monoclonal antibodies and contributes to their long serum half-life. (1) Antibodies and albumin in the blood undergo fluid-phase endocytosis and are captured within the early endosome of an endothelial cell lining the blood vessel. (2) FcRn binds to antibodies and albumin in the sorting vesicle and either (3) recycles them back to into the blood from the apical side of the cell or (4) transcytoses them through the basolateral side of the cell via exocytosis (5). In either case, the antibodies and serum albumin are protected from lysosomal degradation (6). Reproduced with permission from Bern *et al.*¹³⁷

Figure 2.

The detailed structure of an IgG₁ antibody, adapted from Adumeau *et al.*⁴⁸

Figure 3:
Structures of the different IgG subclasses.

Figure 4:
Re-arrangement of disulfide bonds. (A) Different isoforms of IgG₂ antibodies. (B) Fab arm exchange between IgG₄ antibodies.

Figure 5.

The importance of residualizing radiolabels. (A) After internalization, radioiodinated antibodies are routed to the lysosomes, where most of the catabolism occurs. This results in the extracellular release of monoiodotyrosine. (B) In contrast, radiometal-based catabolites are trapped in the lysosomes and thus remain in the cell.

Figure 6.

Examples of prosthetic groups designed to increase the residualization of radioiodine. (A) Nonmetabolizable carbohydrate-tyramine adducts. In these reagents, the cellobiitol carbohydrate is indigestible by lysosomal glycosidase, whereas the lactitol becomes non-degradable after attachment to a protein; (B) Aromatic acylation agents. SIB (*N*-succinimidyl 3-iodobenzoate), SIPC (*N*-succinimidyl 5-iodo-3-pyridine carboxylate) and SGMB (*N*-succinimidyl 4-guanidinomethyl-3-iodobenzoate) produce positively charged metabolites at lysosomal pH that can escape cellular excretion; (C) Example of a short peptide containing D-amino acids that are — unlike L-amino acids — resistant to proteolytic degradation; (D) Bulky ionic polyhedral boron cluster DABI ([4-iodo-4-phenyl-4-aminobenzylammonio)undecahydro-closododecaborate (1-)]⁻)

Figure 7.
Selected chelators and bifunctional chelators for Cu(II).

Figure 8.

(A) Selected bifunctional chelators for ^{111}In ; (B) conjugation strategies associated with the various bifunctional chelators

Figure 9.
Selected chelators and bifunctional chelators for ^{89}Zr .

Figure 10.

The influence of antibody dose on the *in vivo* biodistribution of ^{89}Zr -DFO-trastuzumab in patients with Her2-positive metastatic breast cancer. (A) PET image of a trastuzumab treatment-naïve patient (cohort 1) co-injected with 1.5 mg ^{89}Zr -DFO-trastuzumab and 8.5 mg of unlabeled trastuzumab, showing non-tumor-specific physiologic uptake of the tracer in the gut; (B) PET image of a trastuzumab treatment-naïve patient (cohort 2) co-injected with 48.5 mg unlabeled trastuzumab and 1.5 mg ^{89}Zr -DFO-trastuzumab, showing significantly reduced non-tumor-specific physiologic uptake in the gut and more widespread distribution of the tracer with the pre-indications of lesions that can be visualized in the liver and thoracic regions; (C) PET image of a patient previously treated with therapeutic doses of trastuzumab (cohort 3) co-injected with 1.5 mg ^{89}Zr -DFO-trastuzumab and 8.5 mg of unlabeled trastuzumab, showing higher signal in the heart (indicative of radioactivity restricted to the blood pool) as well as the visualization of hotspots indicative of tumor lesions in the liver and abdominal region; (D) Relative blood pool activity in the patients of the 3 cohorts over a period of 5 days after the injection of the aforementioned doses of radioimmunoconjugate. Adapted and reproduced with permission from Dijkers *et al.*¹³⁶