**Special Section on DMPK of Therapeutic Proteins**

**Evaluation of Near Infrared Fluorescent Labeling of Monoclonal Antibodies as a Tool for Tissue Distribution**

Kip P. Conner, Brooke M. Rock, Gayle K. Kwon, Joseph P. Balthasar, Lubna Abuqayyas, Larry C. Wienkers, and Dan A. Rock


Received July 30, 2014; accepted September 8, 2014

**ABSTRACT**

The pharmacokinetic (PK) behavior of monoclonal antibodies (mAbs) is influenced by target-mediated drug disposition, off-target effects, antidrug antibody-mediated clearance, and interaction with fragment-crystallizable domain (Fc) receptors such as neonatal Fc receptor. All of these interactions hold the potential to impact mAb biodistribution. Near infrared (NIR) fluorescent probes offer an approach complementary to radionuclides to characterize drug disposition. Notably, the use of IRDye800 (IR800; LI-COR, Lincoln, NE) as a protein-labeling agent in preclinical work holds the potential for quantitative tissue analysis. Here, we tested the utility of the IR800 dye as a quantitative mAb tracer during pharmacokinetic analysis in both plasma and tissues using a model mouse monoclonal IgG1 (8C2) labeled with ≤1.5 molecules of IR800. The plasma PK parameters derived from a mixture of IR800-8C2 and 8C2 dosed intravenously to C57BL/6 mice at 8 mg/kg exhibited a large discrepancy in exposure depending on the method of quantitation [CL\text{\text{plasma}} = 8.4 \text{ ml/d per kilogram (NIR fluorescence detection) versus 2.5 \text{ ml/d per kilogram (enzyme-linked immunosorbent assay})}. The disagreement between measurements suggests that the PK of 8C2 is altered by addition of the IR800 dye. Additionally, direct fluorescence analysis of homogenized tissues revealed several large differences in IR800-8C2 tissue uptake when compared with a previously published study using \(^{125}\text{I}\)8C2, most notably an over 4-fold increase in liver concentration. Finally, the utility of IR800 in combination with whole body imaging was examined by comparison of IR800-8C2 levels observed in animal sagittal cross-sections to those measured in homogenized tissues. Our results represent the first PK analysis in both mouse plasma and tissues of an IR800-mAb conjugate and suggest that mAb disposition is significantly altered by IR800 conjugation to 8C2.

**Introduction**

Monoclonal antibodies (mAbs) embody an attractive therapeutic modality for disease intervention as they combine high affinity and specificity toward their target with biologic half-lives conducive to schedules of infrequent dosing. In part, the success of mAb therapeutics has sparked efforts to harness the favorable pharmacokinetic properties of the antibody scaffold by fusing the fragment-crystallizable domain (Fc) of an IgG with novel biologic proteins (Korth-Bradley et al., 2000; Fast et al., 2009; Herzog et al., 2014). However, the absorption, distribution, metabolism, and excretion of mAb-based proteins are complicated owing to interactions with the pharmacological target(s) (e.g., target-mediated drug disposition), the potential influence of antidrug antibodies, and interactions with Fc receptors. The aforementioned interactions create uncertainty when characterizing the pharmacokinetic and pharmacodynamic responses for mAbs and associated fusion proteins.

Knowledge of the disposition of engineered mAb-based therapeutic proteins that result from complex in vivo interactions provides insight into biologic activity and/or off-target effects, and can lead to design strategies that maximize the intended therapeutic response. Importantly, if the biodistribution of a candidate therapeutic is sufficiently resolved at the organ level during the design and testing stages of drug discovery, then this information can be useful for defining the parameters that best predict sites of target-mediated drug disposition and/or toxicity.

Radiolabeling of proteins represents an informative technique to study mAb distribution to antigen-specific tissues as well as overall organ exposure. For decades, mAb-based therapeutics have incorporated radionuclide labels for use as diagnostic tools (Sands, 1990; Duncan and Welch, 1993). Different radio-label tracers possess unique properties upon endogenous processing of the conjugated proteins. Notably, proteins conjugated with \(^{111}\text{In}\) and \(^{89}\text{Zr}\) exhibit increased mean residence time inside the cell, and as such are coined “residualizing” labels (Duncan and Welch, 1993). Residualizing behavior is believed to arise from a combination of a slower rate of protein catabolism and...
elimination from the cell and is most probably related to intrinsic physical properties of the linker and metal (Geissler et al., 1991; Duncan and Welch, 1993; Zhu et al., 1997; Perera et al., 2007; Diagaradjane et al., 2008; Boswell et al., 2010). In contrast, nonresidualizing probes that incorporate 1H, 13C, and 125I eliminate their protein and radiolabel catabolites from the cell more rapidly. This subtle distinction in vivo for various radioisotopes can be exploited to differentiate mAb tissue accessibility versus catabolic fate (Yip et al., 2014).

Several studies have used radiolabels to identify local off-target expression of an antigen, and to assess the impact of protein engineering on biodistribution and catabolism of mAbs (Aerts et al., 2009; Boswell et al., 2013; Steiner et al., 2013). Although the utility of radiolabels for characterization of therapeutic mAb biodistribution is unquestioned, their use is precluded in many laboratories owing to stringent regulatory requirements, in addition to the costly operational overhead. Thus, the availability of a more generally accessible label would be valuable.

Near infrared (NIR) fluorescent probes combined with optical detection offer a complementary approach to radiolabeling that has gained much interest in the clinical setting (Tanaka et al., 2007; Guo et al., 2014). For detection and imaging, NIR optical probes have several advantages over traditional fluorophores. First, the NIR spectrum is less prone to exciting endogenous fluorophores in animal tissue. Second, the long wavelength excitation and emission spectra of NIR probes allow for enhanced tissue penetration of the applied light source and transmittance of light from the specimen. These probe features result in negligible autofluorescence with enhanced signal recovery, and high target-to-background contrast. Last, direct tissue fluorescent measurement has been shown to be quantitative when proper sample dilution is considered (Oliveira et al., 2012). NIR optical probes offer ease of use, are cost effective, and require no specialized equipment or added safety precautions.

Numerous options for fluorescent probes and labeling chemistries exist, but the use of indocyanine-based IRDye800 from LI-COR (Lincoln, NE) allows the translation of preclinical data obtained with NIR conjugates to be used directly for downstream clinical studies. Although characterization of IR800 in tissue has been shown to be quantitative (Oliveira et al., 2012), additional characterization of the pharmacokinetics (PK) of IR800-mAb conjugates is warranted. Specifically, preclinical study of IR800-mAb conjugates that do not contain additional radiolabels has yet to be conducted. We sought to compare the in vivo behavior of an IR800-mAb to both its unlabeled mAb equivalent and that bearing the nonresidualizing 125I radiolabel. The objective of this current work is to evaluate and compare the pharmacokinetics and biodistribution in mouse of separately labeled IR800 and 125I conjugates of the model murine mAb, mouse monoclonal IgG1 (8C2). In addition, the novel combination of whole body sectioning as a tool for imaging biodistribution with IR800 is explored.

Materials and Methods

8C2, a murine IgG1 antitopotecan antibody, was employed as a model mAb for this study. The antibody was produced and purified from the culture of hybridoma cells, as previously described (Chen et al., 2007). Sodium iodide (Na-125I) was obtained from PerkinElmer (Waltham, MA). Chloramine-T, sodium metabisulfite, calcium sulfate (CaSO4), and carboxymethyl cellulose were from Sigma Life Science (St. Louis, MO). Potassium iodide was obtained from Fisher Scientific (Pittsburgh, PA). IRDye800 (IR800) was purchased from LI-COR. All other materials were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

8C2 Labeling. For conjugation of mAb 8C2 to IR800-NHS, 10 mg of antibody was buffer-exchanged into 100 mM potassium phosphate (pH = 8.5) using a Zeba 7-kDa MWCO spin-column (Pierce/Thermo, Rockford, IL). Prior to dilution to a final reaction concentration of 1.5 mg/ml as determined by UV absorbance, 20 mM Na-metabisulfite (1 mg/ml in methanol) was added from the reaction mixture via multiple rounds of buffer exchange on a Zeba 7k-Da MWCO spin column pre-equilibrated in PBS. Size exclusion chromatography was used to verify conjugate purity and the predominance of a monomeric mAb aggregation state monitored by UV-visible detection (215-, 280-, and 780-nm channels). Chromatography was conducted using an 4.6 × 300 mm Bio SEC-3 [30-μm pore, 3-μm diameter particle; Agilent Technologies (Santa Clara, CA)] with mobile phase containing 100 mM KPi (pH = 7.2) plus 150 mM NaCl. Initial characterization of the degree of labeling was conducted by UV-visible absorbance analysis, using a molar extinction for the IR800 provided by the manufacturer (ε770nm = 270,000 in PBS/MEOH 1:1) and a correction factor of 0.03 to account for the contribution of the dye to absorbance at 280 nm. As a secondary confirmation of final protein concentration, the conjugate was subjected to IR analysis using a Direct Detect FTIR spectrometer (Millipore, Billerica, MA) to measure the characteristic amide-stretch frequency (1600−1700 cm−1) previously calibrated with bovine serum albumin (BSA) standards. Final confirmation of the labeling was performed by liquid chromatography–mass spectrometry on an AB Sciex 5600 Triple TOF (Framingham, MA) coupled with a Shimadzu LC20 HPLC (Kyoto, Japan).

8C2 was radiolabeled with 125I using a modified chloramine-T method, as described in prior work (Garg and Balthasar, 2007). Briefly, 10 μl of Na-125I (100 μCi/ml) was added to the antibody solution, and 20 μl of potassium iodine (1 mg/ml in phosphate buffer) was then added to the mixture. The reaction was stopped after 90 seconds by the addition of 25 μl Na-metabisulfite (1 mg/ml in phosphate buffer), followed by 40 μl of potassium iodide (10 mg/ml). Iodinated protein was purified by loading the reaction mixture unto a Sephadex G-25 column (GE Healthcare, Pittsburgh, PA). The purity of the iodinated IgG was assessed using instant thin layer chromatography (PE Sil-G, Whatman Ltd., Kent, England) (Garg and Balthasar, 2007). For all experiments, the purity of the iodinated preparation was higher than 99%.

8C2 Pharmacokinetics. Male C57BL/6NHSed mice (Harlan Laboratories, Indianapolis, IN), age 6–9 weeks, weighing between 20 and 40 g, were cared for in accordance with Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). All research protocols were approved by the Institutional Animal Care and Use Committee and studies were in compliance with the Animal Welfare Act Regulations (9 CFR3) and the guidelines published by the National Institutes of Health.

8C2 tissue distribution and plasma PK were studied in C57BL/6 mice (20–40 g). To formulate the test article, unlabeled 8C2 and IR800-8C2 (degree of labeling = 1.2) were mixed for a final average degree of labeling of 0.2 and total 8C2 concentration of 1.4 mg/ml in PBS. The conjugate-containing mixture was dissolved at 8 mg/kg intravenously via tail vein injection. Blood was collected from three animals per time point sacrificed by exsanguination at 1, 2, 6, 12, 24, 48, 96, 168, and 240 hours. After exsanguination, organs were harvested at each time point (liver, lung, GI, heart, kidney, bone, fat, muscle, skin, and spleen), rinsed with saline, blotted dry, and weighed, after which the tissues were frozen and stored at −70°C. Immediately prior to starting the homogenization procedure, the tissues were removed from the −70°C freezer, weighed, and along with 5-mm stainless steel grinding balls placed into a 96-Well plate on ice. Chilled lysis buffer (50 mM Tris HCl, 100 mM NaCl, 0.1% Triton X-100, pH 7.4) containing a protease inhibitor cocktail (Roche, Indianapolis, IN) was then added at a volume equal to 2-fold the tissue mass (final conc. 1 g of tissue/2 g of buffer). The samples were homogenized using a Geno/Grinder 10 (SPEX SamplePrep, LLC, Metuchen, NJ).

IR800-8C2 conjugate levels were quantitated by fluorescence in both serum and tissue homogenates using the following general assay: Briefly, standard curves (ranging from 7.5 to 0.001 μg/ml) were prepared with IR800-8C2 test article spiked in control-tissue homogenate or serum diluted severalfold to assess matrix effects on the linear fluorescence response. Fluorescence (ex. = 784 nm, em. = 800 nm) of each 20-μl sample was measured in 384-well small-volume plate format with an Infinite M1000 PRO plate reader (Tecan, Morrisville, NC). Separate QC samples were prepared to validate each curve. To quantify levels in tissues, the samples were diluted directly in PBS to match the tissue-specific dilution matrix of each standard curve and quantified accordingly. The tissue concentrations were normalized to liver exposures for each individual animal. The final concentrations are reported in nanomolarity assuming a sample density of 1 g/ml and an 8C2 molecular weight of 150 kDa.
Plasma concentrations of 8C2 were also determined with the use of an antigen-capture enzyme-linked immunosorbent assay (ELISA). Briefly, 8C2 capture was performed with use of a cationized BSA-topotecan conjugate (cBSA-top), synthesized through the use of a carbodiimide-catalyzed amide bond reaction. Microplate wells (VWR International, Bridgeport, NJ) were coated overnight with cBSA-top dissolved in 0.02 m Na2HPO4, pH 9 (10 μg/ml, 250 μl/well). Plates were then washed with PB-Tween (0.05% Tween in 0.02 m Na2HPO4), followed by two washes of double-distilled water. Plates were then incubated with standards and samples in triplicate (200 μl) for 2 hours at room temperature. At the end of incubation, the plates were washed and then incubated with 100 μl of goat anti-mouse-Fab alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature (1:500 dilution of the conjugate with PB-Tween with 30% BSA). After washing, p-nitrophenyl phosphate solution (Pierce/Thermo), 4 mg/ml in diethanolamine buffer, pH 9.8, was added to each well (250 μl/well). The change in absorbance at 405 nm with respect to time (dA/dt) was measured (Spectra Max 340; Molecular Devices, Sunnyvale, CA), 5 minutes. Plasma fractions were collected and radioactivity was counted using a gamma counter (LKB Wallac 1272; Wallac, Turku, Finland). Radioactive counts were corrected for decay and background, and 8C2 plasma concentration was determined.

The pharmacokinetics of 125I-labeled 8C2 was conducted previously (Abuqayyas and Balthasar, 2012). Briefly, 8C2 was combined with a tracer dose of [125I]8C2 (10 μCi/mouse) and administered to a final dose of 8 mg/kg. Blood samples (20–40 μl) were collected from the retro-orbital plexus or from the submandibular vein at 1, 3, 8, 24, 48, 96, and 240 hours. Blood samples were centrifuged at 13,000 rpm for 5 minutes. Plasma fractions were collected and radioactivity was counted using a gamma counter (LKB Wallac: 1272; Wallac, Turku, Finland). Radioactive counts were corrected for decay and background, and 8C2 plasma concentration was determined.

The catabolite of IR800-8C2, IR800-L-lysine was formulated in 80% water and 20% DMSO to a total concentration of 2.0 mg/ml. The conjugate-containing mixture was dosed at 1 mg/kg intravenously via tail vein injection. Blood was collected from animals via retro-orbital sinus punctures for each time point; at 0-, 0.25-, 0.5-, 1-, 3-, 5- and 8-hour time points. Blood was transferred into Microtainer treated serum resulted in no detectable fluorescent signal. The pharmacokinetic parameters by ELISA were similar to those measured previously by 125I-labeled 8C2 directly (Abuqayyas and Balthasar, 2012).

### Tissue Quantitation with IR800-8C2 and [125I]8C2

8C2 tissue concentrations were evaluated by fluorescence detection in the liver, spleen, heart, lung, GI, kidney, muscle, skin, and bone. The AUC for each tissue was calculated using noncompartmental analysis. The parameters are reported in (Table 2). Previously, tissue distribution for 125I-labeled 8C2 was reported (Abuqayyas and Balthasar, 2012), and these results are also included in Table 2. A comparison of IR800-8C2 and [125I]8C2 tissue pharmacokinetic profiles is captured inFig. 3. Comparative analysis revealed consistently decreased exposure for the IR800-8C2 mAb compared with [125I]8C2. On average maximum tissues had 35% lower exposure compared with tissues measured with [125I]8C2 (excluding the liver). In contrast, the liver exhibited a 3.8-fold increase Cmax for IR800-8C2 relative to [125I]8C2, at 409 nm ± 36 Nm versus 108 nm ± 20 Nm, respectively. The corresponding AUC0–10 was 32% greater in the liver for IR800-8C2 compared with [125I]8C2.

The kidney, lung, and spleen all had similar initial concentrations between the two tracers. For example, the kidneys showed similar initial tissue concentration of 164 nM ± 34 nm for IR800-8C2 compared with 139 nM ± 16 nM for [125I]8C2, but then the IR800-8C2 concentrations fell sharply after 24 hours leading to an overall 3.9-fold lower tissue AUC0–10. A similar trend was observed for lung and spleen (Fig. 2). All other tissues measured using IR800-8C2 showed substantial decrease in exposures relative to [125I]8C2 ranging from 10 to 33% of the AUC0–10, respectively (Table 2). Upon correcting for exposures of 8C2 in the blood compartment, the tissue exposures for IR800-8C2 were not substantially increased with the exception of the liver (Fig. 5).

### Whole Body Cross-Sectioning with IR800-8C2

Whole body cross-sections (sagittal plane) were taken at specific time points to capture tissue exposures and for comparison with quantitative organ homogenization. Representative images are displayed in Fig. 4. In total, five levels were taken with level 1 being the outermost cross-section (closest to the skin) and level 5 representing the cross-section closest to the midline of the animal (Fig. 4, left panel). A representative time course at level 2 is shown in Fig. 4, right panel, and provides a time course of exposure to the major organs. At the initial time point it was evident IR800-8C2 was well distributed. In specific, the liver and bone marrow retained high levels of mAb. Organs of interest were quantified using LI-COR software (Image Studio v2.1). An attempt to use the whole body cross-sections to semiquantitatively measure tissue exposure was made by delineating the
individual organs in the software prior to measurement of the integrated fluorescence intensity, which was then normalized against that of the liver. A comparison of the whole body sections to the homogenized organs is shown in Fig. 5. The kidney, heart, muscle, bone, and GI were evaluated. In all tissues except the GI the levels determined from whole body cross-sections were lower than those derived directly from tissue homogenization. However, there were no statistically significant differences observed between the two methods.

Discussion

The differential serum exposure observed upon dosing IR800-8C2 depending on the quantitation assay employed (ELISA or direct IR800 fluorescence) was unexpected. Previous work (Oliveira et al., 2012) highlights the utility of the IR800 dye attached to antibodies as a method for evaluating mAb biodistribution. In that study, the authors employed a dual-labeling approach wherein cetuximab was labeled with desferal-chelated positron emitter zirconium (\(^{89}\)Zr) and IR800 on the same mAb molecule to yield \([^{89}\text{Zr}]\text{cetuximab-IR800}\) (Oliveira et al., 2012). This approach yielded similar patterns in biodisposition of the two tracers. Unfortunately, the comparison of dual-labeled \([^{89}\text{Zr}]\text{cetuximab-IR800}\) to \([^{89}\text{Zr}]\text{cetuximab}\) rather than to unlabeled cetuximab confounds any interpretation of changes in drug disposition induced independently by either tracer.

Previously, Cohen et al. (2011) had highlighted the limitations of IR800 with regard to altered liver uptake, yet that study also drew conclusions from the use of mAb containing both \([^{89}\text{Zr}]\text{desferal}\) and IR800 tracers. The results reported therein describe increased liver uptake when IR800 dye to mAb levels exceeded 1.5. With these previous results in mind, our aim was to keep the degree of labeling to minimize the potential impact of IR800 on pharmacokinetics. However, even with these precautions, an effect was observed with respect to IR800-8C2 serum exposure. It is plausible that the differences in physicochemical properties of the 8C2 and cetuximab antibodies contribute to their unique biodistribution. Alternatively, the rather moderate alteration in blood clearance and biodistribution into tissues observed by Cohen et al. when using 1–2 molecules of IR800 may have been influenced by the coconjugation of the desferal chelate moiety, which may have masked some of the effects of IR800 tracer in the case of \([^{89}\text{Zr}]\text{cetuximab-IR800}\).

Previously, our laboratory has evaluated the IR800 dye to characterize biodistribution for several therapeutic proteins and mAbs. For example,
a 55-kDa mAb fragment bearing the IR800 dye as the tracer displayed a PK profile indistinguishable from the unlabeled molecule (Supplementary Fig. 1A). An important distinction between this fragment and IR800-8C2 mAb is that it has much faster clearance compared with a full mAb (hours versus days). One possibility for the apparent lack of IR800 label-related effects during our previous study is that the molecule exhibits an inherently high clearance and short half-life that could mitigate any impact of IR800 tracer on the PK. Thus, we hypothesize that low clearance and long mean residence time are requisites to observe deviations in clearance from the parent molecule (i.e., the stark IR800-dependent increase in CL will be sufficiently resolved). In support of our hypothesis, we have since examined other IR800-labeled mAbs and have observed results similar to those reported herein (Supplemental Fig. 1B). These observations suggest that the effects of IR800 protein disposition are not mAb-specific, which may markedly limit the utility of this probe for quantitative tissue biodistribution studies, at least for mAb-based therapeutics. Work in our laboratory is ongoing to test whether the short half-lives of IR800-labeled mAb fragments, such as Fab and F(ab)2, mask the effects of IR800 on the observed pharmacokinetics and tissue distribution.

A benefit of using mouse mAb 8C2 as a model IgG1 is the lack of endogenous antigen, and it therefore displays linear pharmacokinetics, as previously demonstrated (Abuqayyas and Balthasar, 2012). Importantly, these properties facilitate the comparison with previously reported PK studies of 8C2 with 125I as the labeling agent. The pharmacokinetic parameters observed with IR800-8C2 were distinct from the 125I-8C2 data when measured using IR800 fluorescence. However, when the IR800-8C2 was measured by ELISA, the data were comparable to those determined previously by gamma counting via the attached 125I label (Fig. 2). The distinction in analytical methods is that IR800 fluorescent detection measures only the conjugated 8C2, whereas the ELISA measures both the conjugated and unconjugated mAb. As such, the different results could arise via several mechanisms. The IR800 label on the mAb could be unstable yielding unreliable quantitation by fluorescence. In vitro incubations in plasma over 48 hours did not produce any loss in fluorescent signal (data not shown). In addition, analysis of tricarboxylic acid–precipitated serum supernatant revealed no fluorescent signal (<1 ng/ml), implying that IR800-8C2 circulates intact, and is consistent with previous stability experiments (Oliveira et al., 2012). Fluorescence quenching is another possible rationale for a discrepancy in PK parameters, but the sensitivity of IR800 allowed for rather large dilutions that minimize this risk. Furthermore, if quenching was to occur, one would expect this effect to be variable across different tissues and this is inconsistent with the tissue PK that exhibits similar elimination rates for all sample types.

To further investigate if tissue-specific uptake was responsible for the accelerated clearance of IR800-8C2, quantitative tissue analysis was performed. The 125I-8C2 tissue distribution data previously generated was used as a baseline of what might be expected for IR800-8C2 (Fig. 3). The liver showed higher 8C2 concentrations for the first 5 days with the IR800 tracer versus 125I-8C2. Given that the liver is one of the primary clearance organs for mAbs, this increase is probably responsible for the lower serum exposures of IR800-8C2. It is important to recognize that the higher concentrations of IR800-8C2 could also be related to the residualizing nature of the IR800 tracer compared with 125I. However, upon analysis of the other tissues the IR800 levels were consistently lower than those obtained with 125I. These findings are at odds with what would be expected for a residualizing tracer that is anticipated to have greater tissue exposure relative to a nonresidualizing 125I (Table 2; ratio of IR800 T:S/125I T:B). Therefore, the increased liver concentrations for IR800-8C2 do not appear to be a result of differences in residualizing properties between the two tracers. The

### TABLE 1.

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Analytical Technique</th>
<th>AUC (125I)</th>
<th>CL</th>
<th>Vss</th>
<th>C0</th>
<th>t1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nM*day</td>
<td>ml/d per kg</td>
<td>ml/kg</td>
<td>nM</td>
<td>days</td>
</tr>
<tr>
<td>IR800-8C2</td>
<td>ELISA</td>
<td>1628 (68)</td>
<td>2.5 (0.2)</td>
<td>30 (1)</td>
<td>624 (142)</td>
<td>7.4 (0.8)</td>
</tr>
<tr>
<td></td>
<td>Fluorescence</td>
<td>875 (115)</td>
<td>8.4 (1.1)</td>
<td>33 (4)</td>
<td>672 (81)</td>
<td>2.7 (0.3)</td>
</tr>
<tr>
<td>[125I]8C2</td>
<td>Gamma count</td>
<td>2145 (51)</td>
<td>1.8 (0.2)</td>
<td>26 (2)</td>
<td>638 (61)</td>
<td>10.1 (3.1)</td>
</tr>
<tr>
<td>Lysine-IR800</td>
<td>Fluorescence</td>
<td>104 (27)</td>
<td>21.3 (4.1)</td>
<td>592 (8)</td>
<td>2027 (289)</td>
<td>0.05 (0.01)</td>
</tr>
</tbody>
</table>

*Dosed separately. IR800-L-lysine is the major protein catabolite of NHS-IR800-labeled proteins.

### TABLE 2.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AUC (IR800)*</th>
<th>AUC ([125I])</th>
<th>AUC (IR800)/AUC ([125I])</th>
<th>TB (IR800)/TB ([125I])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM*day</td>
<td>nM*day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>636.1 (69)</td>
<td>483.7 (57.1)</td>
<td>1.32</td>
<td>3.24</td>
</tr>
<tr>
<td>Spleen</td>
<td>117.1 (15.5)</td>
<td>560.5 (56.9)</td>
<td>0.21</td>
<td>0.52</td>
</tr>
<tr>
<td>Lung</td>
<td>213.6 (13.9)</td>
<td>656.4 (65.1)</td>
<td>0.33</td>
<td>0.80</td>
</tr>
<tr>
<td>Heart</td>
<td>410.0 (6.6)</td>
<td>406.8 (33.3)</td>
<td>0.10</td>
<td>0.25</td>
</tr>
<tr>
<td>Kidney</td>
<td>130.1 (23)</td>
<td>505.5 (45.6)</td>
<td>0.26</td>
<td>0.63</td>
</tr>
<tr>
<td>Skin</td>
<td>80.1 (0.5)</td>
<td>630.8 (27.8)</td>
<td>0.13</td>
<td>0.31</td>
</tr>
<tr>
<td>Muscle</td>
<td>21.3 (1.4)</td>
<td>1889.9 (7.9)</td>
<td>0.01</td>
<td>0.28</td>
</tr>
<tr>
<td>Bone</td>
<td>61.2 (3.3)</td>
<td>324.9 (15.2)</td>
<td>0.19</td>
<td>0.46</td>
</tr>
<tr>
<td>GI</td>
<td>56.2 (3.1)</td>
<td>350.3 (101.3)</td>
<td>0.16</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*AUC determined directly by IR800 fluorescence.

**TB** represents the tissue-to-blood ratio. Parameters are derived from triplicate measurements with average and (standard deviation) reported.
increased liver signal is most probably attributable to increased specific uptake and elimination of IR800-8C2. Previous studies demonstrated specific uptake of several proteins, including IgG, into mouse liver nonparenchymal cells via scavenger receptors after modification with succinic acid to alter the negative charge density (Yamasaki et al., 2002). By extension, it is possible that conjugation of IgG with IR800, which bears several sulfonic acid groups, may influence the disposition of IR800-8C2 in a similar fashion.

Fig. 3. Tissue pharmacokinetic profiles for IR800-8C2 and [125I]8C2. Conc, concentration; GI, gastrointestinal tract.
Previously, parallels between IR800 tissue concentrations to those observed with $^{89}$Zr provide evidence (Oliveira et al., 2012) to support that IR800 can behave as a residualizing tracer. However, as mentioned previously, that study evaluated a dual-labeled ($^{89}$Zr and IR800) molecule. It is plausible that the dual labeling approach interferes with the inherent properties of the IR800. Specifically, the $^{89}$Zr chelate desferal moiety could impact the cellular disposition of the IR800 catabolite. We have identified the major catabolite from IR800 protein conjugation to be IR800-L-lysine (data not shown). Therefore, in a separate mouse study, we administered an intravenous dose of IR800-L-lysine and observed rapid clearance from serum (Fig. 2). The rapid elimination of IR800-L-lysine ($t_{1/2} < 1$ hour) is consistent with the molecule’s zwitterionic character that should lead to low permeability. To this end, the formation of the IR800-L-lysine in the cell upon catabolism of IR800-8C2 could lead to reduced cellular efflux. These findings support further investigation into the tracer properties of IR800.

Lastly, an additional aim of this work was to evaluate the ability of IR800 tracer in combination with whole body imaging using a fluorescent scanner as an affordable, rapid method to perform tissue biodistribution studies. The technique is capable of generating quality images using a basic laboratory infrared scanner and can be used to detect exposures to the major organs (Fig. 4), similarly to quantitative whole body autoradiography techniques. Ideally, the whole body imaging could represent the tissue concentrations that were determined by homogenization. Figure 5 compares the tissue homogenized AUC_{0-10} values compared with the organ quantified by region-of-interest at the 48-hour time point, demonstrating the feasibility of this approach. The tissue levels were normalized to the liver values determined from each analytical technique. The two methods did not yield any statistical difference in the subset of tissue analyzed illustrating the potential value of this approach. Several advantages of this technique are the ability to rapidly sample across the entire organ in addition to specific regions of interest. This method also allows strict boundaries to be drawn between tissues. One drawback is that not all organs are clearly identifiable within the cross-sections. Further optimization of the technique should be employed in an attempt to improve the translation of data from whole body imaging to that from homogenization using a fluorescent tracer that does not impact protein disposition. For example, it is possible to vary the chosen tissue slice thickness, and this may allow less heterogeneous tissue sampling in a thicker slice; alternatively, one could aim for less variability in fluorescent signal via signal quenching if the slice thickness is reduced.

On the basis of the findings herein, the IR800 as a tracer for proteins clearly impacts the pharmacokinetics of mAbs and is not ideal for determining absolute tissue distribution in mice. However, the sensitivity and selectivity of the IR800 tracer is obvious and the potential to provide exquisite contrast from background enables clear distinction of antigen-expressing tissues in mouse xenograft models and can serve as a useful tracer for intraoperative imaging (Kovar et al., 2009; Keereweer et al., 2012). Investigations into the mechanism(s) leading to the accelerated serum PK and enhanced liver uptake are ongoing. Defining the translation of these findings from the mouse to humans will be important to developing quantitative optical imaging techniques and can provide additional characterization of fluorescent tracers for use as clinical diagnostic tools.
Authorship Contributions

Conducted experiments: Conner, B. Rock, Kwon, D. Rock.

Contributed new reagents or analytic tools: Balthasar.

Performed data analysis: Conner, D. Rock.

Wrote or contributed to the writing of the manuscript: Conner, B. Rock, D. Rock.

References


Address correspondence to: Dr. Dan A Rock, 1201 Amgen Court West, Seattle, WA 98119. E-mail: drock@amgen.com
Supplemental Figure

Evaluation of Near IR Fluorescent Labeling of mAbs as a Tool for Tissue Distribution

Kip P. Conner, Brooke M. Rock, Gayle K. Kwon, Joseph P. Balthasar, Lubna Abaquayyas, Larry C. Wienkers and Dan A. Rock

Biochemistry and Biophysics Group in Pharmacokinetics and Drug Metabolism, Amgen Inc. Seattle, WA (KPC, BMR, GKK, LCW, DAR)

Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY (JPB)

Quantitative Pharmacology Group in Pharmacokinetics and Drug Metabolism, Amgen Inc. Thousand Oaks, CA (LA)
Supplemental Figure 1: (A) Pharmacokinetics of unlabeled representative protein and conjugated 800IR representative protein (B) Pharmacokinetics of unlabeled human IgG1 protein and conjugated 800IR human IgG1 protein.