Intravital microscopy reveals unforeseen biodistribution within the liver and kidney mechanistically connected to the clearance of a bifunctional antibody

Amita Datta-Mannan†1, Bruce A. Molitoris2, Yiqing Feng3, Michelle M. Martinez2, Ruben M. Sandoval2, Robin M. Brown4, Daniel Merkel3, Johnny E. Croy3 and Kenneth W. Dunn†2

1 Exploratory Medicine and Pharmacology (A.D-M.), 4 Clinical Laboratory Services (R.B.), Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46225, USA.

2Department of Medicine, Division of Nephrology, Indiana University School of Medicine, 950 W. Walnut St., Indianapolis, IN 46202

3Biotechnology Discovery Research (Y.F., D.M., and J.C.), Lilly Research Laboratories, Lilly Technology Center North, Indianapolis, IN 46221, USA
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Corresponding authors:

Amita Datta-Mannan, Eli Lilly and Company, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46225 USA, Tel.: (317) 276-2000; Email:datta_amita@lilly.com

Kenneth W. Dunn, Department of Medicine, Division of Nephrology, Indiana University School of Medicine, 950 W. Walnut St., Indianapolis, IN 46202

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Non-standard abbreviations:

- BfAb – bifunctional antibody
- mAb – monoclonal antibody
- ECD – extracellular domain
- IVM – intravital microscopy
- TMDD – target-mediated drug disposition
ABSTRACT

Bifunctional antibody therapeutics offer the potential for novel functionalities beyond those of the individual mono-specific entities. However, combining these entities into a single molecule can have unpredictable effects, including changes in pharmacokinetics that limit the compound’s therapeutic profile. A better understanding of how molecular modifications impact in vivo tissue interactions could help inform bifunctional antibody design. The present studies were predicated on the observation that a bifunctional antibody (BfAb) designed to have minimal off-target interactions cleared from the circulation twice as fast as the monoclonal antibody (mAb) from which it was derived. The present study leverages the spatial and temporal resolution of intravital microscopy (IVM) to identify cellular interactions that may explain the different pharmacokinetics of the two compounds. Disposition studies of mice demonstrated that radiolabeled compounds distributed similarly over the first 24 hours, except that BfAb accumulated ~2-3-fold more than mAb in the liver. IVM studies of mice demonstrated that both distributed to endosomes of liver endothelia, but with different kinetics; whereas mAb accumulated rapidly within the first hour of administration, BfAb accumulated only modestly during the first hour, but continued to accumulate over 24 hours, ultimately reaching levels similar to those of the mAb. Although neither compound was freely filtered by the mouse or rat kidney, BfAb (but not mAb) was found to accumulate over 24 hours in endosomes of proximal tubule cells. These studies demonstrate how IVM can be used as a tool in drug design, revealing unpredicted cellular interactions that are undetectable by conventional analyses.
SIGNIFICANCE STATEMENT

Bifunctional antibodies offer novel therapeutic functionalities beyond those of the individual mono-specific entities. However, combining these entities into a single molecule can have unpredictable effects, including undesirable changes in pharmacokinetics. Studies of the dynamic distribution of a bifunctional antibody and its parent monoclonal antibody presented here demonstrate how intravital microscopy can expand our understanding of the in vivo disposition of therapeutics, detecting off-target interactions that could not be detected by conventional pharmacokinetics approaches nor predicted by conventional physicochemical analyses.
INTRODUCTION

Multi- or bi-functional antibodies (BfAbs) are an emerging class of biotherapeutics. Unlike monoclonal antibodies (mAbs) which typically target one epitope, BfAbs recognize at least two different epitopes or antigens within a single moiety often through an obligate physical linkage of the binding components. These interactions can expand and prolong BfAb efficacy. Advances in protein engineering have enabled significant innovations of BfAb formats through leveraging the modular structure of their counterpart mAbs (Labrijn et al. 2019; Ma et al. 2021). The IgG-like BfAbs, including IgG-extracellular domain (ECD) formats, consist of target binding subunits attached a mAb (Sergey E Sedykh 2018).

Although BfAbs have immense therapeutic promise and structural tractability, the translation of these modalities as medicines has been relatively slow compared to mAbs, with approvals for amivantamab, emieizumab and blinatumomab occurring recently. Like most therapeutics, the causalities of BfAb slow clinical success can be generally related to several factors, including incomplete understanding of the biological mechanism of action and exposure-response profiles, insufficient safety margins, strategic industry decisions and immunogenicity. The higher structural diversity of the BfAbs also results in greater uncertainty in their pharmacokinetic (PK) and disposition profiles that could limit their potential advantages (Boswell et al. 2012; Boswell et al. 2013; Datta-Mannan 2019; Khawli et al. 1996; Khawli et al. 2010; Tibbitts et al. 2016; Rock and Foti 2019; Datta-Mannan et al. 2019; Datta-Mannan A 2016 ; Datta-Mannan et al. 2021). As such, consideration of dynamic evaluations of BfAb tissue and cellular biodistribution profiles and how these are connected to their clearance are warranted.

Integration of quantitative whole-body biodistribution and tissue level imaging into the development can provide informative readouts regarding the disposition (Boswell et al. 2012; Boswell et al. 2013; Datta-Mannan 2019; Khawli et al. 1996; Khawli et al. 2010). Towards this, a handful of imaging approaches including, computed tomography (CT), magnetic resonance imaging, whole-body imaging bioluminescence/radiolabeling and positron emission tomography (PET) have been leveraged (Boswell et al. 2013; Datta-Mannan 2019; Datta-Mannan et al. 2021; Datta-Mannan A 2016 ; Williams et al. 2016; Boswell et al. 2019; Datta-Mannan et al. 2019).
While these imaging techniques offer a high degree of sensitivity and enable some quantitative measurements of the pharmacokinetic-pharmacodynamic (PK-PD) relationship, they lack spatial and temporal resolution; thus, multiple approaches are required to characterize the connectivity of disposition to pharmacology (Wang et al. 2018). Often, for cellular distribution resolution this requires orthogonal histological evaluations. These evaluations involve sacrificing animals and can be laborious in terms of identifying reagents, unidimensional and have the potential for artifactual findings (Wang et al. 2018).

Advances in the field of non-linear microscopy have made intravital multiphoton microscopy (IVM) an important tool, combining high sensitivity and high spatiotemporal resolution to provide a unique window into the dynamics of cell biology in living animals (Dunn and Day 2017; Nobis et al. 2018). IVM provides a novel approach to examine real-time molecular behavior within intact organs, providing insights into cellular and subcellular transport that complement radiolabel biodistribution approaches. In the present study, we combined quantitative whole-body radiolabel studies with IVM to gain insight into the real-time dynamic disposition and uptake/elimination of a BfAb and its counterpart parental mAb within major organs of elimination as a means to dissect the mechanisms influencing the in vivo tissue, cellular and subcellular distribution of the molecules.

An IgG-ECD BfAb architecture made with ECD and mAb units targeting two distinct soluble ligands which have negligible peripheral concentrations in normal animals was used for the present work, so disposition and cellular trafficking dynamics could be evaluated without target mediated drug disposition (TMDD). This BfAb, deemed G₄₁-C-HC, has the D2 domain of vascular endothelial growth factor receptor 1 (VEGFR1) as the ECD fusion partner, connected via a flexible Glycine-Serine linker to the C-terminal end of the heavy chain (HC) of an IgG₄-based parent (G₄₁-) mAb (Supplemental Figure 1) (Datta-Mannan et al. 2019). In earlier studies, G₄₁-C-HC showed inferior PK properties in mice relative to the parental G₄₁- mAb (Datta-Mannan et al. 2019). Herein, studies with a radiometal chelating tissue residualizing probe (which can accumulate within tissues if constructs are catabolized intracellularly) showed the PK differences were connected to early enhanced association and accumulation of the G₄₁-C-HC construct within liver and kidney. While the tissue concentration disparities between the molecules was observed at times <24 hours post administration, the blood exposures at
≤24 hours were perplexingly similar between the molecules. Thus, the mechanistic basis of the later differences in clearance was further addressed using fluorescence-based dynamic IVM in kidneys and liver.

The IVM studies revealed three key insights: 1) fluorescent conjugates of G₄₁-C-HC BfAb do clear faster from the circulation than corresponding fluorescent conjugates of G₄₁ mAb during the first 24 hours post-dose; 2) both compounds are internalized into sinusoidal endothelia in the liver, but with strikingly different kinetics; 3) G₄₁-C-HC BfAb (but not G₄₁ mAb) accumulates dramatically in endosomes of the proximal tubule epithelia. While the basis for these differences are unclear, they appear to reflect differences in how the two compounds interact with the endocytic systems of the liver and kidney. Taken together, these results highlight the importance of exploring dynamics of tissue, cellular and subcellular disposition for understanding the mechanistic basis of biologics clearance and elimination to improve drug-ability.

MATERIALS AND METHODS

Expression and purification of monoclonal and bispecific-antibody molecules

The mAb and BfAb described within this report were expressed in either transient HEK-293 or stably-transfected Chinese hamster ovary cells that were generated at Eli Lilly and Company (Indianapolis, IN, USA) as described elsewhere (Datta-Mannan et al. 2019; Datta-Mannan A 2016).

DyLight594 (DL594) fluorescent probe labeling of monoclonal and bispecific-antibody molecule

G₄₁ mAb and G₄₁-C-HC BfAb were both labeled with DyLight594 NHS Ester (Thermo Fisher, Waltham, MA). The DyLight594 dye was dissolved in DMSO to a concentration of 9.3 mM. A 3x molar excess of dye was added to either G₄₁ mAb or G₄₁-C-HC BfAb and the reaction mixtures were incubated at room temperature for 1 hour to achieve an average dye to G₄₁ mAb or G₄₁-C-HC BfAb ratio of 1.6 and dye to G₄₁-C-HC BfAb ratio of 1.0. Removal of excess dye was accomplished using preparative size exclusion chromatography (GE 16/60 HiLoad Superdex 200pg) while buffer exchanging into Gibco 1xPBS pH 7.2 buffer.

In vitro characterization of the fluorescently labeled-monoclonal and bispecific-antibody
Electrostatic interactions of G41 mAb or G41-C-HC BfAb were measured using a heparin chromatographic method as described in Datta-Mannan et al. (Datta-Mannan A 2016). The extent of heparin sulfate binding was assessed by the observed column retention time and the corresponding sodium chloride concentration to elute the bound protein from the column.

Global hydrophobicity of the molecules evaluated in this study was assessed using hydrophobic interaction chromatography methods as described previously (Datta-Mannan A 2016). The hydrophobicity of the molecules was inferred based on the observed column retention (measured in minutes) and the corresponding minimal ammonium sulfate concentration required for column retention.

The binding of fluorescently labeled G41- mAb or G41--C-HC BfAb to rat FcRn was measured at pH 6 in a microscale thermophoresis (MST) assay as previously described (Wagner et al. 2016).

Exposure evaluation of DL594 labeled- and unlabeled monoclonal and bispecific-antibody molecules in rats

A Sprague Dawley (SD) rat exposure study was conducted in accordance with Standard Operating Procedures (SOPs) and the protocol as approved by the Covance IRB (now Labcorp, Madison, WI, USA) and in compliance with the requirements of Eli Lilly and Company. The exposure study was performed with male rats (~300-350 g) with the DL594 labeled and unlabeled version of the G41 mAb and G41-C-HC BfAb molecules. Three rats were assigned to each study group and all animals received a single intravenous (IV) bolus dose of either DL594-labeled or unlabeled G41 mAb and DL594-labeled or unlabeled G41--C-HC BfAb dissolved in PBS (pH ~7.4) at 1.0 mg/kg. Each animal had blood samples collected via a jugular vein at 0.083, 1, 6, 12 and 24 hours after administration of the dose. Additionally, blood samples for the DL594-labeled G41 mAb and DL594-labeled G41-C-HC BfAb were also collected at 48, 72 and 96 hours post dose. All the blood samples were collected into tubes containing K3EDTA maintained in chilled cyroracks and centrifuged to obtain plasma. Concentrations of the labeled and unlabeled G41 mAb and G41-C-HC BfAb molecules in rat plasma were determined using anti-human IgG ELISAs as previously described (Datta-Mannan et al. 2019). Plasma concentration-time data following IV administration for the labeled constructs was described using a
noncompartmental method according to the statistical moment theory using Phoenix® WinNonlin® software package (Pharsight, A Certara™ Company, St. Louis, MO). The parameters calculated included the maximum serum concentration ($C_{\text{max}}$) and area under the curve ($AUC_{0-\infty}$), clearance (CL), and elimination half-life ($t_{1/2}$).

**Murine biodistribution study with radiolabeled $G_4l$ mAb and $G_4l$-C-HC BfAb using a tissue residualizing agent**

A murine tissue distribution study was conducted in accordance with SOPs and the protocol as approved by Eli Lilly and Company and in compliance with the requirements contained in the MPI Research Radioactive Materials License Number 21-11315-02 (now Charles River, Mattawan, MI, USA), and all applicable regulations issued by the Nuclear Regulatory Commission (NRC) as described previously (Datta-Mannan et al. 2019). Briefly, the non-radiolabeled $G_4l$ mAb and $G_4l$-C-HC BfAb were conjugated to diethylene triamine pentaacetic acid (DTPA) and radiolabeled with $^{111}$In at MPI Research, Inc. (now Charles River, Mattawan, MI, USA) to target a low specific activity using previously published radiochemistry approaches (Boswell et al. 2010). The dosing formulations were administered once via IV injection into the tail vein to a target dose level of 2 mg/kg (15 μCi/animal). Blood samples were collected at 1-, 6-, 12- and 24-hours post-dose (cohorts of two animals per group), processed to plasma and analyzed as reported in earlier studies (Datta-Mannan et al. 2019). Tissues including adrenal gland, bladder (urinary), bone (femur), bone marrow (femur), brain, muscle (gastrocnemius, both quadriceps, and scapular region), heart, kidney, large intestine/cecum with contents, liver, lung, lymph nodes (mesenteric), pancreas, skin (ventral and upper and lower dorsal), small intestine with contents, spleen, stomach with contents, testes, thymus, thyroid, and fat pad were also collected and analyzed from two animals per time point (1, 6, 12, and 24 hours postdose) as reported previously (Datta-Mannan et al. 2019). Individual gamma radioactivity counts were utilized for image reconstruction and analysis.

**Intravital Microscopy studies in mice and rats**

Intravital microscopy studies of mice used either Lys-EGFP (Faust et al., 2000) or C57BL/6N (Jackson Labs, Bar Harbor, Maine, USA) strains (23-30 grams, 7-10 weeks of age). The Lys-EGFP mice express EGFP under the control of the Lys promoter, resulting in expression of EGFP by myelomonocytic cells. Intravital microscopy studies of rats used Munich-Wistar-Fromter rats (9-12 weeks of age), from a colony maintained at...
Indiana University that was originally derived from animals generously provided by Dr. Roland Blantz (UC San Diego). All animals were maintained at the Indiana University LARC facility and were provided with food and water *ad libitum*. All animal experiments were approved and conducted according to the Institutional Animal Care and Use Committee guidelines of Indiana University and adhered to the guide for the care and use of animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals. et al., 2011).

Fluorescent probes used for intravital microscopy studies included Hoechst 33342 trihydrochloride trihydrate (2 mg/kg) (labels nuclei, Thermo Fisher Scientific, Eugene OR, USA), rhodamine 123 (5 µg/kg) (labels functional mitochondria in vivo, Thermo Fisher Scientific, Eugene OR, USA) and fluorescent rat albumin (for studies of glomerular permeability). Rat albumin (Sigma) was conjugated to Texas Red-X succinimidyl ester (AAT Bioquest, Sunnyvale, CA, USA) using standard procedures to achieve a final 1:1 protein to dye ratio and then extensively dialyzed using a 10kDa cutoff filter prior to use (Sandoval et al. 2012). The preparation and validation of DL-594 conjugates of G41 mAb and G41-C-HC BfAb are described in the results section. Immunofluorescence studies were conducted using rat anti-mouse F4/80 (Invitrogen, Carlsbad, CA, USA) and goat anti-mouse MMR (Macrophage Mannose/CD206 receptor, R&D systems, Minneapolis, MN, USA). Primary antibodies were fluorescently labeled using Alexa Fluor 488 goat anti-rat and Alexa Fluor 647 donkey anti-goat antibodies (Invitrogen, Carlsbad, CA, USA).

Intravital microscopy studies were conducted either 24 hours after intravenous injection of DL-594 conjugates G41-C-HC BfAb or G41 mAb (6.5 mg/kg), or during and for the first two hours following intravenous injection. Studies of mouse liver were conducted generally as described previously(Dunn and Day 2017; Ryan et al. 2018). Surgical preparation was started approximately one hour prior to imaging. The mouse was placed on an induction chamber connected to an anesthesia isoflurane circuit at 2-4% of Isoflurane with 1% O2. Once stabilized under anesthesia, the ventral abdominal side of the body and the right side of the neck were shaved and cleaned. Prior to exposing the liver, a jugular catheter was prepared for probe injections. A 1 cm right ventral incision was made in the neck, the jugular was exposed and all fat and fascia surrounding were cleared. The
anterior end of the jugular was tied using 4-0 silk suture to prevent bleeding. A tiny nick was made in the jugular vein and a catheter was slid roughly 1cm into the jugular vein and secured at the posterior end using 4-0 suture. The catheter was sutured and secured to the skin in three different places. After the jugular cannula was placed, the liver was exposed for imaging. A 2x2 cm piece of gauze moistened with saline was glued with a drop of cyanoacrylate to the skin just below the sternum. Exposure of the liver was begun with a 1 cm lateral ventral incision below the rib and the skin and muscle layers were removed. The liver was then exposed by gently squeezing through the incision, and placed on the moistened gauze. The liver was placed in a 40 mm cover-glass bottomed dish (WillCo Well) and glued to the cover glass without pressure. The mouse was then transferred to the microscope stage and placed on warming pads under a heat lamp to maintain body temperature at 35-37 degrees Celsius, as monitored using a rectal probe thermometer. The microscope objective was also maintained at 37 degrees Celsius via an objective heater.

For studies of mouse or rat kidney, a jugular catheter was placed as described above. Following jugular catheterization, a 1cm small lateral incision was made over the left kidney, followed by a 0.5cm incision in the peritoneal muscle. The kidney was identified and pulled up through the skin opening. Once the kidney was exposed, the adrenal gland and the renal ligament was separated. The kidney was then placed in a 40 mm cover-glass bottomed dish with a 2x2 cm piece of gauze moistened with saline.

Intravital microscopy imaging studies were conducted using a Leica TCS SP8 DIVE confocal/multiphoton system mounted on an inverted stand. The imaging was performed using either 25X NA 0.95 water immersion or 63X NA1.3 glycerol immersion Leica HCX APO objective lenses. Images were collected using multiphoton fluorescence excitation at 800 nm. Three channels of fluorescence were collected in non-de-scanned detectors using emission bandpass filters of 405-450 nm (blue), 500-550nm (green) and 600-650 nm (red). Image volumes were collected using 600 Hz bidirectional scanning, at different zoom factors and a vertical spacing of 1.5 microns. Before imaging of the liver, mice were injected IV with Hoechst (2mg/kg) to label nuclei. For studies of the early stages of antibody disposition, images were collected prior to, during and every twenty minutes for 2 hours following intravenous injection of either G41 mAb and G41-C-HC BfAb (6.5 mg/kg). For studies of
mice 24 hours after intravenous injection of antibodies, several image volumes and mosaics were collected following IV injection of Hoechst. In both sets of studies, mice were injected with Rhodamine123 to verify the vitality of the liver (mitochondrial function). At the end of imaging, the mouse was perfuse-fixed and liver tissue was collected. Intravital microscopy studies of kidney were conducted similarly, with the exception that Rhodamine123 was omitted, and, in the case of rat studies, fluorescent albumin was injected at the times indicated.

**Immunofluorescence studies of mouse liver sections**

Immunofluorescence was conducted on mouse liver tissue following intravital microscopy. Tissue cut to a thickness of 50 microns a placed in blocking buffer (10% donkey serum, 0.1% Triton X100 in PBS) for three hours, then incubated with anti-mouse F4/80 and Anti-mMMR (1:50 in blocking buffer) in an orbital shaker at room temperature overnight. The following day, tissues were washed 4x in PBS, then incubated with Alexa Fluor 488 goat anti-rat and Alexa Fluor 647 donkey anti-goat (1:200 in blocking buffer) in an orbital shaker at room temperature overnight. The next day the tissue was washed 5x in PBS and post-fixed in 4% fresh paraformaldehyde for 10 minutes. The tissue was then rinsed 3x in PBS and mounted in Fluoromount aqueous mounting medium (Sigma). Image volumes were collected using confocal microscopy (Leica SP8 DIVE) and a Leica40X NA 1.3 oil immersion objective. Image volumes were collected over a range of zoom values, with an axial spacing of 0.7 microns.

**Quantitative image analysis of liver and kidney microscopy data**

Quantitative image analysis was conducted using Metamorph image processing software (Molecular Devices, Downington, PA). Kidney endosome red-green fluorescence ratios were calculated by first subtracting background from both the red and green channels (measured as the median intensity of a 64 by 64 region around each voxel, Maxfield and Dunn, 1990), and then measuring the mean fluorescence of each channel in regions of interest drawn over 3-9 proximal tubules. Liver punctate microvascular fluorescence was quantified by first subtracting the blue channel from the red channel for each image of the volume (to eliminate crosstalk of Hoechst fluorescence). Diffuse fluorescence (i.e., from freely circulating probes) was removed first by subtracting the median intensity of an 8 by 8 region from each voxel, followed by thresholding images to a value that eliminated
residual diffuse fluorescence. Images of 17 focal planes from each 3D volume were then summed and fluorescence then measured in each of four 400 by 400 voxel regions in the summed images. Images from all conditions were processed and quantified identically. Glomerular sieving coefficients (GSCs) were determined using our previously published method (Sandoval et al. 2012). A series of publications have described in detail the proper parameters to set the detector offset (black level), which is crucial for correctly detecting the low-intensity signal coming from fluorescent albumin in Bowman’s space (Sandoval et al. 2012; Sandoval, Wang, and Molitoris 2014; Sandoval and Molitoris 2013; Dickson et al. 2014). Briefly, z-stack images of the glomerulus before fluorescent albumin infusion were collected to enable background fluorescent levels of Bowman’s space and glomerular capillaries to be quantified. These values were subtracted from the same region after the fluorescent albumin infusion. Quantitative analyses were conducted using raw image data, but micrograph images presented in figures were contrast enhanced in a way that preserved the visibility of both the dim and bright structures of the original images. Images to be directly compared were contrast enhanced identically. Images in figures were processed, assembled and annotated using Adobe Photoshop. Graphs and summary statistics were generated using Kaleidagraph (Synergy Software, Reading, PA).

RESULTS

G41 mAb and G41-C-HC BfAb model system

The studies described herein were performed using the G41-mAb and G41-C-HC BfAb molecules that were previously described (Datta-Mannan et al. 2019). The IgG4-based G41-mAb was developed against an undisclosed soluble target with no measurable concentrations in normal animals (Datta-Mannan et al. 2019). The G41-C-HC BfAb has the D2 domain of VEGFR1, an ~11.5 kDa protein, as the ECD fusion partner, connected via a flexible Glycine-Serine linker to the C-terminal end of the HC of G41 mAb (Supplemental Figure 1). The ECD binds its soluble targets VEGF and PLGF and isoforms thereof, which are known to have low ng/mL and
pg/mL circulating concentrations in the blood of normal animals, respectively (Molskness et al. 2004; Xin et al. 2012). The C-terminal HC position was selected for the fusion configuration of the ECD protein domain based on theoretically allowing the bispecific construct increased target binding accessibility such that the mAb could engage its targets concomitantly without steric hindrance. Mutations were made in both the Fc region of G41-mAb to eliminate effector function and in the hinge region of G41-mAb to remove any potential for in vivo arm exchange with endogenous IgGs (Labrijn et al. 2019; Silva et al. 2015; Labrijn et al. 2009). The mutations in the Fc and hinge regions were transitioned into the mAb component of the G41-C-HC BfAb; thus, the only differences between the molecules are the peptide linker and ECD fusion within G41-C-HC BfAb.

Tissue distribution of radiolabeled G41 mAb and G41-C-HC BfAb in mice

A radiolabel biodistribution study of 111In-labelled DTPA conjugated versions of the G41 mAb and G41-C-HC BfAb molecules was conducted to evaluate the concentrations of the molecules at static timepoints (1, 6, 12 and 24 hours) following intravenous administration to mice. The goal of this study was to analyze the whole body biodistribution of the G41 mAb and G41-C-HC BfAb in multiple tissues using traditional quantitative radiolabel methods to identify the tissues involved in clearance for subsequent dynamic intravital imaging disposition studies. Plasma and tissue concentrations of 111In-labelled DTPA conjugates were measured over 24 hours following intravenous injection in CD-1 mice and reported as a percentage of the injected dose per gram of plasma or tissue.

The G41 mAb and G41-C-HC BfAb displayed similar plasma concentrations up to 24 hours post administration consistent with the exposure profiles for their respective unlabeled counterparts (Supplementary Figure 2). Due to the sparse number of concentrations versus time plasma samples, PK parameters, such as clearance and half-life, were not determined. Tissue concentrations were measured 1, 6, 12 and 24 hours following intravenous administration. The vast majority of the constructs were detected in the liver, kidney, spleen, muscle and skin, whose percent injected dose per gram (%ID/g) concentrations are reported in Figure 1 (along with pooled measurements from the remaining tissues, listed as “other”). When the weight (grams) of the
organs are considered, the highest concentrations were found in two organs of elimination, the liver and kidney, which were thus chosen as the focus of intravitral microscopy studies.

**Description and physiochemical characterization of the DL594 fluorescently labeled G41 mAb and G41-C-HC BfAb molecules**

As our dynamic disposition IVM studies are based upon detection of fluorescently-labeled molecules, we conjugated G41-mAb and G41-C-HC BfAb with Dylight 594 (DL594) at a ratio of 1-2 fluorophore molecules per antibody. Since conjugation of mAbs with fluorescent dyes has sometimes been found to alter their physiochemical properties (Cilliers et al. 2017) in ways that might influence in vivo disposition, we conducted physiochemical characterizations of the fluorescent conjugates with respect to properties that have been shown to have an influence on clearance in vivo (Boswell et al. 2010; Datta-Mannan et al. 2012; Datta-Mannan et al. 2015; Datta-Mannan and Wroblewski 2014; Hotzel et al. 2012; Igawa et al. 2010). The results of these analyses are summarized in Table 1.

A previously developed heparin-based column assay was used to determine the degree of charge-based interaction for the DL594-labeled molecules relative to their respective unlabeled counterparts (Datta-Mannan et al. 2020). In this experiment, the molecules were injected over a column of heparin sepharose and then eluted with a linear gradient of increasing ionic strength. Neither DL594-labeled nor unlabeled G41-C-HC BfAbs showed retention on the heparin column, indicating the DL594 labeling did not affect the overall electrostatic properties (Table 1).

Changes in non-specific interactions of the molecules as a consequence of DL594-labeling driven by hydrophobic association were evaluated using a HIC-based HPLC assay in which molecules were injected onto a solid phase hydrophobic resin pre-equilibrated in high concentrations of salt. DL594-labeling led to a similar shift to becoming more hydrophobic (ie., longer elution time and higher %HIP) for both molecules evaluated. This was not unexpected given the solvent accessible nature of the aromatic-based dye additions to the mAb or BfAb and the relative consistency in retention time shifts were congruent with the similar dye to protein ratio for each molecule (Table 1).
Binding of the DL594 labeled G41-mAb and G41-C-HC BfAb to FcRn was measured using a previously reported MST method (Wagner et al. 2016). Results of these studies show that the two molecules bind to rat FcRn with similar affinities, and that conjugation to DL594 did not significantly alter either the weak or strong affinities of either, indicating a conserved engagement profile with FcRn (Table 1).

Exposure of the DL594 fluorescently labeled G41 mAb and G41-C-HC BfAb molecules in rats after a single IV administration.

Others have reported that labeling of mAbs with fluorescent dyes can alter their exposure, thus potentially confounding the evaluation of their tissue and cellular disposition profiles (Cilliers et al. 2017). Therefore, we compared the exposures of the DL594-labeled and their counterpart unlabeled G41 mAb and G41-C-HC BfAb in rats up to the latest timepoint to be evaluated by IVM (~24 hours post administration) to evaluate the influence of the fluorescent dye conjugation on the behavior of the molecules. Rats were selected for these studies as serial blood sampling is relatively less invasive than in mice. Following a single 1 mg/kg IV administration of each construct, the mean exposures of the DL594-labeled G41 mAb and G41--C-HC BfAb and their respective unlabeled molecules were similar in rats up to 24 hours post administration (Supplementary Figure 3), indicating conjugation of the molecules did not change their in vivo behavior. In addition, the DL594-conjugated G41 mAb and G41-C-HC showed similar blood concentrations up to 24 hours post dose, consistent with previous findings in rodents (Datta-Mannan et al. 2019) (Supplementary Figure 3C). Divergence of the PK profiles of the DL594-conjugated molecules became evident in the elimination phase (ie., post 24 hours following administration) (Supplementary Figure 3D) and resulted in the DL594-labeled G41-C-HC BfAb and DL594-labeled G41 mAb displaying a clearance of ~1.8 mL/hr/kg and ~1.0 mL/hr/kg, respectively (Table 2). The ~2-fold more rapid clearance observed for DL594-labeled G41-C-HC BfAb relative to G41-DL594 is consistent with the pharmacokinetics reported in previous studies for the unlabeled counterparts in mice (Datta-Mannan et al. 2019).

Intravital microscopy studies of the disposition of DL594-labeled G41 mAb and G41-C-HC BfAb in mouse liver.
Intravital microscopy studies of the liver disposition of the DL594-labeled G41 mAb and G41-C-HC BfAb constructs in mice were conducted approximately 1 hour and 24 hours following a single IV dose of each construct (Figure 2). Both constructs were found to accumulate in puncta on the boundaries of the liver capillaries throughout the sinusoid network, although with different kinetics. Whereas the fluorescence of G41-C-HC puncta increased to high levels within the first hour, G41-C-HC BfAb puncta were relatively dim one hour after administration but accumulated to levels similar to those of G41-C-HC over 24 hours. The puncta are more easily appreciated in high-magnification images, (Figures 2 E-H). In order better delineate hepatocytes, mice were also injected with rhodamine123, a green-fluorescing probe that accumulates in active mitochondria and strongly labels hepatocytes in vivo. These and subsequent figures demonstrate that neither compound appears to associate with hepatocytes.

To characterize the dynamics of the accumulation of the G41 mAb and G41-C-HC BfAb molecules in sinusoid puncta, studies were conducted in which the same regions of the liver were repeatedly imaged over an ~2-hour period immediately following intravenous injection. As shown in Figure 3A, fluorescent puncta can be detected in sinusoids within 19 minutes of injection of the G41-C-HC BfAb, and the fluorescence of these puncta increase with time, particularly during the next 30 minutes. Similar results were obtained in mice injected with G41 mAb that, within 11 min of injection could be detected in distinct puncta that subsequently increased in fluorescence over the next hour (Figure 3B). Quantitative analysis of the integrated fluorescence of punctate of G41 mAb and G41-C-HC BfAb measured in replicate mice ~1 hour and 24 hours after injection demonstrate that G41 mAb accumulates more than 2x faster than G41-C-HC BfAb during the first hour. However, G41-C-HC BfAb continues to accumulate, finally achieving comparable levels with 24 hours (Figure 3C). Comparisons of fields collected 1 hour with those collected 24 hours after injection demonstrated that whereas the integrated fluorescence of punctate G41 mAb increased insignificantly (~1.7-fold between 1 and 24 hours), the integrated fluorescence of punctate G41-C-HC BfAb increased more than 4-fold over the same period (p=0.001).

The studies shown in Figure 3 were conducted in Lys-EGFP mice, which express EGFP under control of the lysozyme promoter (Faust et al. 2000). While EGFP is expressed by all myelomonocytic cells, it is
expressed most strongly in neutrophils, which are indicated with arrows. Close comparison of the indicated cells in the green and red channels suggest that only modest amounts of G41 mAb and G41-C-HC BfAb associate with neutrophils immediately following IV injection. Additional studies, conducted to characterize the nature of the structures in which G41 mAb and G41-C-HC BfAb accumulate, and to identify the cells in which they accumulate, are described below.

Images collected from living mice at higher resolution (Figure 4) indicate that the G41 mAb and G41-C-HC BfAb constructs are internalized by the endothelial cells lining the liver sinusoids. Figure 4A shows an example of a field collected from the liver 24 hours after intravenous injection of the G41-C-HC BfAb. Numerous endothelial nuclei can be found throughout the field, each surrounded by a ring of punctate G41-C-HC BfAb fluorescence. The close apposition of the puncta with nuclei in the same focal plane suggests that the G41-C-HC BfAb punctate fluorescence derives from intracellular compartments, likely endothelial endosomes. The apposition of these puncta with endothelial nuclei is even more obvious in the magnified region of the image (Figure 4B), or in the image collected from a different focal plane from the same field (Figure 4C). These apparently internal structures are evident even as early as 36 min after injection (Panels D-F), although they are somewhat less obvious against the background of free G41-C-HC BfAb in the plasma at this early time point. Similar results were obtained in studies of the disposition of the G41 mAb. As with G41-C-HC BfAb, the G41 mAb is also found surrounding endothelial nuclei both at 24 hours (panels G and H) and 2 hours after injection (panel I). These results are consistent with endocytic uptake and accumulation of G41-C-HC BfAb and G41 mAb in the sinusoidal endothelia of the mouse liver.

Consistent with the images collected at early time-points shown in Figure 3, images collected 24 hours after injection demonstrate that only a minor fraction of the accumulated G41 mAb and G41-C-HC BfAb molecules associate with EGFP-expressing neutrophils in EGFP-Lys mice (Supplementary Figure 4). In order to more definitively identify the cells internalizing G41 mAb and G41-C-HC BfAb, we next conducted immunofluorescence studies in which tissues were labeled with antibodies to CD206, a marker of liver endothelia
and F4/80, a marker of Kupffer cells. Consistent with the results shown in Supplementary Figure 4, studies of liver tissues fixed 24 hours after intravenous injection demonstrate that the G41-C-HC BfAb accumulation closely correspond to CD206-positive cells, consistent with internalization by endothelial cells (Supplementary Figures 5A and B). Since CD206 is also expressed at low levels by Kupffer cells, additional studies were conducted in tissues labeled with antibodies against both CD206 and F4/80 (Supplementary Figure 5C). As with the images shown in panels A and B, these images show that G41-C-HC BfAb associates with cells labeled with CD206, but not with cells labeled with F4/80. Similar results were obtained in studies of liver tissues of mice 24 hours after injection with G41 mAb; whose distribution closely corresponded with cells expressing CD206, but not with cells expressing F4/80 (Supplementary Figures 5 D and E, respectively).

**Intravital microscopy studies of the disposition of DL594-labeled G41 mAb and G41-C-HC BfAb in mouse kidney**

Intravital microscopy studies were next conducted on the kidney, the second major organ in which the constructs accumulated. Figure 5 shows images collected from the kidney of living C57BL/6 mice following intravenous injection of DL594 fluorescent conjugated G41-C-HC BfAb (panels A-D) or G41 mAb (panels E-H) at various times after administration. Neither compound was found to accumulate in detectible structures during the first 70 minutes, instead remaining apparently free in the vascular circulation. However, over the next 24 hours, DL594-G41-C-HC BfAb accumulated to very high levels in what appear to be endosomes and/or lysosomes of proximal tubule cells. No comparable accumulation was found in mice injected with DL594-G41 mAb, which appeared to remain free in the vascular circulation.

Similar results were obtained in intravital microscopy studies of the kidney of Munich-Wistar rats. As observed in the murine kidney IVM studies, both G41 mAb and G41-C-HC BfAb are largely retained in the plasma for at least the first hour after injection (Figure 6A and D). However, over the next 24 hours, the distribution of G41-C-HC BfAb dramatically changes, diminishing in the plasma and increasing dramatically in endosomes of the proximal tubule epithelia, as evidenced in the shift in the spectrum of their fluorescence from the yellow characteristic of endogenous lysosomal autofluorescence to red (Figures 6B and C). Quantifications of the red-
to-green fluorescence ratio in each tubule demonstrate that the ratio shifts from an initial mean of 0.95 measured one hour after injection to values as high as 3.68 24 hours later (Figure 6C). In contrast, and consistent with observations in mice, the distribution of G41 mAb on the day following injection (~24 hours post administration) is essentially identical to that at one hour, with only a minor decrease in vascular levels and a modest shift in the spectrum of endosome/lysosome fluorescence (Figures 6E and F).

The presence of G41-C-HC BfAb in proximal tubule endosomes in both mice and rats indicates that, while not filtered rapidly enough to be visually apparent in Bowman’s space, is nonetheless filtered to some degree. Because the glomeruli of Munich-Wistar rats occur at depths that are accessible to microscopy, it was possible to quantify glomerular permeability of the two constructs (Sandoval and Molitoris 2013). Consistent with the visual observations, quantifications of glomerular sieving coefficients (see Methods) demonstrate that both are minimally filtered, with glomerular sieving coefficients of 0.00685 ± 0.00018 for G41-C-HC BfAb and 0.00532 ± 0.00071 for G41 mAb.

**DISCUSSION**

The studies described here were predicated on the observation that the rate of peripheral clearance of G41-C-HC BfAb was roughly double that of the parent G41 mAb. This difference was unexpected since G41-C-HC BfAb was specifically designed to minimize physiological interactions known to influence the PK of biologics (Datta-Mannan 2019; Tibbitts et al. 2016). For instance, while the G41-C-HC BfAb was constructed with an ECD (the D2 domain VEFGR1) fused to the HC C-terminus of the G41 parental mAb, the ECD and mAb targeted two distinct soluble ligands which have negligible peripheral concentrations in normal animals (Molskness et al. 2004; Xin et al. 2012). Moreover, the BfAb had no specific interaction with cell surface receptors thus, eliminating both circulating ligand-mediated and cell surface TMDD as potential mechanisms for the observed clearance. In addition, since the G41-C-HC BfAb used the same IgG4 parental Fc within G41 mAb that has been engineered to eliminate interactions with Fcγ receptors, direct binding with blood cells is not expected to be a major viable clearance mechanism either (Shields et al. 2001). The fact that the two used the same Fc region and showed
similar in vitro FcRn interaction properties, also argues that the difference in clearance was not based upon differences in interactions with FcRn in vivo, as has been observed for other antibody modalities (Datta-Mannan and Wroblewski 2014; Pyzik et al. 2015). Although native IgG4-based antibodies have a strong potential for Fab-arm exchange with endogenous IgG4s that can lead to aberrant and suboptimal PK properties (Labrijn et al. 2009; Silva et al. 2015), the inclusion of the S228P mutation in the hinge region of the G41 mAb and G41-C-HC BfAb eliminated this potential and thereby, also excluded IgG4-related hinge instability differential as a plausible mechanism for the PK differences.

Radio-label disposition and intravital microscopy studies were thus conducted to characterize the tissue and cellular interactions of the G41-C-HC BfAb construct which, despite being engineered for minimal off-target cellular interactions, cleared two-fold faster from the peripheral circulation in mice and rats. Whole-body biodistribution studies demonstrated that the two constructs accumulated to similar degrees in most tissues with the exception of the liver, in which G41-C-HC BfAb accumulated to ~2- to 3-fold higher concentrations. This difference was apparent within the first hour of administration but was maintained throughout the 24-hour time course of the study (Figure 1). The fact that levels of the two in the plasma were essentially superimposable over this period (Supplementary Figure 2A) clearly shows that the G41-C-HC BfAb has higher mean liver: blood accumulation relative to the G41 mAb suggesting G41-C-HC BfAb has a strong liver binding component at early timepoints that may contribute to its faster peripheral clearance relative to the G41 mAb.

Intravital microscopy studies identified profound differences between the cellular interactions of the two molecules. Both constructs were found to accumulate in endosomes of the sinusoidal endothelia of the liver, but with different kinetics. In contrast to the results of the radio-label studies, the fluorescent probe DL594-G41 mAb was found to accumulate more than 2x faster than DL-G41-C-HC BfAb during the first hour, after which time DL-G41-C-HC BfAb continues to accumulate, finally achieving similar levels by 24 hours (Figure 3). The basis for the discrepancy with the results of the radio-label studies may reflect differences in the residualizing properties of Lys-DTPA-111In used in the radiolabel studies versus the DL594 used in the IVM studies. Insofar as IVM demonstrated that both compounds were internalized into the endo-lysosomal system of endothelia, these
differences are likely to be important. To our knowledge, the residualizing properties of probes labeled with fluorescent probe DL594 have not been published. However, they are almost certainly different from those labeled with the residualizing agent DTPA-\(^{111}\)In. This is because DTPA-\(^{111}\)In retains its cellular localization even after degradation of the parent conjugate; thus, its abundance will reflect the abundance of both the intact and degraded conjugate. In contrast, fluorescent probes such as DL594 are free to disperse and diffuse out of cells following degradation of the antibody conjugate. As such the DL594 fluorescence will more closely reflect just the levels of the intact conjugate at the particular time point of imaging. To the degree that some extent of conjugate degradation occurs in endosomal compartments (which include lysosomes), the amount of conjugate that has been directed to these compartments will be more accurately reflected by levels of DTPA-\(^{111}\)In, whereas it will be under-represented by the levels of DL594 fluorescence. It is tempting to speculate that the combined data suggest that the accelerated peripheral clearance BfAb reflects degradation due to increased partitioning to a degradative endocytic pathway in liver sinusoidal endothelia.

Although radio-label distribution studies indicated no differences in how mAb and BfAb interact with the kidney (Figure 1), IVM studies of rats and mice identified profound differences between the two (Figures 5 and 6). While both were observed only free in the plasma flowing through the peritubular capillaries within one hour of administration, BfAb accumulated dramatically in endosomes of the proximal tubule over 24 hours. The accumulation of BfAb, but not mAb in proximal tubule endosomes was somewhat unexpected. Although both visual and quantitative analyses indicated minimal glomerular permeability of either compound in our studies, previous studies have demonstrated that limited amounts of IgG are filtered in the mouse kidney and internalized into endosomes of the proximal tubule(Lawrence et al. 2017). However, one would expect the levels of BfAb in proximal tubule endosomes to be less, rather than more than those of mAb, simply due to its larger size, and thus lower glomerular permeability. The pronounced accumulation of BfAb over 24 hours was likewise unexpected, since it is believed the IgGs are transcytosed back into the circulation following internalization by the proximal tubule, rather than accumulating (Kobayashi et al. 2002; Lawrence et al. 2017). One possible explanation is that the strong endosomal fluorescence observed 24 hours after administration of DL594-BfAb derives from
fluorescently-labeled fragments of the degraded compound, generated in liver endothelia as described above. This provides a satisfying explanation for the much stronger endosomal fluorescence observed in mice 24 hours after injection with DL594-BfAb, whose degradation would result in the continuous generation of fragments that would be freely filtered and internalized by proximal tubule cells. To the degree that the distribution of fluorescent fragments differs from that of the residualizing Lys-DTPA-¹¹¹In probe, this model might also explain the discrepancy of the IVM studies with the radiolabel studies, which detected no difference in the accumulation of the two probes in the kidney.

Another possible explanation for the observation of the accumulation of DL594-BfAb, but not DL594-mAb in proximal tubule endosomes is that while both are filtered and internalized into proximal tubule cells, their subsequent intracellular fates are different. As described above, IgGs internalized into proximal tubule cells are believed to be directed onto a transcytotic pathway for return to the circulation. Accordingly, the steady-state levels of mAb associated with proximal tubule cells would be expected to be limited by the continuous transcytotic efflux. In contrast, if the molecular modifications of BfAb altered its interactions with the endocytic sorting machinery involved in transcytosis, it would be expected to accumulate on a lysosomal pathway.

The molecular basis for the differences in the cellular interactions of the two compounds is unclear. As described above, the BfAb was specifically designed to avoid off-target cellular interactions. Physicochemical analyses revealed only a subtly enhanced propensity of the G₄₁-C-HC BfAb for hydrophobic-related interactions. Increased non-specific binding potential, including those driven by electrostatic (ie., charge) and hydrophobic interactions, have been empirically connected to PK behavior (Boswell et al. 2010; Datta-Mannan et al. 2020; Datta-Mannan et al. 2015; Grinshpun et al. 2021; Hu and D'Argenio 2020; Igawa et al. 2010; Jain T1 2017); thus, it seems reasonable to speculate that the increased propensity of G₄₁-C-HC BfAb for hydrophobic interactions may have altered its interactions with surface receptors mediating endocytosis, or with the molecular machinery mediating endocytic sorting leading to increased clearance relative to the mAb.

Alternatively, the differences in the disposition of the two compounds may reflect differences in their interaction with FcRn. While the FcRn binding affinity of G₄₁-C-HC BfAb at pH 6.0 was only modestly lower
than that of G41 mAb, it is intriguing to speculate that subtle differences in affinity, particularly in the context of the sequence of pH and physical environments experienced in vivo may underlie the observed differences in the in vivo disposition of BfAb and mAb. FcRn has a well-described role in preventing lysosomal degradation of IgG, mediating sorting of IgG onto a recycling pathway in endothelia and a transcytotic pathway in proximal tubule cells (Kobayashi et al. 2002; Roopenian and Akilesh 2007). Thus, while speculative, a model based upon altered interactions with FcRn provides a unifying explanation that is consistent with the results of our IVM and radiolabel distribution studies, and with the contention that the faster peripheral clearance of BfAb is based upon enhanced degradation of BfAb in liver endothelia and kidney proximal tubule epithelia.

In summary, the studies here demonstrate how the spatio-temporal resolution of IVM can be leveraged to expand our understanding of the in vivo disposition of therapeutics, detecting off-target interactions that could not be detected by conventional pharmacokinetics approaches nor predicted by conventional physicochemical analyses.

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**Authorship Contributions**

*Participated in research design:* Datta-Mannan, Molitoris, Feng, and Dunn.

*Conducted experiments:* Martinez, Sandoval, Brown, and Merkel.

*Contributed new reagents or analytic tools:* Croy.

*Performed data analysis:* Martinez, Sandoval, Brown, and Merkel.

*Wrote or contributed to the writing of the manuscript:* Datta-Mannan, Molitoris, Feng, and Dunn.
REFERENCES


Footnotes

a) This work was funded by Eli Lilly & Company. Datta-Mannan, Feng, Brown, Merkel and Croy are employees and recipients of Eli Lilly & Company stock. Molitoris, Martinez, Sandoval and Dunn have no actual or perceived conflict of interest with the contents of this article.

b) n/a

c) Amita Datta-Mannan
Exploratory Medicine and Pharmacology (A.D-M.), 4 Clinical Laboratory Services (R.B.), Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46225, USA.
datta_amita@lilly.com

d) 1Exploratory Medicine and Pharmacology (A.D-M.), 4 Clinical Laboratory Services (R.B.), Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46225, USA.

2Department of Medicine, Division of Nephrology, Indiana University School of Medicine, 950 W. Walnut St., Indianapolis, IN 46202

3Biotechnology Discovery Research (Y.F., D.M., and J.C.), Lilly Research Laboratories, Lilly Technology Center North, Indianapolis, IN 46221, USA
Figure legends

**Figure 1.** Radiolabel biodistribution data for the mAb and BfAb in mice following a single IV administration of ~2 mg/kg (~0.020 µCi/animal). Mean percentage of injected dose/gram of tissue (+/- SE) of $^{111}$In-DTPA-G41 mAb (black columns) and $^{111}$In-DTPA-G41-C-HC BfAb (gray columns) determined by gamma counting radioactive signal. Data for the five organs which displayed the highest tissue concentrations are reported. The sum of the %ID/g for all additional organs collected are reported as ‘other’.

**Figure 2.** Intravital microscopy of mouse liver following intravenous injection of G41-C-HC-DL594 BfAb or G41-DL594 mAb. Low power images of the liver of a living mouse collected 66 minutes (A) and 24 hours (B) after intravenous injection of 6.5 mg/kg G41-C-HC-DL594 BfAb. Red – DL594. Blue - Hoechst 33342 (nuclei). Green - rhodamine123, a fluorescent probe that accumulates in actively respiring mitochondria, particularly in hepatocytes in vivo. C and D, are corresponding images collected 78 minutes (C) and 24 hours (D) after intravenous injection of G41-DL594 mAb. E and F – 4X magnified regions from panels A and B, respectively. G and H – 4X magnified regions from panels E and F, respectively. Scale bars represent 200 microns for low-power images and 50 microns for high-power images.

**Figure 3.** Accumulation of G41-C-HC-DL594 BfAb or G41-DL594 mAb in the liver vasculature of Lys-EGFP mice. (A) Series of images collected from the liver of a living mouse over the 2 hr period following after intravenous injection of 6.5 mg/kg G41-C-HC-DL594 BfAb (red). As in Figure 3, blue signal derives from the nuclear probe Hoechst 33342, and green signal derives from rhodamine123, which strongly labels hepatocytes in vivo. First row – all probes. Second row – Hoechst 33342 and G41-C-HC-DL594 BfAb (red). Third row – as in the second row but magnified 2 times. (B) Series of images collected from the liver of a living mouse over the 2 hour period following intravenous injection of G41-C-HC-DL594 BfAb. First row – all probes. Second row – Hoechst 33342 and G41-C-HC-DL594 BfAb (red). Third row – as in the second row but magnified 2 times. (C) Results of quantification of field-wise fluorescence of G41-C-HC-DL594 BfAb or G41-DL594 mAb 1 hour (top) or 24 hours (bottom) after injection. Graphs indicate means and standard errors of analyses of 4 mice injected.
with G.1-C-HC-DL594 BfAb and 3 mice injected with G.1-DL594 mAb. Scale bars represent 100 microns for low-power images and 40 microns for high-power images.

**Figure 4 – Accumulation of G.1-C-HC-DL594 BfAb or G.1-DL594 mAb in sinusoidal endothelia in mouse liver.** (A) Image collected from the liver of a living mouse 24 hours after intravenous injection of 6.5 mg/kg G.1-C-HC-DL594 BfAb (red). (B) 2X magnified region of the image shown in panel A. (C) Image collected at higher resolution from the same field. As in Figure 3, blue signal derives from the nuclear probe Hoechst 33342, and green signal derives from rhodamine123, which are clearly resolved in these high-resolution images. (D) Image collected from the liver of a living mouse 36 min after intravenous injection of 6.5 mg/kg G.1-C-HC-DL594 BfAb (red). (E, F) 2X and 4X magnified regions, respectively, of the image shown in panel D. (G) Image collected from the liver of a living mouse 24 hrs after intravenous injection of 6.5 mg/kg G.1-DL594 mAb (red). (H) 2X magnified region of the image shown in panel G. (I) Image collected from the mouse 2 hours after intravenous injection of 6.5 mg/kg G.1-DL594 mAb. Scale bars represent 40 microns (A, D, G), 20 microns (B, E, H) and 10 microns (C, F, I)

**Figure 5 – Intravital microscopy of mouse kidney following intravenous injection of G.1-C-HC-DL594 BfAb or G.1-DL594 mAb.** (A) Low power image of the kidney of a living mouse collected 75 minutes after intravenous injection of 6.5 mg/kg G.1-C-HC-DL594 BfAb (red). Green signal derives from tissue autofluorescence and blue signal derives from the fluorescence of the nuclear probe Hoechst 33342 introduced 30 minutes prior to G.1-C-HC-DL594 BfAb injection. (B) Low power image of the kidney of a living mouse collected 24 hours after intravenous injection of G.1-C-HC-DL594 BfAb. (C, D) Corresponding high-power images collected at 75 min and 24 hours, respectively. (E) Low power image of the kidney of a living mouse collected 75 minutes after intravenous injection of 6.5 mg/kg G.1-C-HC-DL594 mAb (red). (F) Low power image of the kidney of a living mouse collected 24 hours after intravenous injection of G.1-C-HC-DL594 mAb. (G, H) Corresponding high-power images collected at 75 min and 24 hours, respectively. Scale bars represent 200 microns for low-power images and 50 microns for high-power images.
Figure 6 – Intravital microscopy of rat kidney following intravenous injection of G.1-C-HC-DL594 BfAb or G.1-DL594 mAb. (A) Image of the kidney of a living Munich Wistar Fromter rat collected 66 minutes after intravenous injection of 6.5 mg/kg DL594-G.1-C-HC BfAb (red). Green signal derives from tissue autofluorescence and blue signal derives from the fluorescence of the nuclear probe Hoechst 33342 introduced 30 minutes prior to G.1-C-HC-DL594 BfAb injection. (B) Low power image of the kidney of a living rat collected 24 hours after intravenous injection of G.1-C-HC-DL594 BfAb. (C) 2X magnified image collected from the same field shown in B. (D) High power image collected from a different region of the kidney shown in panels A-C. (E) Image of the kidney of a living rat collected 63 minutes after intravenous injection of 6.5 mg/kg G.1-DL594 mAb (red). (F, G and H) Images collected at different magnifications, from different focal planes from the kidney of a living rat 16 hrs after intravenous injection of G.1-DL594 mAb. Values shown in panels C and G represent mean red-to-green fluorescence ratios of the different tubules. Scale bars represent 40 microns (panels A, C, E and G), 80 microns (B and F) and 20 microns (D and H).
# Tables

## Table 1: Properties of the molecules

<table>
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<tr>
<th>Molecule</th>
<th>Heparin binding ([NaCl] at main peak elution apex, mM)</th>
<th>HIC chromatography</th>
<th>Rat FcRn binding K_d (μM)</th>
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<tr>
<td></td>
<td></td>
<td>Retention Time (min)</td>
<td>%HIP</td>
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<tr>
<td>G1 mAb</td>
<td>None</td>
<td>10.3</td>
<td>36.1</td>
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<tr>
<td>G1 mAb-DL594</td>
<td>None</td>
<td>12.1</td>
<td>43.9</td>
</tr>
<tr>
<td>G1-C-HC BfAb</td>
<td>None</td>
<td>14.7</td>
<td>55.2</td>
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### Table 2: Mean (+/- SD) Pharmacokinetic parameters of the molecules

<table>
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<th>Molecule</th>
<th>$C_{max}$ (μg/mL)</th>
<th>$AUC_{0-\infty}$ (h* μg/mL)</th>
<th>CL (mL/hr/kg)</th>
<th>$T_{1/2}$ (hours)</th>
<th>$V_{ss}$ (mL/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G41 mAb–DL594</td>
<td>39.8 (3.0)</td>
<td>1019 (180)</td>
<td>1.00 (0.19)</td>
<td>15.2 (2.7)</td>
<td>27.8 (5.5)</td>
</tr>
<tr>
<td>G41-C-HC BFab–DL594</td>
<td>39.4 (3.6)</td>
<td>585 (35)</td>
<td>1.81 (0.10)</td>
<td>24.9 (2.5)</td>
<td>26.1 (2.6)</td>
</tr>
</tbody>
</table>

SD, standard deviation. $C_{max}$, maximal observed serum concentration; $AUC_{0-\infty}$, area under the plasma concentration curve from time zero extrapolated to infinite time; CL, clearance; $T_{1/2}$, elimination half-life. N=3 rats/time point. All PK parameters were determined from non-compartmental pharmacokinetic analyses unless otherwise noted.
Figure 1

![Graphs showing the distribution of injected dose per gram in different organs](image-url)
Figure 5

(A) 70 minutes
(B) 24 hours

BfAb

(C) 70 minutes
(D) 24 hours

mAb

(E) 70 minutes
(F) 24 hours

BfAb 4x

(G) 70 minutes
(H) 24 hours

mAb 4x
Figure 6
**Intravital microscopy reveals unforeseen biodistribution within the liver and kidney mechanistically connected to the clearance of a bifunctional antibody**

Amita Datta-Mannan†1, Bruce A. Molitoris2, Yiqing Feng3, Michelle M. Martinez2, Ruben M. Sandoval2, Robin Brown4, Daniel Merkel3, Johnny Croy3 and Kenneth W. Dunn†2

1 Exploratory Medicine and Pharmacology (A.D-M.), 4 Clinical Laboratory Services (R.B.), Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46225, USA.
2Department of Medicine, Division of Nephrology, Indiana University School of Medicine, 950 W. Walnut St., Indianapolis, IN 46202
3Biotechnology Discovery Research (Y.F., D.M., and J.C.), Lilly Research Laboratories, Lilly Technology Center North, Indianapolis, IN 46221, USA

**Supplementary Figure 1**: Schematic representation of (A) G41 mAb and (B) G41-C-HC BfAb molecules evaluated in this study. The ECD moieties are represented as ovals fused to a Glycine-Serine linker region connected to the C-terminal end of the HC of the BfAb.
Supplementary Figure 2: Radiolabel biodistribution data for the G₄₁ mAb and G₄₁-C-HC BfAb in mice following a single IV administration of ~2 mg/kg (~0.020 μCi/animal). (A) Mean (+/- SD) plasma concentrations of ¹¹¹In-DTPA G₄₁ mAb and ¹¹¹In-DTPA G₄₁-C-HC BfAb determined by gamma counting radioactive signal. (B) Mean (+/- SD) plasma concentrations of G₄₁ mAb and G₄₁-C-HC BfAb determined by ELISA. Data are n=2/time point. The standard deviation (SD) is reported for all plasma samples.
Supplementary Figure 3. The mean exposure profiles of (A) G41 mAb compared with DL594-labeled G41 mAb, (B) G41-C-HC compared with DL594-labeled G41-C-HC and (C) DL594-labeled G41-C-HC compared with DL594-labeled G41 mAb up to 24 hours post administration to rats. The mean pharmacokinetic profile of (D) DL594-labeled G41-C-HC compared with DL594-labeled G41 mAb up to 96 hours post administration to rats. Data are the mean for three animals/timepoint for all molecules.
Supplementary Figure 4 – Accumulation of $G_{4,1}$-C-HC-DL594 BfAb or $G_{4,1}$-DL594 mAb in mouse liver shows minimal association with neutrophils. (A) Image collected from the liver of a living mouse 24 hours after intravenous injection of 6.5 mg/kg $G_{4,1}$-C-HC-DL594 BfAb (red). (B) 2X magnified region of the image shown in panel A. Blue signal derives from the nuclear probe Hoechst 33342, the relatively dim green fluorescence derives from rhodamine123 in hepatocytes and the bright green fluorescence derives from EGFP in neutrophils. Examination of the red fluorescence (right) shows that little, if any of the accumulated $G_{4,1}$-C-HC-DL594 BfAb associates with neutrophils. (C) Image collected from the liver of a living mouse 24 hours after intravenous injection of 6.5 mg/kg $G_{4,1}$-DL594 mAb (red). (D) 2X magnified region of the image shown in panel C. As with $G_{4,1}$-C-HC-DL594 BfAb, little $G_{4,1}$-DL594 mAb is found to associate with neutrophils (circled), but arrows also indicate a few examples of cells expressing GFP at somewhat lower intensities, which may represent Kupffer cells. Scale bars represent 100 microns (A, C) and 50 microns (B, D).
Supplementary Figure 5: Immunofluorescence of CD206 and F4/80 in mouse liver tissues collected 24 hrs after intravenous injection of G41-C-HC-DL594 BfAb or G41-DL594 mAb. A – 3-plane projected image of immunofluorescence of CD206 (green) in mouse liver tissue collected 24 hours after intravenous injection of 6.5 mg/kg DL594- G41-C-HC BfAb (red). B – 2X magnification of projection of a different set of 3 focal planes of the field shown in panel A. C – Dual immunofluorescence of CD206 (green) and F4/80 (blue) of mouse liver tissue collected 2 hours after intravenous injection of 6.5 mg/kg DL594-G41-C-HC BfAb (red). Circles indicate F4/80-positive Kupffer cells. D – Immunofluorescence image of CD206 (green) in mouse liver tissue collected 24 hours after intravenous injection of 6.5 mg/kg DL594-G41-mAb (red). Fluorescence of Hoechst 33342 is shown in blue. D – Immunofluorescence image of 15-plane projected image of F4/80 (green) in mouse liver tissue collected 24 hrs after intravenous injection of 6.5 mg/kg DL594-G41-mAb (red). Fluorescence of Hoechst 33342 is shown in blue. Scale bars represent 40 microns (A, C) or 20 microns (B, D, E).