

## Avidity-Mediated Enhancement of *In vivo* Tumor Targeting by Single-Chain Fv Dimers

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**Abstract** Radiolabeled single-chain Fv (sFv) molecules display highly specific tumor retention in the severe combined immunodeficient (SCID) mouse model; however, the absolute quantity of sFv retained in the tumors is diminished by the rapid renal elimination resulting from the small size of the sFv molecules ( $M_r$ , 27,000) and by dissociation of the monovalent sFv from tumor-associated antigen. We previously reported significant improvement in tumor retention without a loss of targeting specificity on converting monovalent sFv into divalent [(sFv)<sub>2</sub>] dimers, linked by a disulfide bond between COOH-terminal cysteinyl peptides engineered into the sFv'. However, our data for enhanced dimer localization in tumors could not distinguish between the contributions of enhanced avidity and increased systemic retention associated with the larger size of 54 kDa [(sFv)<sub>2</sub>] dimers relative to 27-kDa sFv. In this investigation, we have compared tumor targeting of divalent anti-c-erbB-2/HER2/*neu* 741F8-1 (sFv')<sub>2</sub> homodimers with monovalent 741F8/26-10 (sFv')<sub>2</sub> heterodimers ( $M_r$ , 54,000) and 741F8 sFv monomers (741F8 sFv has binding specificity for erbB-2/HER2/*neu* and 26-10 sFv specificity for digoxin and related cardiac glycosides). These studies allowed us to distinguish the dominant effect of valency over molecular weight in accounting for the superior tumor retention of 741F8-1 (sFv')<sub>2</sub> homodimers. Each of the radioiodinated species was administered i.v. to SCID mice bearing SK-OV-3 human tumor xenografts and tumor localization at 24 hours post i.v. injection was determined for <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> (3.57 %ID/g), <sup>125</sup>I-741F8/26-10 (sFv')<sub>2</sub> (1.13 %ID/g), and <sup>125</sup>I-741F8-1 sFv (1.25 %ID/g). These findings substantiate that the improved tumor retention of (sFv')<sub>2</sub> homodimers over sFv monomers results from the availability of dual binding sites rather than from the slower systemic clearance of homodimers.

Antibody engineering permits the design of new antibody-based proteins that can potentially address the shortcomings of intact antibodies as cancer therapeutics (1). One approach has been to produce the minimal antibody binding site in the form of a single-chain Fv (sFv), comprising the heavy and light chain variable domains of an antibody, joined by an appropriate linker peptide (2, 3). These 26- to 27-kDa sFv antibody species

can be engineered from monoclonal antibodies or selected from phage-antibody libraries to obtain monovalent sFv species with high affinity and specificity for a target of choice. We have previously investigated the advantage of targeting tumors with sFv dimers, prepared as disulfide-linked (sFv')<sub>2</sub> (4) or non-covalent diabodies (5), and in both cases dimeric/divalent species showed significant improvement over monomers. This investigation addresses the specific contributions of valence and molecular size to the *in vitro* binding characteristics and *in vivo* tumor targeting of sFv monomers and dimers.

This investigation was based on our preparation of 741F8-1 sFv monomers, dimers, and bispecific heterodimers (4, 6) reactive with the tumor-associated antigen c-erbB-2/HER2/*neu* that is overexpressed in a number of cancers, including those of the breast (7–9), ovaries (10), and gastrointestinal tract (11–13). In mouse models, the small size of these molecules ( $M_r$  ~ 27,000 for monomers and  $M_r$  ~ 54,000 for dimers) facilitates their rapid penetration into tumors (14) and their rapid elimination from blood and all tissues devoid of target antigen (4, 15, 16). We observed significantly greater tumor retention with radioiodinated 741F8-1 (sFv')<sub>2</sub> dimer than with sFv monomer (13). We also report that the dimers exhibited better tumor retention than enzymatically produced 741F8 Fab fragments of similar size. However, the comparison to monovalent Fab fragments was an imperfect control because a Fab fragment has a rigid conformation comprising the Fv region fused to constant domains, which necessarily differ from

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the flexible back-to-back conjugation of two sFv regions within the (sFv)<sub>2</sub>. The present studies were designed to conclusively examine how size and valence affect the tumor targeting properties of engineered sFv monomers and dimers.

We previously developed the sFv with cysteinyl residues in its COOH-terminal tail, designated sFv' (4, 16, 17), and constructed divalent disulfide-bonded 741F8-1 (sFv')<sub>2</sub> molecules, heterodimeric disulfide-bonded 741F8-1/26-10-1 (sFv')<sub>2</sub> molecules (monovalent for HER2/*neu*), and control 26-10-1 (sFv')<sub>2</sub> molecules, where the designations 741F8-1 and 26-10-1 refer to a COOH-terminal (Ser-Gly<sub>4</sub>-Cys) tail, with all molecules containing a 14-residue serine-rich linker (-Ser<sub>4</sub>-Gly-Ser<sub>4</sub>-Gly-Ser<sub>4</sub>) bridging the variable domains of the sFv' constructs (refs. 6, 17; Table 1). The Gly<sub>4</sub>-Cys tail used for making covalent back-to-back (sFv')<sub>2</sub> dimers was also shown to be useful as the first genetically engineered chelation site for <sup>99m</sup>Tc. Gamma camera imaging studies were conducted on mice treated with the 741F8-1 sFv'- [<sup>99m</sup>Tc] and displayed clear tumor localization at times as early as 6 hours following i.v. administration to the same mouse tumor model used in this investigation. The Gly<sub>4</sub>-Cys peptide formed a square planar chelate with oxotechnetium using a standard coordination kit with radiometal in the form that is routinely available in radiology departments (18). The 741F8-1 (sFv')<sub>2</sub> molecule employed here was also conjugated to the γ-emitting radioisotope <sup>111</sup>In and effectively employed to image established c-erbB-2-overexpressing human tumor xenografts growing in immunodeficient mice (19).

In the present study, we have characterized the (sFv')<sub>2</sub> species with respect to their binding affinity for recombinant, purified c-erbB-2/HER2/*neu* extracellular domain, their cell-surface retention on SK-OV-3 cells, and *in vivo* tumor localization in human tumor xenograft models in SCID mice. The present results show that there is a dominant contribution of the (sFv')<sub>2</sub> divalency to the enhanced binding of 741F8-1 (sFv')<sub>2</sub> to HER2/*neu* whereas there is a negligible effect of size on the tumor localization of monovalent sFv' monomers and monovalent (sFv')<sub>2</sub> heterodimers.

## Materials and Methods

**sFv and extracellular domain production.** The 741F8-1 sFv', which is specific for the tumor-associated antigen c-erbB-2 (HER2/*neu*), and the 26-10-1 sFv', which recognizes digoxin and related cardiac glycosides,

such as ouabain, were produced with a COOH-terminal Gly<sub>4</sub>-Cys peptide as described (6, 17, 20). The sFv constructs employed in this study are described in Table 1. These proteins were expressed in *Escherichia coli* as cytoplasmic inclusion bodies that were denatured in guanidine HCl, refolded, and purified as described (20). 741F8-1 (sFv')<sub>2</sub> homodimers, 741F8-1/26-10-1 (sFv')<sub>2</sub> heterodimers, and 26-10-1 (sFv')<sub>2</sub> homodimers were produced as described (6). Briefly, 741F8-1 (sFv')<sub>2</sub> and 26-10-1 (sFv')<sub>2</sub> homodimers were produced through a disulfide-linkage between cysteine residues on the COOH-terminal tail of each sFv' by air oxidation following dialysis removal of the reducing agent (2 mmol/L DTT). 741F8-1/26-10-1 (sFv')<sub>2</sub> heterodimers were produced following the procedure that Brennan et al. (21) employed to prepare heterobispecific F(ab')<sub>2</sub>. Free sulfhydryls on 26-10-1 sFv' were blocked with thionitrobenzoic acid, which facilitated a nucleophilic attack by the COOH-terminal cysteine of reduced 741F8-1 sFv'. Typical final yields following refolding, purification, and dimerization, based on a liter of fermented cells, were 87 mg/L for the 741F8-1 sFv' monomer, 15 mg/L for the 741F8-1 (sFv')<sub>2</sub> homodimer, and 87 mg/L for the 26-10-1 (sFv')<sub>2</sub> homodimer. Production of the 741F8-1/26-10-1 (sFv')<sub>2</sub> heterodimer typically resulted in an overall yield of 40% (mg heterodimer / mg reactants × 100).

The extracellular domain, with a COOH-terminal hexahistidine purification tag, was produced by mammalian cell culture and purified to homogeneity from the extracellular medium in two steps, by immobilized metal affinity chromatography and by size exclusion chromatography, as previously described (4).

**Affinity and kinetic measurements.** The binding and dissociation kinetics of extracellular domain association with 741F8-1 sFv', 741F8-1 (sFv')<sub>2</sub>, 26-10-1 (sFv')<sub>2</sub>, and 741F8-1/26-10-1 (sFv')<sub>2</sub> were determined using a BIACore surface plasmon resonance instrument (Pharmacia, Uppsala, Sweden) as described (22). Briefly, 1,400 RU of HER2/*neu* extracellular domain (25 μg/mL in 10 mmol/L sodium acetate, pH 4.5) were covalently bound through amino groups to a CM5 sensor chip. Association and dissociation of the sFv species (100-800 nmol/L) were measured under continuous flow (5 μL/min). The determination of *k*<sub>on</sub> was done by plotting (ln(dR / dt)) / t versus concentration whereas *k*<sub>off</sub> was determined from the dissociation part of the sensogram at the highest sFv concentration analyzed. Based on these kinetic measurements, the dissociation constant, *K*<sub>d</sub>, and association constant, *K*<sub>a</sub>, were calculated.

**Radioiodinations.** All samples were labeled with <sup>125</sup>I using the chloramine-T method (4). The sFv or (sFv')<sub>2</sub> (200 μg) was combined with <sup>125</sup>I (17.4 mCi/μg; NEZ 033H, Dupont NEN, Wilmington, DE) at an iodine-to-protein molar ratio of 1:6 in a 12 × 75-mm plastic test tube. Ten microliters (1 mg/mL) of chloramine-T (Sigma, St. Louis, MO) per 100 μg of protein were added and the reaction was allowed to proceed for 3 minutes at room temperature. The reaction was quenched with the addition of 10 μL (1 mg/mL) of sodium metabisulfite (Sigma) per 100 μg of protein. The radiolabeled protein was immediately separated from remaining free radioiodine by the G-50-80 centrifuged column method (4). The specific activities of labeled products were ~2 mCi/mg.

**Table 1.** Details of sFv' monomer and (sFv')<sub>2</sub> dimer sequences used and their designations

sFv	Valence	Construct layout (tail or linkage)	Reference
741F8-1 sFv'	1	(741F8 sFv)-GGGGC	(6, 20)
26-10-1 sFv'	1	(26-10 sFv)-SGGGGC	(6, 20)
741F8-1 (sFv') <sub>2</sub>	2	(741F8 sFv)-GGGGCCGGGG-(741F8 sFv)	(6, 20)
26-10-1 (sFv') <sub>2</sub>	2	(26-10 sFv)-SGGGGCCGGGG-(26-10 sFv)	(6, 20)
741F8-1/26-10-1 (sFv') <sub>2</sub>	2*	(741F8 sFv)-GGGGCCGGGG-(26-10 sFv)	(6)

\*The 741F8-1/26-10-1 (sFv')<sub>2</sub> has a valence of one for each antigen.

**Table 2.** Affinity constants of the sFv species determined on a BIAcore instrument

Construct	Valence for HER2/ <i>neu</i>	Concentration (nmol/L)	$K_d$ (mol/L)	$k_{on}$ (mol/L/s)	$k_{off}$ ( $s^{-1}$ )
741F8-1 (sFv) <sub>2</sub>	Divalent	400	$9.2 \times 10^{-9}$	$3.7 \times 10^5$	$3.4 \times 10^{-3}$
741F8/26-10 heterodimer	Monovalent	800	$3.4 \times 10^{-8}$	$7.6 \times 10^4$	$2.6 \times 10^{-3}$
741F8-1 sFv'	Monovalent	800	$4.8 \times 10^{-8}$	$2.0 \times 10^5$	$9.5 \times 10^{-3}$
26-10 (sFv) <sub>2</sub>	None	400	—*	—	—
26-10 sFv'	None	800	—	—	—

NOTE: Binding properties of 741F8-1 sFv and 26-10-1 sFv derived species were determined using a BIAcore with 1,400 RU of c-erbB-2/HER2/*neu* extracellular domain bound to a CM5 sensor chip as described in the text.  
\*No binding observed.

The quality of the radiolabeled sFv was evaluated by high-performance liquid chromatography, SDS-PAGE, and a live cell binding assay (4). High-performance liquid chromatography analysis was done using a Superdex-75 column (Pharmacia) on a Rainin Dynamax High-Performance Liquid Chromatography system. Eluted fractions (0.25 mL) were collected and counted on a gamma well counter (Beckman, Irvine, CA). High-performance liquid chromatography consistently showed that >99% of the eluted activity was associated with the protein peak. The radioiodinated sFv monomers and dimers were also evaluated by SDS-PAGE. Nonreduced forms were run on 12% gels (10 × 10 cm; Jule, New Haven, CT) and the migration of the proteins was detected by autoradiography (Kodak X-OMAT scientific imaging film with X-omatic regular intensifying screens; Rochester, NY). Of the nonreduced dimers, >95% migrated as  $M_r \sim 54,000$  bands whereas a similar percentage of the 741F8-1 sFv' monomer migrated as  $M_r \sim 27,000$  bands.

The immunoreactivity of the radioiodinated 741F8-based sFv species was determined in a live cell-binding assay using SK-OV-3 cells (HBT 77; American Type Culture Collection, Rockville, MD) that overexpress HER2/*neu*. Ten nanograms of labeled sFv in 100  $\mu$ L of PBS (0.154 mol/L NaCl, 10 mmol/L phosphate, pH 7.2) were added in triplicate to  $5 \times 10^6$  cells in 15-mL polypropylene centrifuge tubes. After incubating for 30 minutes at room temperature, the cells were washed with 2.0 mL of PBS and centrifuged for 5 minutes at  $500 \times g$ . The supernatants were separated from the pellets and both were counted in a gamma counter. The percentage of the activity associated with the cell pellets was usually >70% (>50% for the heterodimer in one assay). The radioiodinated 26-10-1 (sFv)<sub>2</sub> did not bind significantly to the cells.

**Cell-surface retention studies.** To assess the effect of sFv valence on association with cell-bound HER2/*neu*, an *in vitro* cell-surface retention assay was done. In this assay, 460 ng of radioiodinated 741F8-1 sFv, 741F8-1 (sFv)<sub>2</sub> homodimer, or 741F8/26-10 (sFv)<sub>2</sub> heterodimer were incubated with  $11.5 \times 10^6$  SK-OV-3 cells in a total volume of 4.6 mL of PBS (for a final concentration of 1.9 nmol/L for the sFv and 3.7 nmol/L for the homodimers and heterodimers) for 30 minutes on ice. These concentrations are approximately the  $K_d$  of the individual 741F8 sFv complexed to extracellular domain. The cells treated with the various sFv species were centrifuged at  $500 \times g$  for 5 minutes at 4°C, washed with 10 mL of ice-cold PBS twice, and then resuspended gently in 4.6 mL of PBS at 37°C. The cell suspensions were then incubated at 37°C in a rotating water bath. To decrease the rebinding of dissociated radioiodinated sFv species to the cells, the suspensions were pelleted at  $500 \times g$  for 5, 15, and 30 minutes after commencing the incubation; the supernatants were aspirated and the cells were gently resuspended in the same volume of fresh PBS at 37°C. Immediately after each round of pelleting and resuspension, 0.2-mL aliquots containing  $5 \times 10^5$  cells were removed in triplicate (i.e., at 0, 5, 15, and 30 minutes) and immediately centrifuged at  $500 \times g$  for 5 minutes at 4°C. Cell pellets and supernatants were counted in a gamma well counter (Beckman) to determine the quantity of radiolabeled species retained on the cells over time.

**Biodistribution studies.** Animal studies were done in accordance with institutional animal welfare guidelines. Four- to six-week-old C.B17/Icr-*scid* mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. SK-OV-3 cells ( $2.5 \times 10^6$ ) were implanted s.c. on the abdomens of the mice. Six to eight weeks later, when the tumors had achieved a size of ~100 to 200 mg, Lugol's solution was added to the drinking water to block thyroid accumulation of radioiodine. The biodistribution studies were initiated 3 days later.

Radiolabeled sFv preparations were diluted in PBS to a concentration of 200  $\mu$ g/mL and cohorts of five to six mice were given 100  $\mu$ L of each radiopharmaceutical by tail vein injection. The injected dose was determined by counting each mouse immediately after injection on a Series 30 multichannel analyzer/probe system (Probe model 2007, Canaberra, Meridian, CT). Whole body counts and blood samples (retro-orbital) were obtained and the mice were sacrificed at 24 hours following the injections. The tumors and organs were removed, weighed, and counted in a gamma counter along with the blood samples and standards to determine the percent injected dose per gram of tissue (%ID/g; refs. 4, 23). The mean and SE for each group of data were calculated, tumor-to-organ ratios were determined, and the significance of the results was determined by Student's *t* test.

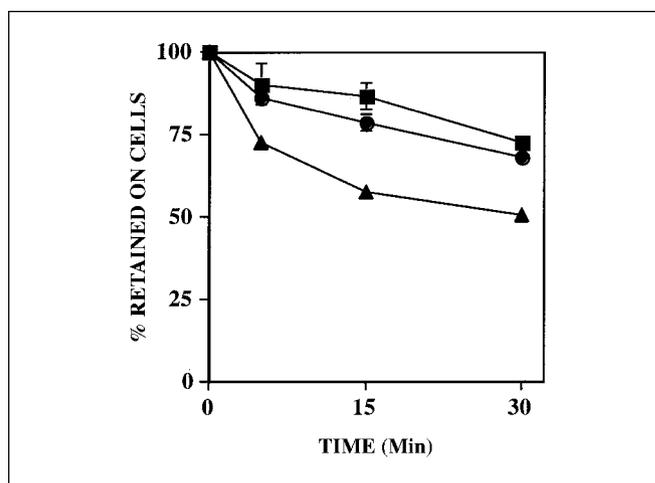
## Results

**Surface plasmon resonance extracellular domain-binding measurements.** The binding of the sFv species to HER2/*neu* extracellular domain was evaluated by surface plasmon resonance measurements with extracellular domain immobilized to methylcarboxylated dextran chip surfaces in the BIAcore. The binding kinetics were determined using concentrations of 100 to 800 nmol/L of sFv as presented in Table 2. The highest extracellular domain affinity was measured for 741F8-1 (sFv)<sub>2</sub>, which was found to have a  $K_d$  for its complex with HER2/*neu* extracellular domain of  $9.2 \times 10^{-9}$  mol/L. Whereas the  $K_d$  of the 741F8/26-10 (sFv)<sub>2</sub> for HER2/*neu* ( $3.5 \times 10^{-8}$  mol/L) reflected a lower affinity than was observed for the 741F8-1 (sFv)<sub>2</sub> dimer, it was the same within experimental error as that exhibited by the 741F8-1 sFv' monomer ( $4.8 \times 10^{-8}$  mol/L). Thermodynamic considerations cause the first binding site of a divalent homodimer and antigen to associate with twice the intrinsic association constant,  $K_{av}$ , for a monovalent binding species whereas the second site binds with  $0.5K_a$ . The BIAcore analysis allows apparent affinities of the 741F8 species for extracellular domain to be calculated: homodimer  $K_{a,app} = 1.09 \times 10^8$

(mol/L)<sup>-1</sup>, heterodimer  $K_{a,app} = 0.3 \times 10^8$  (mol/L)<sup>-1</sup>, and sFv monomer  $K_{a,app} = 0.2 \times 10^8$  (mol/L)<sup>-1</sup>. The deviations from thermodynamic predictions for these binding species may result from technical limitations and experimental errors of the BIAcore methodology. Another source for the deviation between  $K_{a,app}$  for monomer and heterodimer may be related to the observation that (sFv)<sub>2</sub> dimers are structurally more stable than sFv monomers based on unfolding and refolding studies of 26-10 species.<sup>7</sup> The 26-10 (sFv)<sub>2</sub> negative control did not bind the HER2/*neu* on the BIAcore chip.

**Cell-surface retention studies.** Following incubations of radioiodinated sFv (4 nmol/L of monomer or 2 nmol/L of homodimer or heterodimer) with human SK-OV-3 cells overexpressing the HER2/*neu* target antigen, the rate of dissociation of sFv species from the surface of cells was determined. As presented in Fig. 1, the 741F8-1 (sFv)<sub>2</sub> homodimer and 741F8-26-10 (sFv)<sub>2</sub> heterodimer exhibited similarly prolonged dissociation rates from the SK-OV-3 cells compared with the more rapidly dissociating 741F8-1 monomer. The behavior of sFv monomer is consistent with its faster dissociation rate,  $k_{off}$ , in BIAcore analysis. However, the basis of similar dissociation rates for the homodimer and the heterodimer is not predicted from their intrinsic binding properties or target localization *in vivo*. Possible contributing factors may include the formation on cell surfaces of 741F8-26-10 (sFv)<sub>2</sub> tetramers (dimers of the heterodimer), which were observed to form in equilibrium at moderate concentrations in solution with this molecule (4), or it may reflect the diffusion rates of heterodimer and homodimer away from the cell surface, which would be slower than for sFv monomers and would favor rebinding of dimers.

**Biodistribution studies.** Twenty-four-hour biodistribution studies were done in SCID mice bearing s.c. human SK-OV-3 tumor xenografts to determine the effect of valence on tumor retention. The results from an experiment representative of two such studies are presented in Table 3 and Fig. 2. In these studies, we observed that significantly greater tumor retention was associated with the divalent <sup>125</sup>I-741F8-1 (sFv)<sub>2</sub> than with the monovalent heterodimeric <sup>125</sup>I-741F8/26-10 (sFv)<sub>2</sub> (3.57 versus 1.13 %ID/g, respectively;  $P = 0.0264$ ). Furthermore, the tumor retention of the monovalent heterodimeric <sup>125</sup>I-741F8/26-10 (sFv)<sub>2</sub> was very similar to that of the monomeric <sup>125</sup>I-741F8-1 sFv (1.13 versus 1.25 %ID/g, respectively) and significantly less than that of the <sup>125</sup>I-741F8-1 (sFv)<sub>2</sub> (3.57 %ID/g,  $P = 0.0153$ ). Finally, the tumor retention of the anti-digoxin <sup>125</sup>I-26-10 (sFv)<sub>2</sub> (representing nonspecific retention, digoxin being an irrelevant antigen in this model) was 0.36 %ID/g, significantly less than that of the anti-HER2/*neu* extracellular domain (sFv)<sub>2</sub> species. The tumor-to-organ ratios of the divalent 741F8-1 (sFv)<sub>2</sub> were superior in all organs to those observed with either the monomer or the heterodimer, indicating increased specificity of tumor retention when two binding sites were present for HER2/*neu* (Table 3). For example, the 741F8-1 (sFv)<sub>2</sub> homodimer exhibited tumor/blood ratios of 19:1 at 24 hours postinjection that were greater than the tumor/blood ratios of 7.3:1 and 10.6:1 observed with the heterodimer and monomer, respectively, which are the same within experimental error.



**Fig. 1.** Retention of radioiodinated 741F8-1 (sFv)<sub>2</sub> (■), 741F8/26-10 (sFv)<sub>2</sub> (●), and 741F8-1 sFv (▲) on the surface of SK-OV-3 cells overexpressing c-erbB-2/HER2/*neu*. Assays were done as described in the text. Points, mean of three samples; bars, SE.

Given the similar blood levels of the 26-10 homodimer, heterodimer, and 741F8 monomer, these results are consistent with the *i.v.* administered 741F8-1/26-10 (sFv)<sub>2</sub> existing predominantly as nonassociated, monovalent heterodimers. If they existed as aggregates, their larger size would exceed the threshold for first pass renal elimination or elicit enhanced elimination by organs of the reticuloendothelial system, leading to altered retention in the blood that would be apparent at the observed 24-hour time point. The similar blood levels for all three constructs suggests that the greater tumor retention observed with the divalent homodimers was not due to the increased blood retention of the dimer as compared with the monomer, but that dual binding sites present in the homodimer provide a competitive advantage over the monomeric sFv. These results suggest that the dual combining sites in the homodimer configuration provide a greater chance for tumor binding and rebinding, which enhances tumor retention as a function of time when compared with the monovalent 741F8 species and the nonbinding control. Despite the propensity for self-association of the 741F8 sFv (a monomer-dimer equilibrium)<sup>8</sup> and the related dimerization of the 741F8-1/26-10 (sFv)<sub>2</sub> that we previously detailed (6), all the current *in vivo* data suggest that this equilibrium is significantly modulated by dilution into the blood of tumor-bearing SCID mice, thus acquiring the properties of a monovalent, nonaggregated heterodimer. If the association into noncovalent heterotetramers were dominant, the level of binding should be at least as great as that exhibited by homodimer instead of less than that of the 741F8-1 sFv.

## Discussion

The localization of antitumor antibodies in tumors expressing their cognate antigen is dictated by the accessibility of the antibody to the tumor antigen, the affinity of the antibody binding sites for target antigen, and the avidity of the divalent

<sup>7</sup> M-S. Tai, H. Oppermann, J.S. Huston, unpublished results.

<sup>8</sup> W.F. Stafford, unpublished results.

**Table 3.** Twenty-four hour biodistribution study

Organs	741F8-1 (sFv') <sub>2</sub>		741F8/26-10 (sFv') <sub>2</sub>		741F8-1 sFv'		26-10 (sFv') <sub>2</sub>	
	% ID/g	T/O	% ID/g	T/O	% ID/g	T/O	% ID/g	T/O
Tumor	3.57	1.00	1.13	1.00	1.25	1.00	0.22	1.00
Blood	0.28	19.09	0.15	7.25	0.13	10.62	0.15	1.49
Bone	0.08	65.09	0.06	19.13	0.05	27.89	0.06	3.85
Heart	0.24*	31.25	0.07	16.36	0.07	19.46	0.07	3.20
Intestine	0.36*	18.42	0.11	14.56	0.18	7.75	0.16	1.61
Kidneys	0.38	10.81	0.64	1.79	0.45	2.79	0.17	1.30
Liver	0.23	23.36	0.21	5.69	0.13	10.55	0.08	3.01
Lung	0.23	19.77	0.24	4.88	0.18	9.20	0.15	1.86
Muscle	0.10	89.13	0.04*	51.10	0.03	54.42	0.03	7.43
Spleen	0.32	18.27	0.32	3.56	0.16	9.15	0.19	1.33
Stomach	0.98	10.40	0.35	3.74	0.55	3.52	0.47	0.56

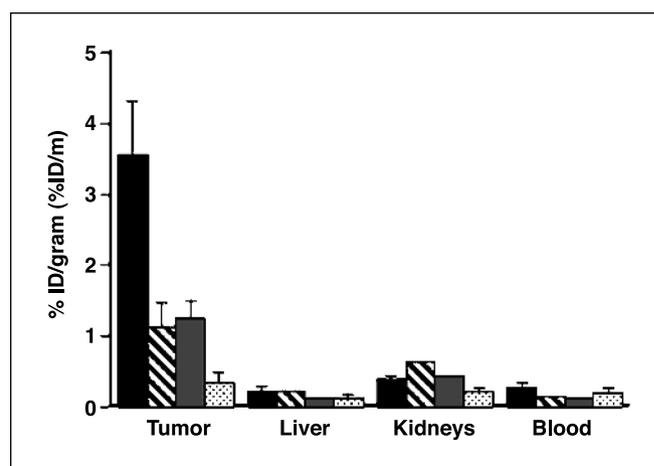
NOTE: Twenty micrograms of <sup>125</sup>I-sFv were injected into human SK-OV-3 tumor – bearing SCID mice as described in the text. Cohorts of six mice were sacrificed in each group [five in the 26-10 (sFv')<sub>2</sub> group] and the mean %ID/g of tissue and corresponding tumor-to-organ ratios are presented. Unless otherwise indicated, the SE was <30% of the corresponding value.  
Abbreviation: T/O, tumor-to-organ ratio.  
\*SE ≤ 33%.

antibody for target antigen within the complex three-dimensional epitope space of a tumor. Accessibility is dependent on a number of factors, including the size of the antibody-based molecule, which affects its rate of tumor penetration (24), and systemic concentration, which is dependent on the dose administered and the rate of clearance (16). The physiologic properties of the tumor, such as its size, degree of necrosis, vascularity, and antigen expression level, also affect the ability of the antibody to access the tumor (25). We used a previously validated dose of 20 μg of antibody in the setting of small, rapidly growing tumors in an attempt to control these variables. The 20-μg dose was selected as it was previously extensively employed in the examination of the *in vivo* targeting properties of the 741F8 (sFv')<sub>2</sub> homodimer (4, 26, 27). We and others have found that increasing or decreasing the dose of divalent sFv molecules 10-fold from the dose employed here does not significantly alter the tumor targeting specificity or the quantity localized in tumor and normal organs, determined as the percentage of the injected dose localized per gram of tissue (27, 28).

The association constant of an antibody for its target antigen is a thermodynamic measure of the stability of the antigen-antibody complex. Finally, the avidity of the interaction is dependent on the intrinsic affinity of single binding sites, the number of binding sites present per molecule (valence), and the mobility or density of the target antigen on tumor cells as well as their shape (29). From the thermodynamic viewpoint, Jacqueline Reynolds has developed theoretical analyses for the different scenarios governing the interaction of monovalent or divalent antibody with mobile cell-surface antigens (29). Of particular importance for this study, it should be noted that a monovalent antibody fragment in solution has the same simple binding equation for association with a cell-bound monomeric antigen as it does if the monomeric antigen is in solution. Thus, a monovalent 741F8 sFv and corresponding monovalent 741F8/26-10 (sFv')<sub>2</sub> heterodimer should have the same

affinity for the monomeric HER2/*neu* antigen on an idealized cell surface. If one takes the BIAcore chip as a model for such a simplified system, these two species were experimentally observed to have the same binding affinity within experimental error. However, the surfaces of cells in tissue culture present a sufficiently nonideal system that this generalization starts to break down when measured in terms of cell-surface retention as a function of time (Fig. 2).

When a divalent antibody binds to a soluble, monomeric antigen, its first interaction has an effective microscopic binding constant,  $k_1$ , which is  $2K_a$ , where  $K_a$  is the association of the monovalent binding site. The second site binds with  $k_2$ , which is  $0.5K_a$ . When the divalent antibody species binds to monomeric antigen on a cell surface, the equations become



**Fig. 2.** Comparative tumor retention of <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> homodimer or <sup>125</sup>I-741F8/26-10 (sFv')<sub>2</sub> heterodimer and sFv molecules with varying degrees of valence for binding to c-erbB-2/HER2/*neu*. Groups of six mice were sacrificed at 24 hours following the i.v. administration of 20 μg of radioiodinated 741F8-1 (sFv')<sub>2</sub> (■), 741F8/26-10 (sFv')<sub>2</sub> (▨), 741F8-1 sFv' (▩), or 26-10 (sFv')<sub>2</sub> (□). The %ID/g retained in tumor was calculated as described in the text. Columns, mean values; bars, SE.

quite complex and various assumptions must be made, including antigen density and the radius of curvature of the cell surface, which preclude a rigorous interpretation of the antigen-antibody association in tumor xenograft models. Analysis of tumor localization of a monovalent antibody species in serum may be considered from a simplified viewpoint, with the caveat that one is ignoring many complexities.

The three-dimensionality of the antigen matrix that constitutes tumor epitope space in a solid tumor imposes additional boundary conditions that lead to a reduced diffusion rate of antibody caused by the entrapment of antibody species within the interstices of cells that constitute a solid tumor. Furthermore, the slower diffusion out of solid tumors results in a markedly increased probability of rebinding if the antibody species is divalent. There are also physiologic factors that reduce the localization of divalent antibody species in solid tumors, including antigen density, the heterogeneity of antigen localization within the tumor, and intratumoral interstitial pressure (25, 30, 31). Overall, the binding of divalent antibody species within a solid tumor must be significantly higher than to the surface of cells dispersed in solution. Of course, the Law of Mass Action always governs binding phenomena; thus, increasing the sFv or (sFv)<sub>2</sub> concentrations will increase their localization within tumors until all available antigenic sites are saturated or some other binding site effect becomes limiting (25, 30, 32).

We examined the tumor-targeting properties of three divalent (sFv)<sub>2</sub> molecules with zero, one, or two binding sites specific for the targeted tumor antigen. As all three molecules were of the same covalent size, we could focus on differences in tumor retention that result from the number of specific binding sites present. In these studies, 3-fold greater tumor retention was achieved with the divalent <sup>125</sup>I-741F8-1 (sFv)<sub>2</sub> than with either the 741F8/26-10 heterodimer or the 741F8-1 sFv monomer, both of which have monovalent binding sites for c-erbB-2/HER2/neu (3.6 %ID/g versus 1.13 and 1.25 %ID/g, respectively). Furthermore, observed differences in their tumor retention were not due to differential clearance and processing as the blood and normal organ retentions of the three species with molecular weights of  $M_r \sim 54,000$  [741F8-1 (sFv)<sub>2</sub> homodimer, the 741F8/26-10 (sFv)<sub>2</sub> heterodimer, and the 26-10 (sFv)<sub>2</sub> control] were very similar. Clearly, increased tumor localization of the species that have monovalent binding sites for c-erbB-2 can be achieved by increasing the dose of antibody administered. However, increased tumor localization achieved in this manner would also be associated with increased localization to normal tissues (27). Our observations that the degree of tumor retention at 24 hours following administration correlated with valence, rather than with molecular weight, represents the first fully controlled analysis of how binding site valence directly affects the sFv targeting and tumor localization.

When examined by BIAcore surface plasmon resonance, the binding of the different anti-c-erbB-2/HER2/neu species to immobilized HER2/neu extracellular domain paralleled the *in vivo* setting. The divalent 741F8-1 (sFv)<sub>2</sub> bound with the greatest affinity,  $K_{a,app} = 1.09 \times 10^8$  (mol/L)<sup>-1</sup> ( $K_d = 9.2 \times 10^{-9}$ ); the monovalent species bound with very similar affinities, 741F8-1 sFv  $K_{a,app} = 0.2 \times 10^8$  (mol/L)<sup>-1</sup> and 741F8/26-10 heterodimer  $K_{a,app} = 0.3 \times 10^8$  (mol/L)<sup>-1</sup> ( $K_d =$

$4.8 \times 10^{-8}$  and  $3.5 \times 10^{-8}$ , respectively); whereas the 26-10 sFv and 26-10 (sFv)<sub>2</sub> negative controls failed to bind to the extracellular domain in BIAcore experiments. The greater effective affinity of the divalent 741F8-1 (sFv)<sub>2</sub>, compared with the monovalent binding of sFv and heterodimer, results from avidity effects rather than improved intrinsic affinity of a single binding site, but suggests contributions from both sites would be consistent with the observed level of binding enhancement. In contrast with the BIAcore results, the *in vitro* cell-surface retention study revealed similar retention patterns for the homodimeric 741F8-1 (sFv)<sub>2</sub> and heterodimeric 741F8/26-10 (sFv)<sub>2</sub>, both greater than 741F8-1 sFv monomer. These results are consistent with our previously published observations on the production of monomeric and dimeric sFv species (4, 6, 33). When examined in solution at a low concentration, 741F8-1 (sFv)<sub>2</sub> was capable of binding a maximum of two HER2/neu extracellular domain molecules, and each 741F8/26-10 homodimer and 741F8-1 sFv monomer bound a maximum of one HER2/neu extracellular domain. Size exclusion chromatography at high concentrations of the 741F8-26-10 (sFv)<sub>2</sub> heterodimer formed tetramers, each capable of binding to two HER2/neu extracellular domain molecules. Our *in vivo* studies were conducted well below the threshold for dimerization; the 20 μg i.v. injection of 741F8/26-10 (sFv)<sub>2</sub> was diluted into an ~2-mL blood volume of a mouse, which rapidly resulted in an upper limit blood concentration of 200 nmol/L. Whereas we have observed very similar tumor retention for 741F8-1 sFv and monovalent 741F8/26-10 heterodimer, both decrease beneath the renal threshold and are cleared very rapidly from circulation. This reinforces the conclusion that the heterodimer did not form tetramers *in vivo* and could only associate with one HER2/neu molecule on the tumor cell surface.

The importance of the rapid elimination of the molecules in evaluating the effect of avidity on tumor retention is illustrated by a similar study done by Van Dijk et al. (30) in which divalent and bispecific F(ab')<sub>2</sub>, which exceeds the renal threshold, and monovalent Fab, which is beneath the renal threshold, were compared in tumor-bearing mice. In their study, the divalent and bispecific F(ab')<sub>2</sub> species had similar tumor retentions at 6, 24, and 48 hours postadministration whereas the monovalent Fab consistently exhibited 3- to 5-fold lower accumulation. These results support our findings that binding avidity has a substantial effect on the tumor retention of small rapidly cleared antibody-based molecules, and it is a factor that can now be considered to be distinct from size below the renal threshold as well as a function of molecular size and clearance rate. It is likely that through the use of flexible, divalent, high-affinity engineered antibody species, such as the (sFv)<sub>2</sub>, the therapeutic potential of this class of sFv-based molecules may be realized.

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