

Editorial

Metabolism of Radiometal-Labeled Proteins and Peptides: What are the Real Radiopharmaceuticals *in vivo*?

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RADIOMETAL-LABELED MONOCLONAL ANTIBODIES AND PEPTIDES

The radiolabeling of monoclonal antibodies (mAbs) for the detection of cancer in the 1970's¹⁻³ marked the beginning of the use of radiolabeled biological molecules for targeting antigens and receptors that are upregulated in tumors. Initially, radiolabeled antibodies were labeled with iodine radionuclides; however, the development of radiometal-bifunctional chelator-mAb conjugates has become more prevalent over the last two decades, and this topic has been a subject in many reviews published in the last 10 years.⁴⁻¹²

The increase in the use of radiometals for labeling to mAbs is largely due to their longer retention in tumors than iodinated mAbs. The rapid clearance of conventionally radioiodinated mAbs is attributed to the dissociation of radioiodine from the mAb *in vivo*.^{13,14}

Intact mAbs are large proteins with a MW of 160 kDa, and because of their large size, they have very slow biological clearance and are excreted through the hepatobiliary system. To cir-

cumvent these drawbacks, mAb fragments have been produced that have molecular weights ranging from 10–100 kDa. Among the many metal radionuclides that have been labeled to mAbs (both intact and fragments) for diagnostic imaging and radioimmunotherapy (RIT) include ¹¹¹In, ⁹⁰Y, ⁶⁷Ga, ^{99m}Tc, ¹⁸⁶Re, ¹⁸⁸Re, ⁶⁴Cu and ⁶⁷Cu. A large number of chelators have been developed to attach these radiometals to the mAbs and peptides.

WHY IS METABOLISM IMPORTANT?

The definition of metabolism is the “sum total of the chemical processes that take place in living cells.”¹⁵ These processes include catabolism, the enzyme-catalyzed breakdown of molecules, and anabolism, the build-up of larger molecules from smaller ones. In the pursuit of designing new radiometal-labeled mAbs and peptides for diagnostic imaging and targeted radiotherapy of cancer, the issue of metabolism of the radiopharmaceutical is often overlooked. When evaluating a nuclear medicine image of a radiolabeled mAb or peptide (taken by either gamma scintigraphy or positron emission tomography (PET), a question arises as to what compound is actually being observed in the image? We can be certain of the purity of the radiopharmaceutical that was injected into the patient, but most of the time we are not certain of how rapidly the com-

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pound is metabolized. Nor do we know the exact chemical form of the radiolabeled metabolite that is present at the various imaging times post-injection. The fate of the radionuclide, i.e., whether it remains bound to the mAb or peptide or whether it is metabolized, is of great significance, since this is what will ultimately determine the absorbed dose of the radiopharmaceutical to the tumor and normal tissues.

WHAT HAVE WE LEARNED FROM METABOLISM STUDIES OF RADIOMETAL-LABELED MABS SO FAR?

Understanding how radiometal-labeled proteins and peptides are metabolized requires taking several factors into consideration. These include: 1) the enzymatic breakdown of a protein or peptide into smaller peptide fragments that may or may not be attached to the radiometal chelate; 2) the enzymatic breakdown of the protein or peptide followed by acetylation or addition of another functional group; and/or 3) dissociation of the radiometal from the chelator. The metabolism of radiometal-labeled mAbs is highly dependent on the radiometal. For example, the metabolism of ^{111}In - and ^{90}Y -labeled chelator-mAb/peptide conjugates has been shown to be significantly different than that of similar $^{64/67}\text{Cu}$ -labeled protein and peptide conjugates. The metabolism of ^{111}In - and ^{90}Y -labeled mAbs has been investigated in animal models and in humans.^{16,17} Rogers et al.¹⁶ focused on the metabolism of the anti-colorectal carcinoma mAb ^{111}In -DTPA-1A3 (where DTPA is diethylenediaminepentaacetic acid) and ^{111}In -DTPA-1A3-F(ab)₂ in the liver and kidneys of Sprague-Dawley rats. In the liver and kidneys low MW metabolites were present, most likely ^{111}In -DTPA-Lys, but to a much greater extent in the kidneys than in the liver. Little or no evidence was observed for the dissociation of ^{111}In from DTPA in these tissues. The results of DeNardo et al.¹⁷ showed a significant amount of lower MW metabolites (non-identified) of ^{111}In - and ^{90}Y -labeled 2IT-BAD-Lym-1 (where 2IT-BAD is 2-iminothiolane-2-[*p*-(bromo-acetamido)benzyl]-1,4,7,10-tetraazacyclodecane-*N,N',N'',N'''*-tetracetic acid) in the blood in non-Hodgkin's lymphoma patients and normal volunteers (36% at 3 d post-injection); however, they also determined that there was no dissociation of these radiometals from the BAD chelator. In addition to

studies with ^{111}In -labeled mAbs, *in vivo* metabolism studies have been carried out with ^{111}In -DTPA-D-Phe¹-octreotide (^{111}In -DTPA-OC).¹⁸⁻²⁰ In these studies, similar to those with ^{111}In -labeled mAbs, no significant amount of dissociation of ^{111}In was reported.

The *in vivo* behavior of radiocopper-labeled mAbs and peptides is very different than that of the analogous ^{111}In - and ^{90}Y -labeled compounds. In the mid-1980's, Meares and colleagues reported that it was necessary to use a macrocyclic chelator to "stably" attach ^{67}Cu to mAb Lym-1, since bifunctional chelators based on EDTA or DTPA allowed the copper to rapidly become bound to albumin in human serum.²¹ Meares et al. developed the bifunctional chelator, BAT (6-[*p*-(bromoacetamido) benzyl]-1,4,7,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetracetic acid), which has been used extensively to attach ^{67}Cu and ^{64}Cu to mAbs for radioimmunotherapy²²⁻²⁵ and radioimmuno-PET imaging.^{26,27} The peptide octreotide (OC) has been labeled with ^{64}Cu via the chelator TETA (1,4,7,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetracetic acid), which was conjugated to the *N*-terminus of OC by converting one of the -COOH groups to an active ester, and forming an amide bond.²⁸ This agent has also been evaluated in clinical trials for PET imaging of somatostatin-receptor positive tumors.²⁹ Metabolism studies have been reported for ^{67}Cu -2IT-BAT-Lym-1 in patients, as well as for $^{64/67}\text{Cu}$ -2IT-BAT-1A3.^{30,31} In both of these studies, significant dissociation of the radiocopper from the BAT chelator was observed in the blood of patients and rat liver, with the $^{64/67}\text{Cu}$ binding to proteins such as ceruloplasmin and superoxide dismutase (SOD). It was also observed that ^{64}Cu -TETA-OC dissociates in rat liver *in vivo*, with all ^{64}Cu in the liver binding nearly completely to SOD by 20 h post-injection.³² Although significant dissociation of $^{64/67}\text{Cu}$ from chelator-mAb/peptide conjugates was demonstrated in the liver and blood in animal models and humans, in the kidneys only low MW metabolites were observed, which were likely $^{64/67}\text{Cu}$ -labeled chelator-amino acid moieties.³⁰

COPPER-LABELED MABS WITH PEPTIDE LINKERS: HOW IS METABOLISM ALTERED?

In this issue of *Cancer Biotherapy and Radiopharmaceuticals*, Novak-Hofer and colleagues

present a study that is a prime example of the importance of chelators and linkages on the metabolism of radiometal-labeled antibody fragments.³³ A strategy is presented to maximize tumor uptake and minimize normal organ uptake of ⁶⁷Cu-labeled antibody fragments for RIT using peptide linkages between the metal chelate and the mAb fragments. Previous studies by this group showed that ⁶⁷Cu-labeled mAb fragments using the CPTA (4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl] benzoic acid) chelator had very high kidney uptake that precluded its use in clinical trials.³⁴ By changing the CPTA chelator, which forms a positively-charged Cu(II) complex, to analogs of the DOTA chelator (where DOTA is 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid), which form negatively-charged Cu(II) complexes, the biodistribution of the ⁶⁷Cu-labeled mAb chCE7 fragments showed a shift in accumulation of ⁶⁷Cu from the kidneys (CPTA) to the liver (DOTA).³⁵ In the study presented in this issue of *CBR*, analogs of the negatively charged DOTA chelator were conjugated to mAb35-F(ab')₂ using four different tripeptide linkages. The linkages were designed to either be substrates for liver proteases or to be control peptides, which were not cleavable by the liver enzymes. The data demonstrated that the ⁶⁷Cu-labeled conjugate with the peptide linkage that is a substrate for the liver proteases (R2) showed the greatest initial liver accumulation, but significant clearance of about four-fold over 48 hours. This agent also showed the lowest amount of activity in the tumor and most rapid blood clearance compared to the other ⁶⁷Cu-labeled conjugates evaluated.

There are many interesting aspects of the study by Novak-Hofer et al., as well as previous studies published by this group, that raise worthwhile questions. In the study by Zimmerman et al.,³⁵ the first question is why was there such a large difference in mouse biodistribution between the mAb fragments with the positively charged ⁶⁷Cu-CPTA chelate and the fragments conjugated to the negatively-charged ⁶⁷Cu-DOTA analogs? This raises the question of how the charge of the metal complex attached to a mAb fragment affects biodistribution. A second observation is that there appears to be significant differences in mouse biodistribution between the ⁶⁷Cu-labeled DO3A-conjugated mAb F(ab')₂ fragments of chCE7 and mAb35.^{33,35} The kidney uptake of ⁶⁷Cu-DO3A-chCE7-F(ab')₂ is considerably greater than ⁶⁷Cu-DO3A-mAb35-F(ab')₂ (21.0 ±

11.5 vs 4.6 ± 0.7%ID/g respectively at 4 h). The liver uptakes between the two agents are also dramatically different, with the uptake of ⁶⁷Cu-DO3A-mAb35-F(ab')₂ being about 2-fold greater than that of ⁶⁷Cu-DO3A-chCE7-F(ab')₂. This raises the question of how different mAb fragments affect biodistribution.

The question of how different peptide linkages between ⁶⁷Cu-DOTA and the mAb fragments affect biodistribution was somewhat successfully addressed by Novak-Hofer et al. The linkage that was a substrate for liver enzyme class of cathepsins (gly-phe-gly (R2)) showed the highest liver uptake with clearance over 48 hours, a more rapid blood clearance and lower tumor uptake compared to the conjugates with the other peptide linkages. The other peptide linkages, particularly the tri-glycine linkage (R1), appeared to stabilize the ⁶⁷Cu-conjugate, since the blood clearance was slower, and the tumor uptake increased over time. Questions remain about the use of R2 as a linker, such as why does the ⁶⁷Cu-DOTA-R2-mAb F(ab')₂ conjugate have such a high initial uptake in the liver, and why does it clear the blood so rapidly? Is the R2 linkage a substrate for proteases other than the cathepsins, such as proteases found in the blood, tumor or kidney? There is likely not a simple way to address these questions.

The final question raised concerning these ⁶⁷Cu-DOTA-peptide conjugated mAb fragments is how much of the liver activity was due to dissociation of ⁶⁷Cu from the DO3A/DOTA chelator? Novak-Hofer et al. addressed this issue briefly, stating they do not believe the presence of a ⁶⁷Cu-labeled 31 kDa protein in liver homogenates at 30 minutes post-injection was due to binding of ⁶⁷Cu to SOD (MW = 32 kDa), which is known to be present in rodent livers.^{30,32} Novak-Hofer et al. state that they have observed this 31 kDa fragment in mouse kidneys, and they believe it to be due to a proteolytic fragment of F(ab')₂. This explanation certainly has merit, and is a logical reason for the prominent 31 kDa band in the SDS gels of liver homogenates at 30 minutes post-injection. However, the reasons for the slow or lack of clearance from the liver out to 48 hours for several of the ⁶⁷Cu-labeled mAb conjugates was not addressed in the study.

In summary, the study by Novak-Hofer emphasizes the importance of metabolism studies. In addition, this paper raises the level of awareness of the need for more research groups to per-

form metabolism experiments to better understand the *in vivo* behavior of radiometal-labeled mAbs and peptides.

WHERE DO WE GO FROM HERE?

One drawback of the majority of metabolism studies of radiolabeled proteins and peptides that have been published so far is that most research groups do not have the ability to definitively identify the radiolabeled metabolites that are formed *in vivo*. The majority of published studies (including the Novak-Hofer paper in this issue of *CBR*) identify the molecular weight of the metabolites by size-exclusion HPLC or gel electrophoresis, but they do not fully characterize the metabolites. One way to truly identify metabolites is by tandem liquid chromatography-mass spectroscopy (LC-MS). This technique has only recently become more affordable for individual research labs to have their own LC-MS to evaluate radiolabeled metabolites. A recent paper by Eckelman et al.³⁶ demonstrated the use of LC-MS to identify metabolites of two 5-HT1A antagonists in human and rat hepatocytes. It is the hope that even more rigorous identification of radiometal-labeled metabolites from proteins and peptides can be obtained in the future, providing further knowledge in the search for better radiopharmaceuticals for cancer imaging and therapy.

The reality of the situation concerning *in vivo* metabolism studies is that they are laborious, time consuming and technically challenging. Anyone who has been in the laboratory performing these studies will agree with this statement. Research groups who go to the trouble to perform careful studies, such as the Novak-Hofer group, should be commended for taking on such an onerous task. The payoff for doing these studies is that we are slowly beginning to understand why radiometal-labeled proteins and peptides behave *in vivo* as they do, and we are designing improved radiopharmaceuticals for cancer imaging and therapy. An appreciation for the knowledge gained from metabolism studies will undoubtedly improve progress in this important area of study. As more and more radiometal-labeled mAbs and peptides enter clinical trials for both imaging and therapy, this appreciation will continue to grow, and some day, there will be more answers than questions.

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