Biodistribution and Targeting of Anti-5T4 Antibody-Drug Conjugate Using Fluorescence Molecular Tomography

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Abstract

Understanding a drug’s whole-body biodistribution and tumor targeting can provide important information regarding efficacy, safety, and dosing parameters. Current methods to evaluate biodistribution include in vivo imaging technologies like positron electron tomography and single-photon emission computed tomography or ex vivo quantitation of drug concentrations in tissues using autoradiography and standard biochemical assays. These methods use radioactive compounds or are cumbersome and do not give whole-body information. Here, for the first time, we show the utility of fluorescence molecular tomography (FMT) imaging to determine the biodistribution and targeting of an antibody–drug conjugate (ADC). An anti–5T4-antibody (5T4-Ab) and 5T4-ADC were conjugated with a near-infrared (NIR) fluorophore VivoTag 680XL (VT680). Both conjugated compounds were stable as determined by SEC-HPLC and plasma stability studies. Flow cytometry and fluorescence microscopy studies showed that VT680-conjugated 5T4-ADC specifically bound 5T4-expressing cells in vitro and also exhibited a similar cytotoxicity profile as the unconjugated 5T4-ADC. In vivo biodistribution and tumor targeting in an H1975 subcutaneous xenograft model demonstrated no significant differences between accumulation of VT680-conjugated 5T4-Ab or 5T4-ADC in either normal tissues or tumor. In addition, quantitation of heart signal from FMT imaging showed good correlation with the plasma pharmacokinetic profile suggesting that it (heart FMT imaging) may be a surrogate for plasma drug clearance. These results demonstrate that conjugation of VT680 to 5T4-Ab or 5T4-ADC does not change the behavior of native biologic, and FMT imaging can be a useful tool to understand biodistribution and tumor-targeting kinetics of antibodies, ADCs, and other biologics.

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Introduction

The biodistribution of a therapeutic agent provides significant insights regarding the drug’s therapeutic efficacy, on- and off-target potential toxicities, clearances mechanisms, half-life and metabolism as well as information for designing next-generation therapeutic agents with superior safety and efficacy (1–5). Traditionally, biodistribution studies are performed using radio-labeled compounds in rodents and collecting tissues at various time points after injection to quantitate the drug concentration. This method is labor intensive and requires intensive handling of radio-labeled compounds, specialized facilities to perform studies, and the use of a large number of animals. A major disadvantage of this method is that not every tissue or compartment of the body can be evaluated. In vivo imaging like Positron Emission Tomography (PET), Single-Photon Emission Computed Tomography (SPECT), and optical imaging can address some of these shortcomings (1, 2, 4–6). PET and SPECT have been used to determine biodistribution of small molecules and biologics (4, 5, 7–9), but they too require radio-labeled compounds and specialized facilities to perform studies. The radio-labels decay with time and thus have a relatively short shelf life. In addition, the use of multiple radiotracers in PET or SPECT imaging is challenging due to the crosstalk between energies of multiple windows (5).

An alternative method to study biodistribution of biologics is fluorescence-based imaging. Labeling of biologic molecules with fluorescent probe is relatively easy, efficient, and can be performed in most research facilities. Multiple two-dimensional (2-D) and three-dimensional (3-D) fluorescence imaging technologies are currently available for in vivo evaluation and have been utilized to qualitatively evaluate biomarkers, biodistribution, and targeting of biologic molecules (8, 10–12). Among these technologies, fluorescence molecular tomography (FMT) imaging is the most advanced, permitting acquisition of 3-D tomographic data for localization, detection, and quantitation of the fluorescently labeled drug in multiple organs and at site of interest. FMT technology uses the near-infrared (NIR) spectral region (600–900 nm) which reduces the auto-fluorescence by physiologic molecules and skin (8, 13–15). The wide spectral window in FMT allows for the use of multiple fluorophores simultaneously. In addition, FMT imaging is noninvasive, and the same animal can be monitored longitudinally, thereby reducing the number of animals while maintaining or even improving the relevance and statistical significance of a study.
In this study, we exploited this capability (namely, longitudinal imaging) and evaluated the biodistribution and tumor targeting of a 5T4-antibody (5T4-Ab) and its companion antibody-drug conjugate (ADC: 5T4-ADC) using FMT imaging. An ADC is composed of an antibody that targets a specific antigen at the site of action (i.e., tumor) and is conjugated via a linker to a cytotoxic agent (payload) that elicits cell death (16, 17). Anti-5T4 antibody conjugated to monomethylauristatin F (MMAF), here after referred to as 5T4-ADC, has shown promising results in various in vitro and in vivo cancer models (18, 19). Our goal in this study was twofold, first to develop a systematic method to evaluate the biodistribution of biologic drugs using FMT and second to evaluate whether conjugation of mcMMAF (the linker-payload) had any effect on the biodistribution of the 5T4 antibody. To accomplish these goals, we labeled the naked antibody or the antibody arm of the ADC with NIR fluorophore VivoTag680XL (here after referred to as VT680) and showed that VT680 conjugation did not change the stability, binding ability to the antigen, nor the biological activity of 5T4-Ab or 5T4-ADC. In addition, no significant difference was observed between 5T4-Ab and 5T4-ADC pharmacokinetics (PK), biodistribution, or tumor targeting. This is the first study evaluating the biodistribution and tumor targeting of ADCs using FMT imaging. Because the fluorophore can be conjugated to any protein/peptide, this novel approach can become a platform technology in conducting biodistribution studies of biological drugs.

Materials and Methods

Fluorophore labeling

VT680 was labeled using a VivoTag680XL protein labeling kit as per the manufacturer’s instructions (Perkin Elmer). The excitation and emission maximums (Ex max and Em max) of VT680 were 668 nm and 688 nm, respectively. VT680 has the succinimidyl ester group that reacts with the free amine groups present on lysine residues of the proteins. The reactions were carried out in amine free buffer, i.e., PBS. The antibodies and ADCs used in this study were generated in-house (Pfizer Inc.), and the structural information of 5T4-Ab and 5T4-ADC has been described before (18). Briefly, the 3 mg of Iso type control-ADC (control-ADC), 5T4-Ab, and 5T4-ADC in PBS were incubated with 150 µg of VT680 in bicarbonate buffer for 2 hours (in dark) on a rotating shaker. Following the conjugation reaction, the free fluorophore molecules were removed using a protein purification column (Bio-Gel P-100). Samples were quantified for the conjugation efficiency by measuring the absorbance at 280 nm and 668 nm using a NanoDrop 8000 (Thermo Scientific), thereby providing the relative efficiency (degree of labeling, DOL) of the VT680 conjugation to each antibody or ADC. Reagents with DOL between 2-3 VT680 molecules/protein were used for this study. In order to evaluate if the VT680 conjugation increased aggregation propensity of the reagents, size-exclusion chromatography-HPLC (SEC-HPLC) was performed. Briefly, Ab and ADCs in PBS (1 mg/mL) were separated on YMC-Pack Diol-200 column (Waters Corporation) using HPLC (Agilent Technologies). The mobile phase consisted of 0.02 mol/L phosphate buffer and 0.4 mol/L NaCl, and system was run under isocratic conditions. The elution profiles of separated proteins were detected by UV absorbance at 280 nm.

In vitro plasma stability of Ab and ADCs

Plasma was isolated from blood collected from naïve nu/nu mice by terminal cardiac puncture procedure. In vitro plasma stability studies were carried out by incubating a concentration equivalent to a 10 mg/kg bolus dose of VT680-labeled 5T4-Ab (5T4-Ab-VT680) or 5T4-ADC (5T4-ADC-VT680) with plasma. This mixture was incubated for different time intervals (1, 24, or 48 hours) at 37°C and 4°C. At the end of the incubation period, an aliquot equivalent to 1 µg of VT680-labeled Ab and ADC was separated on the 4% to 12% Bis-Tris gradient gels under reducing conditions to evaluate their degradation profiles. VT680 fluorophore at a concentration corresponding to the loaded protein (equivalent to DOL) was also run in a separate lane as a control. The gels were imaged using an Odyssey Infrared Imaging System (LI-COR) using 700 nm laser.

Flow cytometry studies

H1975 and MDA-MB-468 cells were procured from the ATCC, and cell culture was performed as per their instructions. No cell authentication was separately performed in our laboratory, but we evaluated for rodent pathogens (IMPACT profiling by IDEXX BioResearch) before in vitro studies. Cell surface binding of 5T4-ADC-VT680 and control-ADC-VT680 was evaluated in MDA-MB-468 and H1975 cells using flow cytometry. Briefly, suspended cells (7.5 × 10^5 cells/mL) were prechilled to 4°C on ice and incubated for 1 hour at 4°C with different concentrations of VT680-labeled ADCs in PBS (pH 7.4). After incubation, cells were washed, resuspended in ice-cold PBS, and immediately analyzed using a BD LSRII flow cytometer. For all experiments, the geometric mean fluorescence intensities were recorded for individual treatments. The experiments were performed in triplicates and repeated twice.

Cell binding and internalization studies by fluorescence microscopy

The cell surface binding and internalization of the ADC was evaluated by epifluorescence microscopy. Briefly, MDA-MB-468 cells (5 × 10^5 cells/mL) were cultured in Nunc Lab-Tek II Chamber Slide System in triplicates. Chamber slides prechilled on ice were incubated for 4 hours at 4°C with VT680-labeled ADCs (10 µg/mL). For binding studies, incubated cells were washed twice with ice-cold PBS and fixed using 4% paraformaldehyde at room temperature for 1 hour. For internalization studies, incubated cells were washed twice with ice-cold PBS followed by incubation with prewarmed medium at 37°C for 4 hours to monitor internalization of VT680-labeled ADCs. Later, cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton-X-100 in PBS for 5 minutes. Cells were then washed and stained with anti-human FITC-conjugated secondary antibody (1:100 dilution) and mounted using Vectashield mounting medium containing DAPI (Vector Laboratories). Images were taken with a Nikon TE2000 microscope at 40× magnification.

Cytotoxicity studies

The in vitro activity of VT680-labeled as well as unlabeled 5T4-ADC and control-ADC was tested in MDA-MB-468 cell line using CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation). Cells (5 × 10^3 cells/well) were plated overnight in a 96-well plate followed by treatment with different concentrations...
Throughout the imaging session, the animals were maintained isoelectric tomography imaging system (Perkin Elmer Inc.). During this dose 5T4-ADC showed moderate antitumor activity (18). The dose was normalized to 2 nmol of VT680, as this dose was determined to give good

In vivo studies

All procedures performed on animals in this study were in accordance with established guidelines and regulations, and were reviewed and approved by the Pfizer Institutional Animal Care and Use Committee. Animals were housed under standard 12:12 light:dark cycle in ventilated racks at a room temp of 72°F and relative humidity between 30% and 70%.

Five million H1975 cells in Matrigel were injected into the SQ flanks of the female nu/nu mice (8 weeks old). When the tumors were approximately 400 mm³, the animals were injected with 5T4-Ab-VT680, 5T4-ADC-VT680, control-Ab-VT680, or VT680 alone via the intravenous route. The dose was normalized to 2 nmol of VT680, as this dose was determined to give good in vivo signal (data not shown) and also at this dose 5T4-ADC showed moderate antitumor activity (18). Imaging was performed using an FMT 4000 fluorescence molecular tomography imaging system (Perkin Elmer Inc.). During imaging, the animals were anesthetized using a mixture of isoflurane and oxygen and placed in an imaging cassette. Throughout the imaging session, the animals were maintained under anesthesia in a thermally regulated (37°C) chamber. FMT imaging (whole-body) was performed at 5 minutes and at 6, 24, 48, 96, and 240 hours after injection of probes. Blood samples (20 µL) were also collected at each time point prior to imaging for PK evaluation. The signal observed during in vivo imaging is the sum of signal from the tumor or tissues due to drug accumulation and signal from the vasculature within the tissue due to drug in circulation.

In order to minimize the fluorescence signal from the circulating drug and better determine the amount of drug that has accumulated in the tissues, ex vivo evaluation was done on a subset of animals. At 48, 96, and 240 hours after dosing, selected animals were subjected to whole-body perfusion (via intracardiac route) with heparinized PBS/Saline and euthanized prior to collecting tissues. The tissues (tumor, liver, brain, spleen, kidney, and lungs) were imaged ex vivo by FMT. The FMT data were reconstructed and analyzed using TrueQuant software (Perkin Elmer Inc.). To validate the 3-D quantitation of the probe by FMT, we determined a standard curve using known concentrations (0–2,000 pmol) in a phantom. Linear regression analysis of 3-D quantitation revealed an R² value of 0.97 showing a good linearity of detection (Supplementary Fig. S1). The 3-D region of interest (ROI) was drawn for the whole-body, tumor, and the organs of interest on the in vivo and ex vivo images. Total fluorescence (in pmol) was obtained under a no thresholding setting to minimize the loss of low-fluorescence signals. Percentage injected dose (%ID) for each time point and organ of interest was calculated using the total signal from the whole-body at 5 minutes (100% injected dose) from each mouse. For determining the %ID/g for ex vivo organs, the fluorescence values from each organ were normalized to its weight and the whole-body signal at 5 minutes. After ex vivo imaging, the tumor/liver tissues at the 48-hour time point was flash frozen for quantitation of the Ab, ADC, and released payload using biochemical methods. The data represented are the average (± SEM) of 3 to 6 mice/time point, for each treatment group.

Pharmacokinetic analysis

Quantitation of the 5T4-Ab and 5T4-ADC concentrations in mouse plasma was performed by enzyme-linked immunosorbant assay (ELISA) as described earlier (18). Briefly, 5T4 protein was coated on to 96-well plates to capture the 5T4-Ab and 5T4-ADC. The captured reagents were detected using biotinylated goat anti-human IgG for the antibody and biotinylated anti-MMAF antibody for the ADC. The absorbance values were determined using a spectrophotometer. The released payload (cys-mc-MMAF) on 5T4-ADC was quantified by LC/MS/MS assay using a 550 Qtrap and C18 column with cys-mcMMAD as the internal standard (18). The PK (including the terminal half-lives) of 5T4-Ab and 5T4-ADC from the FMT imaging and plasma were determined by noncompartmental analysis using Phoenix WinNonlin 6.3 (Certara L.P.). The 3-D FMT imaging quantitation of heart (pmol/mL of VT680) was obtained from TrueQuant software and converted into equivalent protein concentrations (µg/mL) for comparison with plasma concentrations.

Results

Biochemical characterization of fluorophore-conjugated 5T4-Ab and 5T4-ADC

Prior to evaluating the biodistribution and targeting of 5T4-Ab and 5T4-ADC in an in vivo tumor model, we followed a stepwise in vitro quality control assessment process (Fig. 1). The compounds of interest were labeled with the NIR fluorophore—VT680. In order to minimize the effects of fluorophore conjugation to the 5T4-Ab or 5T4-ADC, we developed a conjugation protocol that would yield a DOL of approximately 3 VT680 molecules per antibody. Supplementary Fig. S2A shows the absorbance spectrum of VT680, 5T4-ADC, and 5T4-ADC-VT680. The VT680 alone curve showed a major peak at approximately 670 nm, whereas 5T4-ADC showed a major peak at approximately 280 nm. The 5T4-ADC-VT680 conjugate showed two peaks at approximately 280 nm and 670 nm representing the absorbance maximum of antibody (protein) and fluorophore (VT680), respectively. Similar to absorbance characteristics, we saw fluorescence of VT680 and the 5T4-ADC-VT680 conjugate at approximately 690 nm, whereas the 5T4-ADC alone had no fluorescence at 690 nm (data not shown).

The labeled and the unlabeled biologic molecules had similar elution profiles on SEC-HPLC. SEC-HPLC of 5T4-ADC and 5T4-ADC-VT680 had a major peak representing the monomers and a minor peak probably representing protein aggregates (Supplementary Fig. S2B). AUC of the major peak was 98.2% for 5T4-ADC and 97.7% for 5T4-ADC-VT680, whereas the AUC of the minor peak was 1.1% for 5T4-ADC and 1.8% for 5T4-ADC-VT680. These data suggested that labeling of 5T4-ADC with VT680 did not increase the aggregation propensity. Similar elution profiles were also obtained with 5T4-Ab and control-Ab/ADC labeled with VT680 (data not shown).

In order to evaluate the stability of VT680-conjugated biologic molecules in PBS or plasma, in vitro stability studies were performed. Either 5T4-Ab-VT680 or 5T4-ADC-VT680 were incubated with PBS or mouse plasma at 4°C or 37°C for 1, 24, and 48 hours. After incubation, the samples were separated on a gradient gel in reducing conditions to evaluate any degradation or released VT680 from biologic molecules. Because the 5T4-Ab and 5T4-ADC were labeled with a 680 nm fluorophore, we directly imaged the gels using an infrared imaging system. Two distinct bands

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representing the heavy chain and light chain of the antibody were observed in 5T4-Ab-VT680 and 5T4-ADC-VT680 samples (Supplementary Fig. S2C). Interestingly, the heavy-chain fragments in the plasma samples migrated faster than the PBS samples. This may be due to the excess protein present in the plasma samples which in turn is reducing the number of SDS molecules per protein. No degradation bands reflecting the cleaved VT680 were observed in 5T4-Ab-VT680 or 5T4-ADC-VT680 samples incubated in PBS or plasma. VT680 alone showed a distinct band at the lower end of the gel (Supplementary Fig. S2C). These results indicate that VT680 formed a stable conjugation to the Ab and ADC.

Evaluation of binding and functional properties of VT680-conjugated reagents
To determine if the conjugation of VT680 to biological molecules perturbed their binding property, we used flow cytometry and epi-fluorescence microscopy. Previous studies showed that H1975 is a relatively low 5T4-expressing cell line and MDA-MB-468 is a high 5T4-expressing cell line (18). Concentration-dependent binding of 5T4-ADC-VT680 and control-ADC-VT680 was compared using flow cytometry (Fig. 2A). 5T4-ADC-VT680 bound to both H1975 and MDA-MB-468 cells in a concentration-dependent fashion, whereas control-ADC-VT680 had minimal binding. Mean fluorescence intensity showed that 5T4-ADC-VT680 binding was higher in MDA-MB-468 cells compared with H1975 cells (Fig. 2A). The antibody binding capacity, a measure of relative receptor number on each cell surface, revealed that the expression was approximately 31,000 and 63,400 for H1975 and MDA-MB-468, respectively (Supplementary Fig. S3). In order to further confirm the cell surface binding of 5T4-ADC-VT680, we employed epi-fluorescence microscopy. Figure 2B shows the localization of 5T4-ADC-VT680 at the cell surface. The line intensity profile for VT680 (Fig. 2B, red line) showed two sharp peaks depicting high and specific binding at the cell surface. No significant cell surface binding was observed with control-ADC-VT680, which was confirmed by the scattered line intensity profile. The line intensity profile for DAPI (nuclear marker, blue line) showed a single broad peak inside the cell membrane for both 5T4-ADC and control-ADC-VT680 (Fig. 2B).

To further determine whether VT680 conjugation to the 5T4-ADC had any effect on the cellular uptake or activity, internalization of the ADC was assessed using epi-fluorescent microscopy, whereas a cytotoxicity assay was used to verify activity. Internalization was visualized both by directly imaging VT680 fluorophore and indirectly by detection with an anti-human secondary antibody conjugated to FITC. Punctate labeling inside the cytoplasm was observed in both the channels, indicating localization probably in the lysosomes (Fig. 2C). Line intensity profiles confirmed the overlap of VT680 (red) and FITC (green) channels, suggesting the specificity of the signal to 5T4-ADC (Fig. 2C). These data also suggest that the VT680 signal observed was from the VT680 conjugated to the 5T4-ADC and not free VT680 fluorophore.

To further evaluate the cell killing ability of VT680-labeled 5T4-ADC, we performed cytotoxicity assay in MDA-MB-468 cells. Unlabeled 5T4-ADC showed a concentration-dependent effect on the viability of the cells (Fig. 2D). The cytotoxicity profile of 5T4-ADC-VT680 overlapped with that of the unlabeled 5T4-ADC. Although the control-ADC had very minimal effect on the viability of cells, the cytotoxicity profile of unlabeled control-ADC and control-ADC-VT680 overlapped each other (Fig. 2D). These results indicate that VT680 formed a stable conjugation to the Ab and ADC.
In vitro evaluation of binding and activity of 5T4-ADC-VT680.

A, flow cytometric evaluation of cellular binding of 5T4-ADC-VT680 and control-ADC-VT680. H1975 and MDA-MB-468 cells were incubated with different concentrations of VT680-labeled 5T4-ADC or Isotype control-ADC at 4°C for 1 hour and analyzed by flow cytometry. Note a dose-dependent increase in binding of 5T4-ADC in both cell lines. MDA-MB-468 cells had higher 5T4-ADC binding than H1975 cells. No significant specific binding was observed from control-ADC in both cell lines. The inset graphs show the histogram of control-ADC-VT680 and 5T4-ADC-VT680 at 100 μg/mL concentration.

B, evaluation of cellular binding 5T4-ADC-VT680 and control-ADC-VT680 in MDA-MB-468 cell line using fluorescence microscopy. Punctate signal was observed on the surface of cells labeled with 5T4-ADC-VT680, but a weak nonspecific signal was observed in control-ADC-VT680 cells. Pixel intensity along the line on the microscopic image is represented by the line intensity profile. The red line in the line intensity profile represents the VT680 signal, whereas the blue line represents DAPI (nucleus) signal. Two sharp peaks were observed for VT680 signal in 5T4-ADC-VT680-labeled cells, but scattered lines were observed in control-ADC-VT680-labeled cells. The line intensity profile for nucleus (DAPI) was similar (single broad peak) for both treatments.

C, evaluation of cellular internalization VT680 labeled 5T4-ADC in MDA-MB-468 cell line using fluorescence microscopy. Red, VT680 signal; green, FITC (anti-human secondary antibody); blue, DAPI (nucleus). Punctate labeling in the cytoplasm was observed by both VT680 and FITC signals suggesting cellular internalization of 5T4-ADC. Yellow overlay in the microscopy image shows the colocalization of VT680 (direct detection) and FITC (indirect detection) labeling. The line intensity profile shows a good overlap of red line (VT680) with green line (FITC) signal suggesting colocalized signal by direct and indirect labeling.

D, comparison of cytotoxicity of VT680-labeled 5T4-ADC and control-ADC with unlabeled ADC. All experiments were repeated at least twice; ± SEM is represented.
results show that the VT680 labeling of biological molecules at 2 to 3 DOL did not change their functional activity. This is an important observation as it then discards the notion that a relatively large tag interferes with ADC functional activity and opens up their usability.

In vivo biodistribution, clearance, and plasma PK of 5T4-Ab and 5T4-ADC

In vivo biodistribution and tumor targeting were evaluated in the H1975 mouse xenograft model. In vivo whole-body (torso) FMT imaging was performed at various time points (5 minutes and 6, 24, 48, 96, and 240 hours) after injection. Blood was also collected and processed to obtain plasma before each imaging session. A 3-D ROI was created for selected organs of interest as per the protocol described by Vasquez and colleagues, (8). Since there was a minimal signal (<5%) observed in the head region at all time points, the torso ROI was used to depict signal from the whole body. The whole-body data represent the signal from various tissues/ organs in the torso region and also the probe in the circulation. Initially, FMT imaging showed minimal changes in the whole-body signal, which likely reflects the compound’s distribution into the tissues, whereas after 48 hours, the compound’s systemic clearance becomes evident (Fig. 3A). The FMT profile of both 5T4-Ab-VT680 and the 5T4-ADC-VT680 overlapped at all the time points, suggesting no difference in clearance due to linker-payload (mcMMAF) conjugation over the 10-day period. Plasma concentrations determined by detecting the antibody from 5T4-Ab-VT680 and 5T4-ADC-VT680 samples by ELISA also showed an overlapping clearance profile (Fig. 3B). In all cases, an initial distribution phase was observed followed by a sustained rate of clearance at later time points. Interestingly, the payload’s profile as measured by LC/MS assay showed a faster initial distribution compared with the antibody profile (Fig. 3B).

A 3-D ROI around the heart, which was assumed to primarily reflect the blood profile, was also evaluated (Fig. 3C). Similar to the plasma PK data, 5T4-Ab-VT680 and 5T4-ADC-VT680 profiles in heart tissue overlapped, suggesting that the payload conjugation did not change the elimination kinetics. Furthermore, the kinetic profiles obtained from plasma samples and the heart ROI from FMT imaging were in good agreement with one another, supporting the assumption that this ROI reflects blood clearance (Fig. 3C).

Kinetics of tumor targeting and accumulation in the H1975 xenograft model

Figure 4A shows the whole-body FMT images of the same animal at various time points from either the 5T4-Ab-VT680 or 5T4-ADC-VT680 groups. The image panel also illustrates that the distribution and clearance profiles of 5T4-Ab-VT680 and 5T4-ADC-VT680 from the whole-body were similar (Fig. 4A). As anticipated, liver (being a major site for clearance) and tumor (containing the target antigen) were the two major sites of accumulation, whereas other tissues showed minimal accumulation. The images also reveal that the accumulation and elimination of 5T4-Ab-VT680 and 5T4-ADC-VT680 from the liver followed similar profile. Because imaging was performed over a 10-day period, the tumor size was measured according to a standard schedule (twice a week). No tumor growth inhibition was observed in the 5T4-Ab-VT680 group, whereas in the 5T4-ADC-VT680 group, there was trend showing tumor growth inhibition after day 3 after injection (Fig. 4B). This further confirms our in vitro data that the fluorescent labeling do not change the functional activity of the 5T4-ADC or the 5T4-Ab within the 10-day observation period.

To determine the kinetics of targeting and accumulation of 5T4-Ab-VT680 and 5T4-ADC-VT680, the fluorescence signal was...
quantified and normalized to the tumor size. The peak accumulation was observed at 24 to 48 hours after injection, although significant accumulation was observed even at 96 hours after injection (Fig. 4C). There was no statistically significant difference between 5T4-Ab-VT680 and the 5T4-ADC-VT680 group; however, a trend toward increased accumulation in the 5T4-Ab-VT680 group was observed. These differences may be due to the tumor growth inhibition or cell killing observed in the 5T4-ADC-VT680 group.
group but not in the 5T4-Ab-VT680 group. After 96 hours, there was a consistent decline in the signal from both the groups, although there was still a significant fluorescent signal observed at 240 hours after injection of drugs suggesting a prolonged retention of the biological drug in the tumor. Ex vivo imaging of tumors and other organs was performed at 48, 96, and 240 hours after whole-body perfusion. The highest accumulation in tumor for 5T4-Ab-VT680 and 5T4-ADC-VT680 was observed at 48 hours with %ID/g of 9.89 (SEM ± 1.51) and 7.38 (SEM ±2.02), respectively followed by a decline at 96 and 240 hours (Fig. 4D and E). 5T4-Ab-VT680 was significantly higher than 5T4-ADC-VT680 only at 240 hours (4.54 ±0.16 vs. 2.99 ±0.77 %ID/g). In order to confirm that these findings were specific to 5T4-Ab and 5T4-ADC (rather than a VT680 fluorophore-based artifact), we evaluated the tumor accumulation of control-Ab conjugated with VT680 and the free VT680 fluorophore. There was no significant accumulation of control-Ab or VT680 in the tumors compared with the 5T4-Ab or 5T4-ADC (ex vivo imaging at 48 hours; Supplementary Fig. S4A).

Kinetics of accumulation and clearance in the liver and other organs

FMT imaging showed that the liver was one of the major sites for accumulation of the VT680-labeled biologics. The FMT imaging profile for clearance of 5T4-Ab-VT680 and 5T4-ADC-VT680 from liver overlapped each other (Fig. 5A). Although the in vivo concentration profiles in liver tissue followed a similar trend to whole-body clearance up to 48 hours (Fig. 3A), 5T4-Ab-VT680 and 5T4-ADC-VT680 cleared slightly faster from liver after 48-hour time point (Fig. 5A). Figures 5B and C show the ex vivo images and quantitation of the signal from liver. Both groups (5T4-ADC-VT680 and 5T4-Ab-VT680) had good liver signal, suggesting accumulation in liver at 48, 96, and 240 hours. Because liver has no 5T4 expression and the antibody does not cross-react with mouse, this is likely nonspecific accumulation or the signal from metabolized VT680. In order to confirm this nonspecific accumulation of 5T4-Ab-VT680 and 5T4-ADC-VT680 in liver, a separate ex vivo study (48 hours) evaluated the accumulation of both the control-Ab-VT680 and VT680 fluorophore alone. The accumulation of 5T4-Ab-VT680 and 5T4-ADC-VT680 in liver was similar to control-Ab-VT680, whereas the free VT680 fluorophore had very minimal accumulation in the liver (Supplementary Fig. S4B).

In addition, we determined the amount of released payload (cys-mcMMAF) from 5T4-ADC in the tumor and liver by LC-MS/MS method and found approximately 15-fold higher payload concentrations in tumor than liver (Fig. 5D). Ex vivo imaging was also performed and quantified for a few selected organs: brain, spleen, lungs, and kidneys (Table 1). There was minimal accumulation (<1.6 %ID/g) in these tissues, compared with tumor and liver. Quantitation revealed no meaningful differences between 5T4-ADC-VT680 and 5T4-Ab-VT680 in these organs.

Discussion

The goal of this study was to develop a method to evaluate the biodistribution and tumor targeting of an antibody or ADC using FMT imaging. Because FMT acquires 3-D isotropic data, we hypothesized that FMT can provide quantitative information of the fluorophore or its conjugates (8). Structural modification of antibodies can result in dramatic effects on the 3-D arrangement, PK properties, immunogenicity, binding affinity, and thus their function (20, 21). Hence, attachment of any additional entity like a payload, fluorophore, or radiolabel to the antibody could affect
samples or by whole-body FMT. These half-lives were consistent to 3.6 days for the 5T4-ADC-VT680 measured in plasma concentrations (0 time frame as the distribution phase observed in the plasma distribution into the extravascular tissue space is evident in the accumulation in the peripheral tissues. The compounds display the signal from the probe in circulation and also elimination of the compounds. The whole-body FMT data provide complementary information on the distribution and whole-body FMT signal and the plasma PK profile, suggesting that each antibody, peptide, or the modality used for quantitation. These conclusions accompanied by 2-D fluorescence imaging for quantitation of tissues and tumor by FMT imaging (%ID/g).

Table 1. Ex vivo quantitation of tissues and tumor by FMT imaging (%ID/g).

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<td>SEM</td>
<td>6.12</td>
<td>0.79</td>
<td>0.17</td>
<td>0.06</td>
<td>0.058</td>
<td>0.55</td>
</tr>
<tr>
<td>240 h Mean</td>
<td>9.29</td>
<td>0.12</td>
<td>0.62</td>
<td>0.09</td>
<td>0.248</td>
<td>0.26</td>
</tr>
<tr>
<td>SEM</td>
<td>0.88</td>
<td>0.03</td>
<td>0.39</td>
<td>0.03</td>
<td>0.108</td>
<td>0.08</td>
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</tbody>
</table>

Its binding and functional activity. Multiple studies have shown the utility of conjugating either radioisotope, fluorophore, or both to antibodies or their fragments for biodistribution studies. CD105 (endoglin) monoclonal antibody was conjugated with PET probe 64/68Zr and NIR probe CW-800 and found no difference in binding or specificity between conjugated or nonconjugated antibody (22). Hudson and colleagues conjugated NIR probe CE-750 to EGF ligand for multispectral optoacoustic imaging and demonstrated that it did not affect the biological activity significantly (23). Connor and colleagues showed that conjugation of IRdye-800 to mouse monoclonal IgG1 (8C2) significantly changed the PK property of this antibody (1). They adapted whole-body sectioning accompanied by 2-D fluorescence imaging for quantitation. Such dissimilarities in PK can be related to the differences in modality used for quantitation. These conflicting results also suggest that each antibody, peptide, or the fluorophore can have unique outcomes. In order to address these concerns, we adapted a stepwise approach to our biodistribution studies using FMT and performed qualitative in vitro assessment before the in vivo evaluation (Fig. 1).

We labeled ST4-Ab, ST4-ADC, or Isotype control-Ab/ADC with NIR fluorophore VT680 and demonstrated by in vitro studies that conjugating VT680 at a DOL < 3 does not affect the stability or functional property (i.e., binding, internalization, and cytotoxicity) of ST4-Ab or ST4-ADC. In vivo biodistribution and tumor targeting of ST4-Ab and ST4-ADC were compared in an H1975 subcutaneous xenograft model after administering a single intravenous bolus dose of compound. This model was selected because H1975 cells express 5T4 antigen and its released payload have a propensity for accumulating in the tumor and inhibits the tumor growth (18, 24). The whole-body biodistribution profile from FMT and the plasma concentration profiles (from LBA assays) were overlapping for both ST4-Ab-VT680 and ST4-ADC-VT680, suggesting that payload or VT680 conjugation had no major effects on accumulation and clearance of ST4-Ab. Interestingly, the whole-body FMT signal and the plasma PK profile appear to provide complementary information on the distribution and elimination of the compounds. The whole-body FMT data include the signal from the probe in circulation and also accumulation in the peripheral tissues. The compounds distribution into the extravascular tissue space is evident in the early flat whole-body signal that occurs within the same timeframe as the distribution phase observed in the plasma concentrations (0–48 hours). Beyond 48 hours, both samples show similar clearance profiles with estimated terminal half-lives ranging from 3.4 to 4 days for the ST4-Ab-VT680 and 3.3 to 3.6 days for the ST4-ADC-VT680 measured in plasma samples or by whole-body FMT. These half-lives were consistent with the previously reported plasma PK values in naive mice and MDA-MB-435/ST4 tumor model using the ST4-ADC which was not conjugated to VT680 (24).

Previously, it has been shown that plasma input functions and blood time-activity correlation can be determined by noninvasive PET imaging (25, 26). Because FMT data provide 3-D quantitative data, we presumed that the signal from a ROI encompassing the heart could be compared with the plasma concentration profile. Given that ST4-Ab-VT680 or ST4-ADC-VT680 are not expected to have significant uptake in the heart, the majority of the heart ROI signal can be attributed to the blood fraction. Visual comparison of the plasma concentration profiles and the FMT heart imaging profiles shows good agreement, suggesting that the heart ROI is a reasonable surrogate for the plasma kinetics (i.e., the separate plasma PK evaluation may not be needed in future studies).

Tumor growth was also monitored during the imaging period. We observed tumor growth inhibition in the ST4-ADC-VT680 group after day 4, although statistically not significant due to the relatively small group size (P > 0.1). This suggests that VT680-conjugated ADC retained in vivo tumor regression ability corroborating the similar observations made with in vitro cytotoxicity data. Tumor accumulation profiles by FMT imaging showed peak accumulation for both ST4-Ab-VT680 and ST4-ADC-VT680 groups (as the ratio of %ID/tumor size) was at 24 to 48 hours after injection. There was an increased accumulation in the ST4-Ab-VT680 group (P > 0.08), which might be attributed to the bigger tumor size in this group. In addition, the regression of the tumor size in the ST4-ADC-VT680 group may have added to the complexity in targeting and quantitation. Previous studies have demonstrated that the growth rate and doubling time may affect the drug exposure in the tumor (27, 28). To eliminate the contribution from the fluorescence probe in the circulating blood, we performed ex vivo imaging after whole-body perfusion. Results from the ex vivo imaging confirmed that significant amounts of ST4-Ab-VT680 and ST4-ADC-VT680 were accumulated within the tumor, whereas minimal accumulation was observed for the control-Ab. As anticipated, liver was a major site for nonspecific accumulation and clearance of VT680-conjugated ST4-Ab, ST4-ADC, and control-Ab. Evaluation of released payload by LC-MS/MS analysis from the ex vivo tumor and liver samples showed that the tumor had approximately 15 times higher accumulation, an indication that the ST4-ADC went through the target-mediated delivery process, whereas minimal accumulation and clearance were observed in the liver samples. The compounds distribution into the extravascular tissue space is evident in the early flat whole-body signal that occurs within the same timeframe as the distribution phase observed in the plasma concentrations (0–48 hours). Beyond 48 hours, both samples show similar clearance profiles with estimated terminal half-lives ranging from 3.4 to 4 days for the ST4-Ab-VT680 and 3.3 to 3.6 days for the ST4-ADC-VT680 measured in plasma samples or by whole-body FMT. These half-lives were consistent with the previously reported plasma PK values in naive mice and MDA-MB-435/ST4 tumor model using the ST4-ADC which was not conjugated to VT680 (24).

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Disclosure of Potential Conflicts of Interest

All authors are either current or former employees (full time or contract) of Pfizer Inc.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Giddabasappa, V.R. Gupta, R. Norberg, P. Gupta, J. Eswaraka

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Giddabasappa, V.R. Gupta, R. Norberg, M.E. Spiller, J. Wentland, B. Rago, J. Eswaraka, M. Leal

Writing, review, and/or revision of the manuscript: A. Giddabasappa, V.R. Gupta, P. Gupta, M.E. Spiller, J. Eswaraka, M. Leal

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Giddabasappa, R. Norberg

Study supervision: A. Giddabasappa

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