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The Effect of Molecular Weight, PK, and Valency on Tumor Biodistribution and Efficacy of Antibody-Based Drugs[1,2]

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Abstract

Poor drug delivery and penetration of antibody-mediated therapies pose significant obstacles to effective treatment of solid tumors. This study explored the role of pharmacokinetics, valency, and molecular weight in maximizing drug delivery. Biodistribution of a fibroblast growth factor receptor 4 (FGFR4) targeting CovX-body (an FGFR4-binding peptide covalently linked to a nontargeting IgG scaffold; 150 kDa) and enzymatically generated FGFR4 targeting $F(ab)_2$ (100 kDa) and Fab (50 kDa) fragments was measured. Peak tumor levels were achieved in 1 to 2 hours for Fab and $F(ab)_2$ *versus* 8 hours for IgG, and the percentage injected dose in tumors was 0.45%, 0.5%, and 2.5%, respectively, compared to 0.3%, 2%, and 6% of their nontargeting controls. To explore the contribution of multivalent binding, homodimeric peptides were conjugated to the different sized scaffolds, creating FGFR4 targeting IgG and $F(ab)_2$ with four peptides and Fab with two peptides. Increased valency resulted in an increase in cell surface binding of the bivalent constructs. There was an inverse relationship between valency and intratumoral drug concentration, consistent with targeted consumption. Immunohistochemical analysis demonstrated increased size and increased cell binding decreased tumor penetration. The binding site barrier hypothesis suggests that limited tumor penetration, as a result of high-affinity binding, could result in decreased efficacy. In our studies, increased target binding translated into superior efficacy of the IgG instead, because of superior inhibition of FGFR4 proliferation pathways and dosing through the binding site barrier. Increasing valency is therefore an effective way to increase the efficacy of antibody-based drugs.

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Introduction

Effective antibody therapies for targeting solid tumors are limited by poor penetration [1] and very low percent of injected dose (ID) reaching tumor [2]. Limited tumor penetration, caused by heterogeneous antigen expression [3] and blood supply [4], increased interstitial fluid pressure [5,6], as well as a so-called "binding site barrier" caused by high-affinity binding [7,8] are thought to contribute to less effective therapy by leaving viable cells untargeted [6]. As a consequence, alternatives to full-length IgG drugs have been widely investigated as a means of improving penetration [9,10].

Using a fibroblast growth factor receptor 4 (FGFR4) targeting CovX-body (a nontargeting IgG covalently linked to an FGFR4-

of size, pharmacokinetics (PK), and avidity in tumor uptake, penetration, and ultimately efficacy.

Net drug levels in the tumor are driven by the PK properties (influenced by the dose and rate of plasma clearance), diffusion rate

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[2]This article refers to supplementary materials, which are designated by Figures W1 to W4 and are available online at www.transonc.com.

(determined by the size and properties of the biotherapeutic), binding affinity, and rate of consumption of the drug [3,11,12].

IgG drug scaffolds inherently have excellent PK properties compared to other protein therapeutics because of both their molecular weight and ability to bind to the neonatal FcRn receptor, which recycles molecules that bind to them back to the serum maintaining elevated levels. The limited and heterogeneous tumor penetration of IgGs, however, has led to the use of smaller IgG fragments such as Fabs, scFv′s, and diabodies [13–15], which can, in theory, diffuse more efficiently through tumors, translating into more favorable ID ratios at earlier time points [16]. The use of antibody fragments though must be balanced by the shorter serum half-lives of non–Fc-containing constructs and the potential for more rapid distribution to normal tissues.

As well as PK, increased valency may also drive tumor biodistribution and efficacy, although the role of valency in tumor retention has yielded sometimes conflicting data. Increasing the valency increased the tumor uptake of human epidermal growth factor receptor 2 (HER2) binding diabodies [13,17], while increasing the valency of HER2 binding DARPins, decreased tumor uptake [18]. In those studies, increased valency was achieved by doubling the molecular weight, and therefore, the role of increasing the size (and potentially decreasing clearance time) in tumor uptake and retention could not be distinguished from the role of increased valency. However, in other studies using divalent $(scFv')_2$ molecules with zero, one, and two binding sites (same molecular weight), three-fold greater tumor retention was achieved with the construct with two binding sites [19].

A CovX-body is a peptide antibody fusion generated by conjugating a peptide on an azetidinone linker to a nonbinding humanized IgG1 monoclonal aldolase antibody [20]. The CovX-body technology allows the increase in the number of targeting peptides on our scaffolds from two to four on the bivalent IgG and $F(ab)_2$ and from one to two on the Fab using homodimeric FGFR4-targeting peptides. Increasing the valency of the constructs allows for the measurement of the role of increased valency on tumor uptake and penetration without significantly altering the molecular weight of the targeting scaffolds. Increasing the valency of our constructs increased cell binding of the bivalent constructs. It did not significantly increase tumor levels and decreased the penetration of the scaffolds into the tumor after a single dose, presenting a so-called "binding site barrier." The binding site barrier is the phenomenon whereby high-affinity antibodies accumulate around the vasculature and fail to distribute evenly throughout the tumor [8]. This dynamic barrier can be overcome by increasing the dose of the antibody [7,21]. In a multi-dose efficacy study comparing the tumor growth inhibition (TGI) of the IgG homodimer peptide construct *versus* the IgG monomer peptide, superior efficacy is observed with the homodimer IgG.

This current study demonstrates that in a single dose study, PK is the most important driver of maximal tumor levels. While higher levels of Fab were seen in the tumor after an hour than the IgG and $F(ab)_2$, superior maximal tumor concentrations are achieved with the IgG constructs. Increasing the avidity of an IgG is an effective way to maximize the efficacy of our targeting scaffolds.

Materials and Methods

Generating $F(ab)_2$ and Fab Scaffolds

MA) to produce Fab or immobilized pepsin (Thermo Scientific) to produce $F(ab)_2$. Fab fragments were purified by size exclusion followed by binding and elution to Protein L to separate Fab fragments from Fc fragments. $F(ab)_2$ fragments were purified by size exclusion, followed by cation exchange. Final fractions were analyzed on a Bioanalyzer (2100) protein electrophoresis chip (Agilent Technologies, Santa Clara, CA; Figure W1).

CVX-2000, $F(ab)_2$, and Fab constructs were conjugated with a monomeric or homodimeric FGFR4-targeting peptide through an azetidinone linker on the peptide, which reacts specifically with a lysine in the Fab arm [23]. The original FGFR4-targeting peptide was discovered by phage display and synthesized as described previously [20]. Nontargeted controls of the scaffolds were conjugated with a nonbinding peptide.

Direct binding ELISA. High-binding half-well 96-well plates were coated overnight at 4°C with recombinant human FGFR4-Fc (R&D Systems, Minneapolis, MN) or CVX-2000 anti-idiotype antibody for total scaffold measurements. After washing and blocking, titrated compounds were then added to the plates and incubated at room temperature for 1 hour, followed by incubation with goat anti-human IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1 hour. Tetramethylbenzidine substrate solution (KPL, Gaithersburg, MD) was added, and OD_{450} was measured. Half maximal effective concentration (EC_{50}) value was obtained from the dose-response curve from the experiment.

Competitive ELISA. High-binding half-well 96-well plates were coated with goat anti-human IgG-Fc (Bethyl Laboratories, Montgomery, TX) at 4°C overnight. After washing and blocking, plates were incubated with recombinant human FGFR4-Fc for 1 hour at room temperature. Plates were washed, and titrated compounds were added in the presence of 50 ng/ml recombinant human FGF19 (R&D Systems) and 1 μg/ml heparan sulfate (Seikagaku/Amsbio, Lake Forest, CA) and incubated for 2 hours at room temperature. The bound compounds were detected by biotinylated anti-FGF19 antibody (R&D Systems), followed by incubation with streptavidin-HRP (Fitzgerald Industries, Acton, MA). OD_{450} was measured.

Surface plasmon resonance binding analysis. Surface plasmon resonance (SPR) binding analyses of anti-FGFR4 compounds were performed on ProteOn XPR36 instrument (BioRad, Hercules, CA) at 25°C. For kinetic analysis, recombinant human FGFR4-Fc protein (R&D Systems) was immobilized on parallel surfaces of a GLM chip (BioRad) by amine coupling according to the manufacturer's protocol. Running buffer was phosphate-buffered saline (PBS) with 300 mM sodium chloride and 0.05% (vol/vol) Tween 20. FGFR4 immobilization level was 990 RU for Fab binding and 640 RU for $F(ab)_2$ and IgG binding. Compounds were tested for binding to FGFR4 starting at 200 nM at a flow rate of 50 μl/min. Association was monitored for 180 seconds, and dissociation was monitored for 600 seconds. Chip was regenerated with 0.85% (vol/vol) phosphoric acid in water. Data were double-referenced to blank chip surface and buffer injection and fitted to 1:1 binding model with local R_{max} using ProteOn Manager software (BioRad) to determine kinetic rate constants and K_D. Kinetic constants are averaged from three independent experiments.

For evaluation of monomer and homodimer peptide–conjugated

GLM chip (BioRad) to 10,000 RU. Running buffer was PBS with 0.01% (vol/vol) Tween 20; 50 nM Fab, 10 nM $F(ab)_2$, 10 nM IgG monomer and 80 nM Fab, 20 nM $F(ab)_2$, 20 nM IgG homodimer-conjugated constructs were captured with anti-idiotype CVX-2000 antibody. FGFR4-Fc (10 nM; R&D Systems) protein was tested for binding to anti-FGFR4 compounds captured on the chip. Data were double-referenced to chip surface and buffer blank.

Huh-7 cell line. Huh-7 cells were obtained from Japan Health Science Research Resources Bank (Osaka, Japan; Cat. JCRB0403) and were in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% FBS and maintained at 37°C and 5% CO_2.

Flow cytometry (FACS). Huh-7 cells were harvested with cell stripper and resuspended in FACS buffer (PBS + 10% FBS + 1% sodium azide). Cells were incubated with FGFR4-targeting scaffolds for 1 hour on ice. Cells were then washed and incubated with phycoerythrin (PE)-labeled Goat Anti-Human IgG, $F(ab')_2$ Fragment Specific secondary antibody (Jackson ImmunoResearch Laboratories) for 30 minutes. Cell binding was measured using a flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed using FloJo software (TreeStar Inc, Ashland, OR).

PK in rodents. PK properties of constructs were assessed in 5-week-old male Swiss Webster mice weighing 18 to 20 g (Charles River Laboratories, Wilmington, MA). Compounds were administered intravenously (i.v.) at 10 mg/kg (n = 3), and blood samples were collected over a period of 5 days. Serum samples were prepared and analyzed for FGFR4 binding activity in binding ELISAs as described previously. Total scaffold levels were measured by binding to a CVX-2000 anti-idiotype antibody capture ELISA. Data were analyzed using WinNonlin software (Pharsight, St Louis, MO) to generate PK parameter estimates.

Preparation of near-infrared–conjugated constructs. FGFR4 targeting CovX-bodies (IgG) and $F(ab)_2$ and Fab constructs were labeled with IRDye 800CW (LI-COR Biosciences, Lincoln, NE). In brief, constructs were buffer exchanged into 50 mM sodium phosphate buffer (pH 7) and incubated with two equivalents of the dye to the antibody solution overnight at room temperature in the dark. Constructs were buffer exchanged several times in Amicon spin filters [50 kDa molecular weight cut off (MWCO)] to remove free dye. Dye-to-protein ratios were calculated according to the manufacturer's instructions using the A_{780} and A_{280} measurements.

In vivo *xenograft studies.* Xenografts were induced by subcutaneous implantation of Huh-7 tumor cells into 5- to 7-week-old female nu/nu mice (18-20 g at start of experiment) and allowed to grow to a volume of 200 to 400 mm^3 before initiation of treatment. Once tumors were established, mice were randomized to treatment groups on the basis of their tumor volumes for all *in vivo* studies described below.

In vivo *animal imaging.* Near-infrared–conjugated compounds were administered at 3 and 10 mg/kg by intraperitoneal (i.p.) injection (no significant difference in tumor uptake was observed in a pilot study comparing i.p. *vs* i.v. injection). Mice were anesthetized with 5% isoflurane for induction and maintained at 2% during image capture. coupled device (CCD) camera was used to collect the images. The images were analyzed using Living Image Software 4.0 (PerkinElmer). Regions of interest were quantified for mean pixel values.

Biodistribution studies. Mice were dosed at 30 mg/kg i.p. injection, and tumors were harvested at maximal accumulation time points derived from imaging study (1 hour for Fab, 2 hours for $F(ab)_2$, and 8 hours for IgG). Tumors and normal tissue were harvested for biodistribution and histologic evaluation. For total scaffold accumulation, tissues were homogenized using FastPrep Lysing Matrix D Tubes (MP Biomedicals, Santa Ana, CA). Tissues were placed in tubes in a cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). Tubes were then pulse homogenized using a FastPrep-24 instrument (MP Biomedicals) followed by incubation on a shaker at 4°C for an hour. Samples were then spun at 14,000 rpm for 10 minutes, and the supernatant was removed to a fresh tube. Samples were then applied directly to CVX-2000 anti-idiotype capture binding ELISA plates for total scaffold measurement.

In vivo *efficacy study.* Mice were randomized into groups of 10 mice per group. All compounds were administered once weekly at 30 mg/kg by i.p. injection. Tumor volumes were measured once or twice weekly using calipers. Once the mean tumor volume of each treatment group exceeded 2000 mm^3, mice were killed by CO_2 asphyxiation followed by cervical dislocation. Tumors and normal tissue were harvested for histologic evaluation.

Immunohistochemistry

Tumors from the biodistribution study described above were fixed in formalin for 24 hours. Tumors were embedded in paraffin blocks, sectioned, and mounted for immunohistochemistry. After deparaffinization and rehydration, heat-mediated antigen retrieval was performed using antigen retrieval buffer (Abcam, Cambridge, United Kingdom) for 30 minutes. Slides were incubated in 1% H_2O_2 for 10 minutes followed by a blocking step for 30 minutes and primary antibody incubation. Blood vessels were detected using rabbit anti-CD31 antibody (Abcam; ab28364) overnight at 4°C. Sections were washed with PBS containing 0.01% Tween-20 and incubated with biotin anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature followed by alkaline phosphatase–conjugated streptavidin (Jackson ImmunoResearch Laboratories) for 1 hour. Sections were washed and incubated with Vector Red Alkaline Phosphatase Substrate Kit (Vector Labs, Burlingame, CA) for 20 minutes. For dual staining, sections were washed and incubated in HRP-conjugated donkey anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories) overnight at 4°C. Sections were washed and incubated in DAB peroxidase substrate kit (Vector Labs), dehydrated, and mounted. Images were captured using a Leica SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) at ×40, and images were analyzed on Leica SCN400 Image Viewer software. Tumor penetration was quantified using Image-Pro plus software (Media Cybernetics Inc, Rockville, MD). Five isolated vessels were randomly selected per slide with the distance (in μm) the scaffolds penetrated measured twice with each measurement taken on opposite sides. Statistical significance was determined using Prism (GraphPad Software, San Diego, CA). FGFR4 staining was measured using a rat

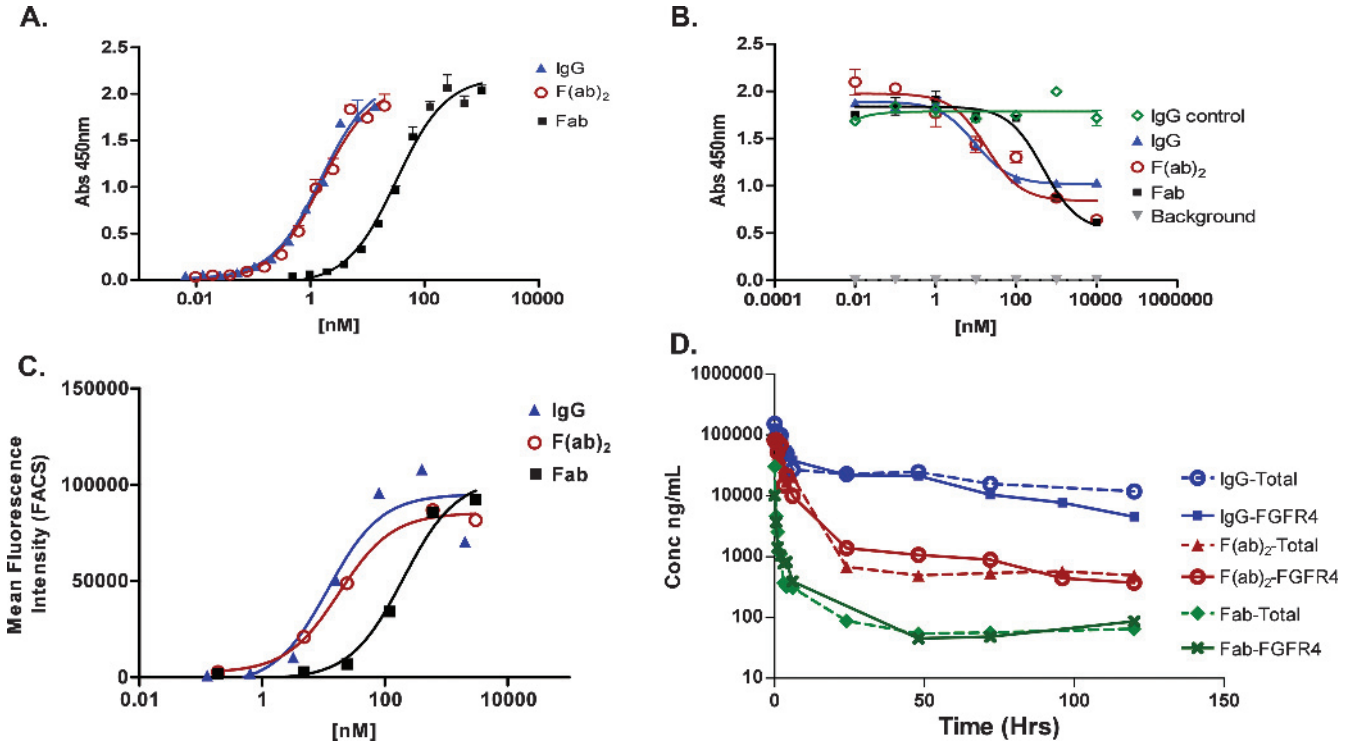

Figure 1. Characterization of FGFR4 binding scaffolds. (A) IgG, $F(ab)_2$, and Fab constructs bind specifically to FGFR4. (B) In an FGF19 competition ELISA, all constructs compete with FGF19 to bind to FGFR4. (C) All constructs bind to Huh-7 cells. (D) PK curves of IgG, $F(ab)_2$, and Fab following a single i.v. dose of 10 mg/kg in Swiss Webster mice. Both total and FGFR4 binding were measured as described in the Materials and Methods section.

Laboratories) for 1 hour at room temperature. Sections were washed and incubated using a DAB peroxidase substrate kit (Vector Labs).

Phospho-p44 Mitogen-Activated Protein Kinase (Extracellular Signal-Regulated Protein Kinase) Assay

Phospho-extracellular signal-regulated protein kinase (Erk1/2) was measured using a PathScan Phospho-p44 mitogen-activated protein kinase (MAPK) Sandwich ELISA Kit (Cell Signaling Technology; Cat. No. 7315). Homogenized tumor lysates from our biodistribution study were used according to the manufacturer's instructions. In brief, 100 µl was placed in microwells and incubated for 2 hours at 37°C. Wells were then washed four times with provided wash buffer and incubated with detection antibody for 1 hour at 37°C. Wells were washed and incubated in HRP-conjugated secondary antibody for 30 minutes at 37°C. Wells were washed and incubated with tetramethylbenzidine substrate for 10 minutes at 37°C, STOP solution was added, and OD_{450} was measured.

Internalization Studies

All scaffolds were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) according to the manufacturer's instructions. Huh-7 cells were harvested using Cellstripper (Cellgro, Manassas, VA) and seeded at 100,000 cells per well in PBS4 (1 mM MgCl, 1 mM $CaCl_2$, 0.2% BSA, 5 mM glucose, and 10% FBS). Compounds were added and cell incubated at 37°C for 0, 15, 30, 60, and 120 minutes. Plates were placed on ice for 5 minutes to stop internalization and washed three times in PBS4. Cells were then incubated in Avidin trifuged, the supernatant was discarded, and cells were solubilized for 30 minutes at 4°C. Total amount of internalized scaffold was measured in supernatant by CVX-2000 anti-idiotype capture binding ELISA followed by incubation with streptavidin-HRP (Fitzgerald Industries).

Statistical Analyses

Data were analyzed either by two-way analysis of variance (ANOVA) with a Bonferroni post-test or by a two-tailed *t* test using Prism (GraphPad Software).

Results

In Vitro *Characterization of Scaffolds*

CVX-2000, $F(ab)_2$, and Fab constructs were conjugated with monomer FGFR4-targeting peptide as described in the Materials and Methods section.

Binding to FGFR4 was measured by ELISA and SPR. The IgG and $F(ab)_2$ constructs bind with an apparent binding affinity of

Table 1. Binding Affinity of IgG, $F(ab)_2$, and Fab Determined by SPR.

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)
Fab monomer	1.1×10^6	9.7×10^{-3}	8.5
Fab homodimer	9.4×10^5	7.1×10^{-3}	7.6
$F(ab)_2$ monomer	1.2×10^6	2.9×10^{-3}	2.5
$F(ab)_2$ homodimer	1.3×10^6	2.9×10^{-3}	2.3
IgG monomer	1.4×10^6	2.4×10^{-3}	1.8

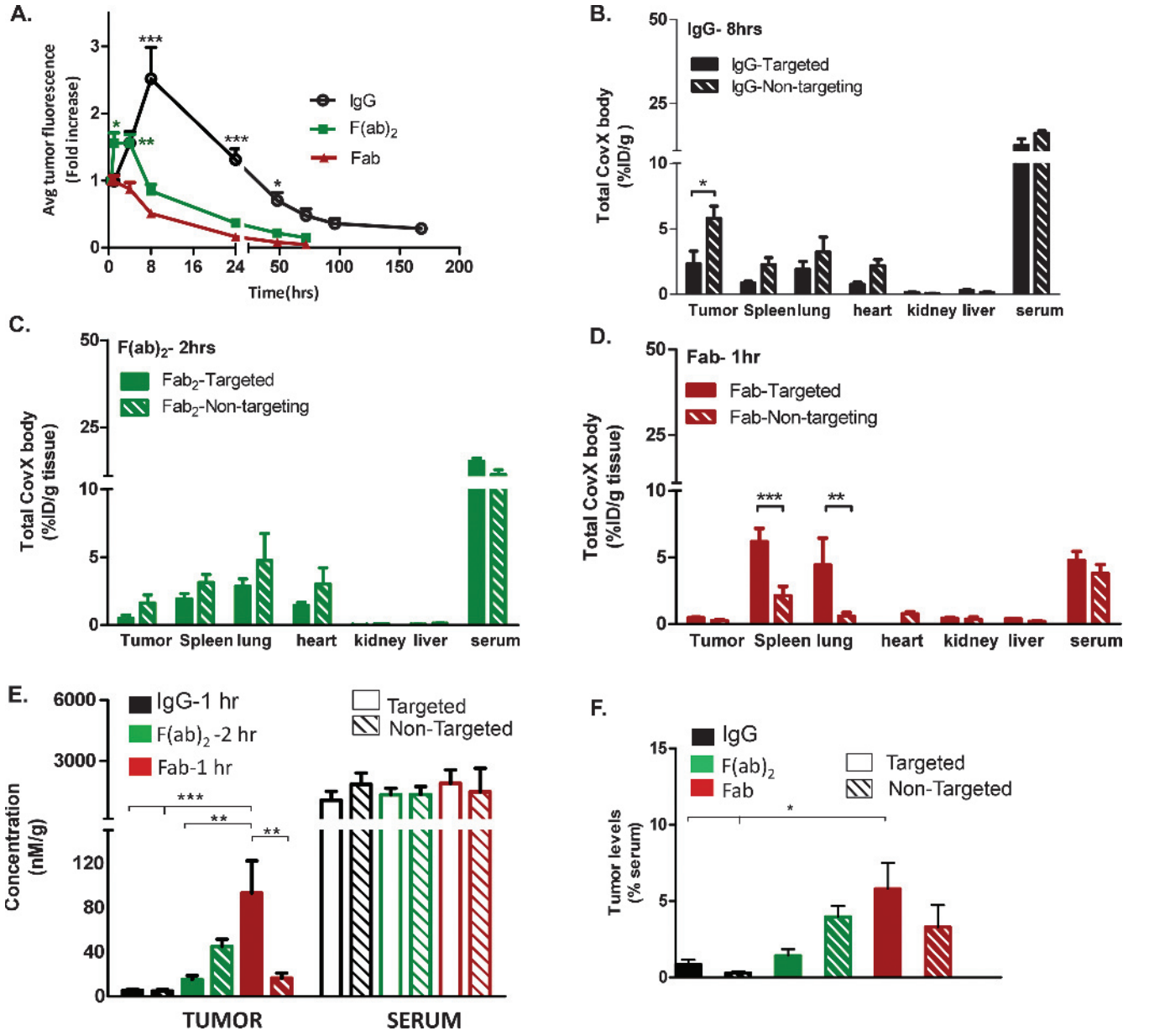

Figure 2. Biodistribution studies. (A) Time-dependent tumor uptake of IgG, $F(ab)_2$, and Fab. *In vivo* optical imaging of near infra-red (NIR)-conjugated constructs. Average signal intensities were quantified using regions of interest (ROIs) from the tumor sites. Data are presented as mean fold increase from initial image capture at 30 minutes ± SEM of eight mice (***$P < .001$, *$P < .05$; IgG *vs* both $F(ab)_2$ and Fab accumulation, *$P < .05$ and **$P < .01$; $F(ab)_2$ *vs* Fab accumulation by two-way ANOVA with Bonferroni post-test). Tumor and normal tissue uptake of (B) IgG 8 hours post dose (*$P < .05$), (C) $F(ab)_2$ 2 hours post dose, and (D) Fab 1 hour post dose (***$P < .001$, **$P < .01$ by two-way ANOVA with Bonferroni post-test). (E) IgG, $F(ab)_2$, and Fab tumor uptakes and serum levels compared at the early time points of 1 hour, 2 hours, and 1 hour, respectively. At this early time point with equivalent serum levels, the targeted Fab shows maximal accumulation levels compared to the $F(ab)_2$ and IgG (***$P < .001$, **$P < .01$ by one-way ANOVA with Bonferroni post-test). (F) Tumor to serum levels further demonstrate that the Fab construct accumulation is significantly higher than the IgG accumulation (*$P < .05$ by two-way ANOVA with Bonferroni post-test).

0.7 and 0.8 nM, respectively, whereas the Fab binds with a binding affinity of 11 nM (Figure 1*A* and Table 1). In a competition ELISA, the IgG and $F(ab)_2$ constructs compete for binding of FGF19 to FGFR4 with an half-maximal inhibitory concentration (IC_{50}) of 9 and 18 nM, respectively, whereas the Fab competes with an IC_{50} of 500 nM (Figure 1*B*).

and $F(ab)_2$ constructs have a cell binding affinity of 9 and 18 nM, respectively, whereas the Fab has a binding affinity of 198 nM (Figure 1*C*).

Removal of the Fc and Reduction in Size Significantly Impact the PK Properties

PK studies were conducted by administering a single i.v. injection of

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