Published in final edited form as:

Mol Pharm. 2015 August 3; 12(8): 3097–3103. doi:10.1021/mp5008579.

Quantitative Correlation of in Vivo Properties with in Vitro Assay Results: The in Vitro Binding of a Biotin–DNA Analogue Modifier with Streptavidin Predicts the in Vivo Avidin-Induced Clearability of the Analogue-Modified Antibody

Shuping Dou[†], John Virostko[‡], Dale L. Greiner[§], Alvin C. Powers^{||,⊥,@}, and Guozheng Liu^{†,*}

[†]Department of Radiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655, United States

[‡]Vanderbilt University Institute of Imaging Science, Vanderbilt University Medical Center, Nashville, Tennessee 37232, United States

[§]Department of Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01655, United States

^{||}Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee 37232, United States

[⊥]Division of Diabetes, Endocrinology, and Metabolism, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee 37232, United States

[@]Veterans Affairs Tennessee Valley Healthcare System, Nashville, Tennessee 37232, United States

Abstract

Quantitative prediction of in vivo behavior using an in vitro assay would dramatically accelerate pharmaceutical development. However, studies quantitatively correlating in vivo properties with in vitro assay results are rare because of the difficulty in quantitatively understanding the in vivo behavior of an agent. We now demonstrate such a correlation as a case study based on our quantitative understanding of the in vivo chemistry. In an ongoing pretargeting project, we designed a trifunctional antibody (Ab) that concomitantly carried a biotin and a DNA analogue

*Corresponding Author. Guozheng Liu, PhD, S2-843, Department of Radiology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655. guozheng.liu@umassmed.edu. Phone: (508) 856-1958. Fax: (508) 856-6363.

The authors declare no competing financial interest.

(hereafter termed MORF). The biotin and the MORF were fused into one structure prior to conjugation to the Ab for the concomitant attachment. Because it was known that avidin-bound Ab molecules leave the circulation rapidly, this design would theoretically allow complete clearance by avidin. The clearability of the trifunctional Ab was determined by calculating the blood MORF concentration ratio of avidin-treated Ab to non-avidin-treated Ab using mice injected with these compounds. In theory, any compromised clearability should be due to the presence of impurities. In vitro, we measured the biotinylated percentage of the Ab-reacting (MORF-biotin)-NH₂ modifier, by addition of streptavidin to the radiolabeled (MORF-biotin)-NH₂ samples and subsequent high-performance liquid chromatography (HPLC) analysis. On the basis of our previous quantitative understanding, we predicted that the clearability of the Ab would be equal to the biotinylation percentage measured via HPLC. We validated this prediction within a 3% difference. In addition to the high avidin-induced clearability of the trifunctional Ab (up to ~95%) achieved by the design, we were able to predict the required quality of the (MORF-biotin)-NH₂ modifier for any given in vivo clearability. This approach may greatly reduce the steps and time currently required in pharmaceutical development in the process of synthesis, chemical analysis, in vitro cell study, and in vivo validation.

Keywords

quantitative correlation; in vitro assay; in vivo properties; trifunctional antibody; antibody conjugate; clearance; morpholino oligomers

INTRODUCTION

Chemical analysis is often performed for quality assurance, but the correlation of the in vitro data with the in vivo properties of a preparation is usually not quantitative. However, if the in vivo chemistry is fully understood, logical and quantitative correlation of the in vivo properties with the analysis data could be possible. This colligation, i.e., the logical correlation of in vitro data with in vivo properties of a preparation, would be important in modifying a synthetic route or controlling a synthetic procedure for pharmaceutical development. Accurate prediction of in vivo results from an in vitro assay would reduce the laborious process of chemical characterization, synthesis, cell evaluation, and in vivo validation. We now report a case study in which we quantitatively correlated the in vitro analysis of a modifier that introduces functions into an antibody (Ab) with the in vivo blood clearability of the modified Ab.

In our ongoing “pretargeting” projects, we are focusing on creating a trifunctional Ab. This trifunctional Ab binds to the antigen in the tissue of interest such as a tumor or islets of Langerhans and simultaneously binds to a clearing agent for background reduction. Background reduction is important for tumor therapy, because this limits radiotherapeutic side effects. For islet imaging, an extremely low background is required because islets constitute only 1–2% of the pancreatic mass. Finally, the trifunctional Ab binds to an “effector” that carries a radiolabel, optical probe, or “warhead” for imaging or therapy. Preferably, the effector will not compete with the clearing agent for the same binding site. A typical pretargeting procedure with clearance will involve three administrations (Figure 1):

the Ab for targeting, the clearing agent to clear the unbound Ab for background reduction, and the effector to bind to the prelocalized Ab in the tissue of interest.

We previously constructed a trifunctional Ab following the conventional route shown in Figure 2.¹ A clearance group (biotin) and an effector-binding group (MORF, a synthetic DNA analogue of morpholino phosphorodiamidate oligomer) were conjugated to an antitumor IgG Ab. Avidin was used as the clearing agent to bind the biotin on the trifunctional Ab. By virtue of the presence of glycosyl groups in the avidin structure, avidin drives (clears) the circulating and unbound Ab to the liver.^{2,3} A radiolabeled complementary MORF (cMORF) was used as the effecting agent (effector).⁴ The radiolabeled cMORF rapidly bound to the MORF-Ab in the target tissue, while the remaining circulating and unbound cMORF was rapidly excreted in urine.⁴

Our modification is different to the Ab modification with one group. In that case, a size exclusion column is commonly used to separate the unreacted small modifier from the Ab. The unmodified Ab impurity usually does not produce significant side effects, and the species with different numbers of modifying groups are usually all useful. However, in our case with double modifications, it becomes more complicated. When two separate groups are conjugated conventionally (Figure 2, left panel), the preparation contains more Ab impurities, including the Ab with only MORF, the Ab with only biotin, and the Ab with neither. The Ab impurities without MORF or with neither MORF nor biotin would not bind the effector, but the Ab impurities carrying a MORF without a biotin are detrimental as they cannot be cleared by avidin but bind the effector. We currently do not have an established method for separating these Ab species. Instead, we have designed a new modification route (Figure 2, right panel) that theoretically prevents this problem.

In this design, prior to conjugation to the Ab, biotin and MORF are fused into one structure denoted as (MORF-biotin) $\rhd$ -NH₂, as shown in the new route of Figure 2 (right panel). The symbol $\rhd$ - indicates that the two preceding entities concomitantly connect to the subsequent entity. Theoretically, if prepared in this manner, any Ab that carries a MORF will also carry a biotin. Therefore, the circulating MORF-Ab can be cleared completely by avidin. Furthermore, the (MORF-biotin) $\rhd$ -NH₂ does not have to be completely pure, as long as the impurities do not react with the NHS-activated aldehyde group that later attaches to the Ab. However, though this design has resulted in improved clearance, as will be shown, there is still considerable variation in clearance among different (MORF-biotin) $\rhd$ -Ab preparations. Theoretically, the formation of the nonclearable MORF-Ab impurities must be due to the impurities in (MORF-biotin) $\rhd$ -NH₂ that do not carry a biotin but can still react with NHS-activated aldehyde and subsequently be attached to the Ab. On the basis of the techniques we recently established,^{5,6} in the current study we have validated this hypothesis by quantitatively and logically correlating the analysis data of the (MORF-biotin) $\rhd$ -NH₂ modifier with the in vivo avidin-induced clearability of the final trifunctional Ab preparation.

In this report, we have used three concepts regarding clearance. The general term “clearance” refers to the removal of a compound from circulation. “Clearance efficiency” refers to the percentage of the molecules of interest that is cleared when using a clearing

agent as compared to without. It measures the clearance effect in a procedure using a clearing agent. "Clearability" refers to the percentage of the molecules of interest that is clearable by a clearing agent. Even if the clearability is 100%, the clearance efficiency may still be lower because it depends not only on the clearability of the molecules of interest in the injectate but also on the in vivo binding process of the clearing agent with the clearable molecules. Herein the clearability is an avidin-induced clearability that measures whether the Ab molecules bearing a MORF can be cleared by avidin. We have established the methodology quantitating the avidin-induced clearability.⁵ For the measurement of the avidin-induced clearability in mice, we added avidin to the (MORF-biotin)⌐-Ab preparation prior to its injection. We compared the clearance of an avidin-added (MORF-biotin)⌐-Ab injectate with that of an identical Ab injectate without avidin. The avidin-bound Ab molecules mimic those formed in the blood pool following avidin administration during the pretargeting process. To follow the Ab molecules bearing a MORF in the (MORF-biotin)⌐-Ab injectate in vivo, a radiolabeled cMORF was added at a tracer level to both preparations. We previously found that, if the Ab was biotinylated, after binding to avidin it was cleared rapidly and completely from the circulation.⁵ Based on this observation, the avidin-induced clearability was quantified.

EXPERIMENTAL SECTION

Materials

The (MORF-biotin)⌐-NH₂ was purchased from Gene Tools, LLC (Philomath, OR). The scientists at Gene Tools, LLC, constructed the (MORF-biotin)⌐-NH₂ by attaching a (biotin-amine)⌐- group to the 3' terminus of the MORF with the previous sequence of 5'-TCTTCTACTTCACAAC₇.⁷ Its structure is illustrated in the bottom of the right panel of Figure 2. The streptavidin and avidin were from Pierce (Thermo Scientific, Rockford, IL). The CC49 Ab, an anti-TAG-72 IgG, was produced by Strategic Biosolutions (Newark, DE) from its murine hybridoma cell line (a gift from J. Schlom, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD). The Sandoglobulin (human immune globulin) was from Sandoz Pharmaceuticals Corp. (East Hanover, NJ). Modification of the Abs with the (MORF-biotin)⌐-NH₂ was achieved using a Hydralink kit of paired agents from Solulink Biosciences (San Diego, CA): NHS-O₂C-(CH₂)₅-Py-NHN=C(CH₃)₂ that is an *N*-hydroxy succinimidyl-activated hydrazinonicotinate acetone hydrazone and hereafter shortened to NHS-Py-NHN=C(CH₃)₂ and NHS-O₂C-(CH₂)₅-Ph-CHO that is an *N*-hydroxy succinimidyl-activated formylbenzoate and hereafter shortened to NHS-Ph-CHO. The NHS-MAG₃ (*S*-acetyl *N*-hydroxysuccinimidyl mercaptoacetyltriglycine) was previously synthesized in house.^{8,9} The MAG₃-cMORF stock solution was prepared from a cMORF with a sequence complementary to the MORF, but the amine derivatization is the same as that for the MORF shown in the left panel of Figure 2. It was labeled following a procedure described previously,¹⁰ identical to that described below for (MORF-biotin)⌐-MAG₃. The Bio-Gel P-4 Gel (medium) and Sephadex G-100 resin (fine) were purchased from Bio-Rad Laboratories (Hercules, CA) and Amersham Biosciences (Uppsala, Sweden), respectively. The PD-10 columns were from NeoRx Corp. (Seattle, WA). The ⁹⁹Mo-^{99m}Tc generator was from PerkinElmer Life Science Inc. (Boston, MA). All other chemicals were reagent grade and used without purification.

Spectrophotometry and High-Performance Liquid Chromatography (HPLC)

The concentrations of MORF and cMORF were measured by UV spectrophotometry using the molar absorbances provided by the vendor. Size exclusion (SE) HPLC was used for their analysis using a system equipped with a superpose-12 10/30 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; optimal separation range of 1×10^3 to 3×10^5 Da), a UV in-line detector, and a radioactivity inline detector. A 0.10 M pH 7.2 phosphate buffer was commonly used as eluant at a flow rate of 0.60 mL/min, but when SA was involved, a 10% ACN/0.10 M NH_4Cl solution was used. Radioactivity recovery was routinely measured and was always greater than 90%.

Conjugation of (MORF-biotin)-NH₂ to Ab

The commercial Hydralink approach was employed as previously described.^{11–13} As shown in Figure 2 (right panel), the Ab and the (MORF-biotin)-NH₂ were conjugated with NHS-Py-NHN=C(CH₃)₂ and NHS-Ph-CHO, respectively. Combination of the Ab-Py-NHN=C(CH₃)₂ and the (MORF-biotin)-Ph-CHO allowed for the formation of a hydrazone bond, linking the (MORF-biotin)-NH₂ to the Ab in the form of (MORF-biotin)-Ph-CH=NNH-Py-Ab. We denote this product as (MORF-biotin)-Ab.

Specifically, 1.88–3.76 mg of NHS-Ph-CHO was dissolved in 0.10 mL of DMF (*N,N*-dimethylformamide), and 3 mg of (MORF-biotin)-NH₂ was dissolved in 0.95 mL of 0.2 M pH 8.0 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. The two solutions were mixed at a MORF:NHS-Ph-CHO molar ratio range of 1:10–20. After incubation for 2 h at room temperature, the reaction mixture was purified over a PD-10 column using 0.1 M pH 5.0 MES [2-(*N*-morpholino)-ethanesulfonic acid] as the eluent. The peak fractions were pooled and stored in a refrigerator. The Ab (either the CC49 or the Sandoglobulin) was modified with NHS-Py-NHN=C(CH₃)₂. Eight milligrams of the Ab in 1.5 mL of 10 mM PB was mixed with 27 μL of a solution of NHS-Py-NHN=C(CH₃)₂ at a concentration of 80 mM in a DMF/PB mixture [1:2 (v/v)]. The molar ratio of Py-NHN=C(CH₃)₂ to Ab is 15:1. After 24 h at 4 °C and 0.5 h at room temperature, the reaction mixture was purified using a PD-10 column. The peak fractions were pooled, mixed with the (MORF-biotin)-Ph-CHO, and reacted at 4 °C for 20 h. A 1 cm $\times$ 50 cm glass Econo column filled with Sephadex G-100 resin was used to remove the unreacted (MORF-biotin)-NH₂ and MORF impurities. The average number of MORFs per Ab was determined as previously described.⁷

Measurement of the Avidin-Induced Clearability of (MORF-biotin)-Ab

Each (MORF-biotin)-Ab was labeled by adding [^{99m}Tc]cMORF (MORF:cMORF molar ratio of 2:1). The labeled Ab in the form of [^{99m}Tc]cMORF/(MORF-biotin)-Ab was purified on a G-100 open column using pH 7.2 PB buffer as the eluent. The fractions were pooled and split into two: one used as is and the other added with excess avidin. The avidin-treated injectate for each mouse contained 30 μg of Ab (40–50 μCi of ^{99m}Tc) and an excess of avidin at an avidin:MORF molar ratio of ~5:1. The injectate without avidin contains only the 30 μg of Ab (40–50 μCi of ^{99m}Tc). Five CD-1 mice (Charles River Laboratories, Wilmington, MA) were used for each group (with or without avidin), and the injection was made via tail vein. The mice were euthanized 3 h post-injection. The organs and tissues of interest were collected, weighed, and counted in an autogamma counter along with the

injectate standards as described previously.⁷ The avidin-induced clearability of (MORF-biotin)-Ab was calculated by the formula of $[1 - (\text{blood concentration})_{\text{with avidin}} / (\text{blood concentration})_{\text{no avidin}}] \times 100\%$. All the experiments involving mice were approved by the Animal Care and Use Committee of the University of Massachusetts Medical School.

In Situ Conjugation of NHS-MAG₃ to (MORF-biotin)-NH₂

We prepared (MORF-biotin)-MAG₃ and (MORF-biotin)-Ph-CHO in the same solution. Specifically, 2.86 mg of NHS-Ph-CHO and 0.8 mg of NHS-MAG₃ were dissolved together in 0.10 mL of DMF. Subsequently, 2.86 mg of (MORF-biotin)-NH₂ in 0.95 mL of 0.2 M pH 8.0 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was added to 0.10 mL of the NHS-MAG₃/NHS-Ph-CHO solution. The MORF:NHS-MAG₃:NHS-Ph-CHO ratios were 1:4:16, and the nominal MORF:NHS-ester ratio was 1:20. After incubation for 2 h at room temperature, 85% of the reaction mixture was purified over a PD-10 column using the MES eluent and used for the subsequent Ab conjugation following the procedure for “conjugation of MORF to Ab”. The (MORF-biotin)-MAG₃ in the (MORF-biotin)-Ph-CHO preparation does not react with Ab. The remaining 15% of the reaction mixture was purified over a P4 column (0.7 cm × 20 cm) using 0.25 M NH₄OAc at pH 5.2 as the eluent. The peak fractions with an optical density (OD) at 265 nm of >5 were pooled. The presence of (MORF-biotin)-Ph-CHO does not interfere with the subsequent processing and labeling of the (MORF-biotin)-MAG₃.

Separate Conjugation of NHS-MAG₃ to (MORF-biotin)-NH₂

Specifically, 1.5 mg of (MORF-biotin)-NH₂ was dissolved in 0.3 mL of 0.2 M pH 8.0 HEPES and the solution mixed with 2.0 mg of NHS-MAG₃ powder. The entire reaction mixture was loaded onto a P4 column for purification like in the process for in situ conjugation.

Processing the NHS-MAG₃ Conjugate for High-Efficiency Labeling

For both the (MORF-biotin)-MAG₃s from the in situ NHS-MAG₃ conjugation and the separate NHS-MAG₃ conjugation, a further step was taken for high labeling efficiency by dissociating unstable MAG₃-(MORF-biotin)-NH₂ conjugates and removing the released MAG₃ chelator as previously described.¹⁰ Specifically, each preparation was mixed with a tartrate buffer (pH 9.2, 50 µg/µL Na₂tartrate · 2H₂O in a solution of 0.5 M Na₂HCO₃, 0.25 M NH₄OAc, and 0.175 M NH₃) and a fresh tin solution (10 µg/µL SnCl₂ · 2H₂O and 1 µg/µL sodium ascorbate in 10 mM HCl). The volume ratios of the preparation/tartrate buffer/tin solution were 15:5:1. The mixed solution was heated at 100 °C for 20 min and purified over a longer P4 column (1.0 cm × 50 cm). The peak fractions with OD values of >5 were pooled as a stock solution.

Streptavidin (SA) Shift on HPLC of ^{99m}Tc-Labeled (MORF-biotin)-MAG₃

^{99m}Tc radiolabeling was performed in triplicate for each conjugate following the protocol established previously.¹⁰ For each labeling, between 5 and 50 µL of [^{99m}Tc]pertechnetate generator eluate was added to a mixed solution of 30 µL of a (MORF-biotin)-MAG₃ solution (0.2–0.4 mg/mL MORF) in pH 5.2 NH₄OAc buffer, 10 µL of tartrate buffer, and 3

μL of $4\text{ }\mu\text{g}/\mu\text{L}$ $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in an ascorbate-HCl solution ($1\text{ }\mu\text{g}/\mu\text{L}$ sodium ascorbate in 10 mM HCl), followed by heating at $100\text{ }^\circ\text{C}$ for 20 min . The radiolabeled (MORF-biotin) γ -MAG₃ preparations were mixed with SA at a SA:MORF molar ratio of 10:1 for the HPLC shift study.

RESULTS

Quantitative Colligation of the SA-Shifted Percentage of (MORF-biotin) γ -MAG₃-^{99m}Tc with the Avidin-Induced Clearability of (MORF-biotin) γ -Ab

As shown in Figure 2, any MORF derivative that reacts with NHS-Ph-CHO will lead to a product that in turn attaches to the Py-NHN=C(CH₃)₂-modified Ab to form either the desired (MORF-biotin) γ -Ab or a MORF-Ab that does not bear a biotin. We reasoned the clearability of the radiolabeled (MORF-biotin) γ -Ab injectate measured in vivo should be equal to the percentage of the (MORF-biotin) γ -Ab among all the Ab species carrying a MORF, assuming the MORF-Ab species share the same pharmacokinetics irrespective of whether they carry a biotin group. Furthermore, this percentage should be equal to the percentage of the NHS-Ph-CHO-modified MORF that carries a biotin, because the NHS-Ph-CHO-modified MORFs react with Ab regardless of whether they bear a biotin.

To measure the percentage of the NHS-Ph-CHO-modified MORF that carries a biotin, we reacted NHS-MAG₃ with the (MORF-biotin) γ -NH₂ sample to trace the reaction of the NHS-Ph-CHO with the (MORF-biotin) γ -NH₂ sample. This is based on the assumption that the reactivity of the NHS-MAG₃ is proportional, if not identical, to that of NHS-Ph-CHO. The advantage of using NHS-MAG₃ is that it can be readily labeled with ^{99m}Tc for sensitive and accurate detection. The percentage of the biotin-bearing MORF can be detected by adding streptavidin (SA) to the (MORF-biotin) γ -MAG₃-^{99m}Tc and measuring the shifted percentage of the radioactive MORF peak on HPLC. This is advantageous because conventional chemical characterizations would not directly provide the percentage of the NHS-Ph-CHO-modified MORF that carries a biotin. As known from our previous work, some MORF impurities that do not bear a biotin also react with NHS-ester although at a much slower rate.⁶

The relationships established above, the calculations of the avidin-induced clearability of the (MORF-biotin) γ -Ab, and the calculations of the SA-shifted percentage of (MORF-biotin) γ -MAG₃-^{99m}Tc on HPLC are summarized in Figure 3.

Validation of Our Colligation Using the (MORF-biotin) γ -MAG₃ Formed in Situ while Preparing (MORF-biotin) γ -Ph-CHO

The relative reactivity with the NHS-esters of the MORF impurities to that of the main content (MORF-biotin)-NH₂ might vary with reaction conditions. To rule out such a possible influence, we ensured identical reaction conditions by performing the NHS-MAG₃ and NHS-Ph-CHO conjugations in the same solution. Three aliquots of the (MORF-biotin) γ -MAG₃ from the “in situ NHS-MAG₃ conjugation” were labeled. Figure 4 shows the radiochromatogram of one labeled (MORF-biotin) γ -MAG₃ (top trace) and its chromatogram after adding SA (bottom trace). The (MORF-biotin) γ -MAG₃-^{99m}Tc after

adding excess cMORF is also included (middle trace) to confirm the radioactive label is on the MORF moiety. The results for the other two labeled (MORF-biotin)⌐-MAG₃ preparations are essentially identical. The SA-shifted percentage of the labeled (MORF-biotin)⌐-MAG₃ was calculated from the bottom traces as $88.8 \pm 1.1\%$ ($N = 3$).

The (MORF-biotin)⌐-Ph-CHO formed in the same solution was subsequently reacted with the Ab-Py-NHN=C(CH₃)₂. Table 1 lists the biodistribution at 3 h of the labeled (MORF-biotin)⌐-CC49 bound or non-bound with avidin. The percentage of the injected dose per gram is denoted as %ID/g. We chose to perform measurements at 3 h as this allows for completion of the clearance process.⁵ The avidin-induced clearability of the (MORF-biotin)⌐-Ab preparation was calculated as $86.9 \pm 1.3\%$ from the blood radioactivity levels bound or non-bound with avidin. The standard deviation was calculated following the uncertainty propagation rule:

$$SD = \left[SD_{\text{no avidin}}^2 \left(\frac{1}{ID\%/g_{\text{avidin-treated}}} \right)^2 + SD_{\text{avidin-treated}}^2 \left(\frac{ID\%/g_{\text{no avidin}}}{ID\%/g_{\text{avidin-treated}}^2} \right)^2 \right]^{1/2}$$

The agreement between the in vivo avidin-induced clearability of $86.9 \pm 1.3\%$ ($N = 5$) and the SA-shifted percentage of $88.8 \pm 1.1\%$ ($N = 3$) (2% difference) validates our colligation that the clearability of the (MORF-biotin)⌐-Ab in mice should be equal to the SA-shifted percentage of the (MORF-biotin)⌐-MAG₃.

Validation of Our Colligation Using the (MORF-biotin)⌐-MAG₃ Formed Separately from the (MORF-biotin)⌐-Ph-CHO Preparation

The “separate NHS-MAG₃ conjugation” was designed to verify that the buffer system used in the in situ conjugation described above would provide sufficiently identical reaction conditions if conjugating NHS-MAG₃ and NHS-Ph-CHO to the (MORF-biotin)⌐-NH₂ in two separate solutions. We compared the SA-shifted percentage of the labeled (MORF-biotin)⌐-MAG₃ from this separate conjugation study with that of the (MORF-biotin)⌐-MAG₃ prepared in situ of preparing (MORF-biotin)⌐-Ph-CHO. We also compared the SA-shifted percentage of the labeled (MORF-biotin)⌐-MAG₃ prepared separately with the avidin-induced clearability of the (MORF-biotin)⌐-Ab that was prepared in a manner independent of the NHS-MAG₃ conjugation process.

As shown in Table 2, two batches of (MORF-biotin)⌐-NH₂ were tested and two Abs (CC49 and Sandoglobulin) were conjugated with the (MORF-biotin)⌐-NH₂s. Batch A is the batch used in the “in situ conjugation”. In agreement with our hypothesis, the SA-shifted percentage of the labeled (MORF-biotin)⌐-MAG₃ ($90.7 \pm 1.4\%$) prepared in the “separate conjugation” agrees well with the value of $88.8 \pm 1.1\%$ reported in the in situ conjugation study mentioned above (difference of ~2%). In a manner independent of the MAG₃ conjugation, we conjugated batch A (MORF-biotin)⌐-NH₂ twice to Sandoz human immune globulin following the procedure of the “conjugation of (MORF-biotin)⌐-NH₂ to Ab”. The avidin-induced clearability was observed to be reproducible (89.4 ± 1.0 and $89.1 \pm 1.4\%$). More importantly, both values agree with the shifted percentage of $90.7 \pm 1.4\%$ and also with the value of $86.9 \pm 1.3\%$ measured in the in situ study using CC49 Ab (3% difference).

As the buffer system used in the in situ conjugation provides sufficiently identical reaction conditions for conjugating NHS-MAG₃ and NHS-Ph-CHO to the (MORF-biotin)⌐-NH₂ in two separate solutions, the conjugation results of NHS-MAG₃ to the (MORF-biotin)⌐-NH₂ should be predictive, because the SA-shifted percentage can be measured without performing the preparation of (MORF-biotin)⌐-Ab. Indeed, as shown in Table 2, batch B, when the SA-shifted percentage is larger ($93.6 \pm 2.1\%$), as predicted, the clearability of the Ab conjugate is higher ($95.0 \pm 1.7\%$). Thus, the conjugation of MAG₃ to (MORF-biotin)⌐-NH₂ can be used to predict the avidin-induced clearability of the (MORF-biotin)⌐-Ab.

DISCUSSION

With the increasing interactions among biology, medicine, and chemistry, many studies of chemical biology have focused on understanding the biological processes at the molecular level by synthesizing or modifying ligands targeting biological sites for diagnosis and therapy. But few efforts have been made to quantitatively correlate in vivo properties (biodistribution and pharmacokinetics) with in vitro assay results, although theoretical studies of structure–activity relationships and pharmacokinetic models contribute to the unification of biology, chemistry, and physics. In the investigation presented here, we have developed and tested an approach that quantitatively correlates the avidin-induced blood clearability of a trifunctional Ab construct with the SA-shifted percentage on HPLC of the labeled (MORF-biotin)⌐-NH₂ modifier. Thus, we are able to predict the required (MORF-biotin)⌐-NH₂ quality for a desired Ab clearability.

In addition to the general importance in methodology for expediting quality assurance and subsequent in vitro and in vivo evaluations, this study furthers our understanding of a particular pretargeting system. For example, our approach has directly correlated the (MORF-biotin)⌐-NH₂ percentage of the Abreacting MORFs in the sample to the in vivo clearability variations that would otherwise be considered in connection with many possible factors both in vitro and in vivo. We now know the Ab modifier does not have to be pure, although it has to be sufficiently pure with regard to the co-presence of biotin and the amine. Our SA-shifting method measures this purity and allows for the prediction of the clearability of a (MORF-biotin)⌐-Ab preparation prior to its synthesis. Conventional chemical characterizations do not allow for this direct assessment. We previously found that approximately 30% of the (MORF-biotin)⌐-NH₂ sample is not bound to biotin.⁶ These molecules do not carry a primary amine but react with NHS-ester (and finally Ab), albeit very slowly. Thus, we learned that more attention should be paid to the coexistence of biotin and amine on the MORF instead of the conventional purity.

A prerequisite of the current approach is the quantitative understanding of the in vivo processes. This investigation derives from our quantitative understanding of the in vivo clearance by avidin. We previously found that a biotinylated Ab, once bound to avidin in the blood pool, will be cleared rapidly and completely by the liver.⁵ Avidin itself is also cleared rapidly into the liver,¹⁴ with minimal diffusion into the extravascular space. On the basis of this understanding, we were able to quantitate the clearability by adding avidin to the injectate prior to injection into mice.

In the area of multiple-step targeting (pretargeting with a clearance step, as in this study), it is recommended that a disease model be used in addition to normal mice for a clearance study, to ensure that the number of binding sites in the disease target is not reduced by the clearance.¹⁵ However, that recommendation is based on the conventional clearance mechanisms in which the clearing agent competes with the effector. With those mechanisms, even though tumor accumulation may appear to be uncompromised,¹⁶ the number of effectors binding to the target may later be found to be reduced.¹⁵ We had previously validated that when the number of binding sites becomes smaller, if the mass dose of the effector is sufficiently low, tumor accumulation in %ID or %ID/g may remain unchanged.¹⁷ In our case where no binding competition is involved, it may not be necessary to use a disease model for a clearance investigation.

CONCLUSION

On the basis of our quantitative understanding of the targeting process and the chemical properties of the targeting agent, it is possible to quantitatively correlate in vitro assay results with in vivo properties, such that in vivo behavior can be quantitatively predicted by the in vitro chemical assay. In particular, the current SA-shifting methodology measures how much of the NHS-ester-reacting MORF derivatives in the (MORF-biotin)-NH₂ sample carries a biotin and therefore the percentage of the MORF groups attached to Ab together with a biotin. In conclusion, the in vivo avidin-induced clearability of the Ab can be predicted from the in vitro assay.

ACKNOWLEDGMENTS

This work was supported by Grants CA94994, DK94199, and DK82894 from the National Institutes of Health as well as a grant from JDRF, a Merit Review grant from the Department of Veterans Affairs, and the Vanderbilt Diabetes Research and Training Center Grant (DK 20593). We thank Dr. Zhihong Zhu for his previous work on MAG3 synthesis, the staff of the Division of Nuclear Medicine, Department of Radiology, University of Massachusetts Medical School, for providing the ^{99m}Tc, and the Gene Tools scientist Dr. Yongfu Li for the inspiring discussion with him during construction of the trifunctional antibody conjugates.

REFERENCES

1. Liu G, Dou S, Chen X, Chen L, Liu X, Rusckowski M, Hnatowich DJ. Adding a clearing agent to pretargeting does not lower the tumor accumulation of the effector as predicted. *Cancer Biother. Radiopharm.* 2010; 25:757–762. [PubMed: 21204772]
2. Green NM. Avidin. *Adv. Protein Chem.* 1975; 29:85–133. [PubMed: 237414]
3. Sinitsyn VV, Mamontova AG, Checkneva YY, Shnyra AA, Domogatsky SP. Rapid blood clearance of biotinylated IgG after infusion of avidin. *J. Nucl. Med.* 1989; 30:66–69. [PubMed: 2911044]
4. Liu G, Mang'era K, Liu N, Gupta S, Rusckowski M, Hnatowich DJ. Tumor pretargeting in mice using technetium-99m labeled morpholinos, a DNA analog. *J. Nucl. Med.* 2002; 43:384–391. [PubMed: 11884499]
5. Dou S, Virostko J, Rusckowski M, Greiner DL, Powers AC, Liu G. Differentiation between temporary and real non-clearability of biotinylated IgG antibody by avidin in mice. *Front. Pharmacol.* 2014; 5:e172.
6. Dou S, Virostko J, Greiner DL, Powers AC, Liu G. A feasible approach to evaluate the relative reactivity of NHS-ester activated group with primary amine-derivatized DNA analogue and non-derivatized impurity. *Nucleosides Nucleotides Nucleic Acids.* 2014; 2015(34):69–78.

7. Liu G, He J, Dou S, Gupta S, Vanderheyden JL, Rusckowski M, Hnatowich DJ. Pretargeting in tumored mice with radiolabeled morpholino oligomer showing low kidney uptake. *Eur. J. Nucl. Med.* 2004; 31:417–424.
8. Winnard P Jr, Chang F, Rusckowski M, Mardirossian G, Hnatowich DJ. Preparation and use of NHS-MAG₃ for technetium-99m labeling of DNA. *Nucl. Med. Biol.* 1997; 24:425–432. [PubMed: 9290078]
9. Liu G, Zhang S, He J, Zhu Z, Rusckowski M, Hnatowich DJ. Improving the labeling of S-acetyl NHS-MAG₃ conjugated morpholino oligomers. *Bioconjugate Chem.* 2002; 13:893–897.
10. Liu G, Dou S, He J, Yin D, Gupta S, Zhang S, Wang Y, Rusckowski M, Hnatowich DJ. Radiolabeling of MAG₃-morpholino oligomers with ¹⁸⁸Re at high labeling efficiency and specific radioactivity for tumor pretargeting. *Appl. Radiat. Isot.* 2006; 64:971–978. [PubMed: 16730997]
11. Liu G, Dou S, Mardirossian G, He J, Zhang S, Liu X, Rusckowski M, Hnatowich DJ. Successful radiotherapy of tumor in pretargeted mice by ¹⁸⁸Re-radiolabeled phosphorodiamidate morpholino oligomer, a synthetic DNA analogue. *Clin. Cancer Res.* 2006; 12:4958–4964. [PubMed: 16914585]
12. He J, Liu G, Dou S, Gupta S, Rusckowski M, Hnatowich DJ. An improved method for covalently conjugating morpholino oligomers to anticancer antibodies. *Bioconjugate Chem.* 2007; 18:983–988.
13. Liu G, Dou S, Pretorius PH, Liu X, Chen L, Rusckowski M, Hnatowich DJ. Tumor pretargeting in mice using phosphorodiamidate morpholino oligomer MORF. conjugated CC49 antibody and radiolabeled complimentary MORF effector. *Quarterly Journal of Nuclear Medicine.* 2010; 54:333–340.
14. Rosebrough SF. Pharmacokinetics and biodistribution of radiolabeled avidin, streptavidin and biotin. *Nucl. Med. Biol.* 1993; 20:663–668. [PubMed: 8358353]
15. Cheal SM, Yoo B, Boughdad S, Punzalan B, Yang G, Dilhas A, Torchon G, Pu J, Axworthy DB, Zanzonico P, Ouerfelli O, Larson SM. Evaluation of glycodendron and synthetically modified dextran clearing agents for multistep targeting of radioisotopes for molecular imaging and radioimmunotherapy. *Mol. Pharmaceutics.* 2014; 11:400–416.
16. Axworthy DB, Reno JM, Hylarides MD, Mallett RW, Theodore LJ, Gustavson LM, Su F, Hobson LJ, Beaumier PL, Fritzberg AR. Cure of human carcinoma xenografts by a single dose of pretargeted yttrium-90 with negligible toxicity. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97:1802–1807. [PubMed: 10677537]
17. Liu G, He J, Dou S, Gupta S, Rusckowski M, Hnatowich DJ. Further investigations of morpholino pretargeting in mice: Establishing quantitative relations in tumor. *Eur. J. Nucl. Med. Mol. Imaging.* 2005; 32:1115–1123. [PubMed: 16133391]

Figure 1.
Three-step pretargeting procedure in a mouse tumor model.

Figure 2. Conventional (left) and new (right) reaction routes to construct clearable trifunctional Ab. Note that the structures of MORF-NH₂ and (MORF-biotin)-NH₂ are depicted.

Figure 3.
Colligation of the avidin-induced clearability of a (MORF-biotin)-Ab injectate with the SA-shifted percentage of the (MORF-biotin)-MAG₃-^{99m}Tc determined via HPLC.

Figure 4. Radiochromatograms of (MORF-biotin)-MAG₃-^{99m}Tc: (a) alone, (b) native cMORF added (at a molar ratio of 55:1), and (c) SA added (at a molar ratio of 10:1).

Biodistribution at 3 h of the [^{99m}Tc]cMORF/(MORF-biotin)3-CC49 Preparation Treated with or without Excess Avidin^a

Table 1

	liver	heart	kidney	lung	spleen	muscle	blood
with avidin	29.6 ± 5.2	0.74 ± 0.09	3.05 ± 0.42	1.47 ± 0.72	4.41 ± 1.02	0.17 ± 0.03	2.03 ± 0.33
without avidin	6.99 ± 1.08	3.53 ± 0.38	6.63 ± 2.72	4.05 ± 0.71	2.73 ± 0.27	0.67 ± 0.07	15.5 ± 0.9

^aValues are means ± the standard deviation in the unit of %ID/g (N = 5).

Table 2

Percentages of the (MORF-biotin) γ -MAG₃-^{99m}Tc Shifted by SA on HPLC (*N* = 3) and the 3 h Clearability of Several (MORF-biotin) γ -Ab Conjugates (*N* = 5)

	batch A		batch B
% shifted by SA	90.7 $\pm$ 1.4%		93.6 $\pm$ 2.1%
Ab	Sandoz	Sandoz	CC49
Ab clearability	89.4 $\pm$ 1.0%	89.1 $\pm$ 1.4%	95.1 $\pm$ 2.7% ^a

^aData at 2 h, but the value at 3 h is expected to be similar as the Ab clearability at 4 h is 95.8 $\pm$ 3.9%.