

# Enhanced Tumor Specificity of 741F8-1 (sFv')<sub>2</sub>, an Anti-c-erbB-2 Single-Chain Fv Dimer, Mediated by Stable Radioiodine Conjugation

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The goal of this study was to determine if the stabilization of the radioiodine-protein bond by the *N*-succinimidyl *p*-iodobenzoate (PIB) method improved the degree and specificity of tumor localization of <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub>, an anti-c-erbB-2 sFv dimer, in an immunodeficient murine model. **Methods:** Gamma camera images were acquired 21 hr after intravenous administration of <sup>131</sup>I-741F8-1 (sFv')<sub>2</sub> labeled by the *p*-iodobenzoate or chloramine T methods. The stability of the radioiodine-protein bond also was assessed in plasma samples after intravenous injection of <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> labeled by either the chloramine T or *p*-iodobenzoate methods. **Results:** By 6 hr postinjection, 97% of the activity associated with the <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> labeled by the *p*-iodobenzoate method was protein bound compared with 61% after labeling with the chloramine-T method. These observations indicate that increasing the stability of the conjugation between the radioiodine and the sFv molecule can significantly increase the degree and specificity of tumor targeting. Significantly greater tumor retention ( $p < 0.005$ ) and lower blood ( $p < 0.001$ ), spleen ( $p < 0.001$ ) and stomach ( $p < 0.005$ ) retention were observed in biodistribution studies when the *p*-iodobenzoate conjugate was used. This resulted in superior tumor-to-organ ratios for all tissue samples studied. **Conclusion:** These observations may have clinical relevance for the use of radiolabeled sFv as imaging agents.

**Key Words:** single-chain Fv molecules; radioiodination, *p*-iodobenzoate; c-erbB-2; tumor localization

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**A**ntibody engineering has renewed interest in the field of radioimmunotargeting by allowing investigators to address a variety of the shortcomings experienced with murine monoclonal antibodies (MAb). Immunoconjugate immunogenicity can now be modified by the production of chimeric (1,2) and humanized antibodies (3). Multiple an-

tigens may be targeted by a single immunoglobulin-based molecule through the use of novel bispecific antibodies (4). The affect of affinity, avidity and size on specific tumor targeting and penetration may be examined conclusively by manipulating the molecular structure of the antibody-derived proteins. Twenty-five kilodalton single-chain Fv molecules (sFv), composed of the variable light and variable heavy domains of an immunoglobulin molecule joined together by an amino-acid spacer sequence, provide one example of engineered immunoglobulin-based molecules (5,6). They retain the targeting specificity of immunoglobulins, but are only one-sixth the size of the parent molecules.

Monovalent anti-tumor sFv molecules exhibit highly specific tumor-targeting in immunodeficient mice bearing relevant subcutaneous tumor xenografts (7-9). Furthermore, this retention is antigen-specific, rather than a result of extravascular pooling (9). The small size of sFv allows for a greater degree of tumor penetration than that observed with IgG (10). They display a rapid systemic clearance, leading to highly specific tumor targeting as early as 4 hr postintravenous administration. By 24 hr postinjection, the specific tumor retention of sFv molecules is associated with negligible normal organ and blood retention, resulting in the ability to visualize small (1-2 cm) tumors by gamma scintigraphy when the molecules are labeled with <sup>131</sup>I (8,9). This rapid clearance from the circulation, however, leads to decreasing immunoconjugate concentrations in tumor, which may lessen the therapeutic potential of these reagents.

A number of strategies to optimize the tumor-specific retention of radiolabeled sFv have been pursued. We have developed divalent sFv species that are retained in tumor to a greater extent than their monovalent subunits without a loss of targeting specificity (9). We also have examined a number of administration strategies and have found that a rapid intravenous injection is the preferred administration method. A 72-hr subcutaneous infusion resulted in a low degree of tumor retention and a loss of targeting specificity (11). In contrast, the use of escalating doses and repetitive intravenous bolus injections increased the absolute reten-

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tion of labeled sFv in tumor (12). The choice of radioisotope and the methodology of its conjugation to the sFv are also significant. Schott et al. have found that radioiodinated and  $^{177}\text{Lu}$ -conjugated CC49 sFv display differential metabolic patterns in tumor-bearing nude mice (13). The observed differences, including significantly greater localization of the radiometal in the liver, spleen and kidneys, are likely due to both a prolonged intracellular retention of the radiometal and the dehalogenation of the radioiodine-labeled CC49 sFv.

In this article, we report that the use of *N*-succinimidyl *p*-iodobenzoate (PIB), a protein radioiodination acylating agent, leads to greater retention of the radiolabel on protein and results in superior selective tumor biodistribution of radioiodinated 741F8-1 (sFv')<sub>2</sub> in tumor-bearing *scid* mice compared to that achieved with the same sFv dimer radioiodinated by the chloramine-T method.

## MATERIALS AND METHODS

### Construction, Production and Characterization of 741F8 sFv and 741F8-1 (sFv')<sub>2</sub>

The 741F8-1 (sFv')<sub>2</sub> was produced as previously described (9,14). Briefly, the V<sub>H</sub> and V<sub>L</sub> genes were cloned from the 741F8 murine hybridoma and linked by Ser<sub>4</sub>-Gly-Ser<sub>4</sub>-Gly-Ser<sub>4</sub> in pH1337 plasmids. The sFv' was produced in *Escherichia coli*. Refolding of the M<sub>r</sub> 27,000 sFv' was performed using a modification of the 3 M urea/glutathione oxidation reduction refolding procedure of Tai (15) to yield stable monomers. Divalent (sFv')<sub>2</sub> were produced through disulfide linkages between carboxyl-terminal cysteine residues on sFv' monomers. The (sFv')<sub>2</sub> preparations were characterized by sedimentation analysis, and association constants were measured using c-erbB-2 extracellular domain (ECD) immobilized on plasmon resonance chips using the BIAcore instrument (Pharmacia, Brussels, Belgium). The overall association constant averaged  $0.2 \times 10^8$ .

### Radiolabeling

The 741F8-1 (sFv')<sub>2</sub> was labeled with radioiodine using either the chloramine-T (16) or *p*-iodobenzoate method (17,18). For the chloramine-T method, 1.0–2.0 mg protein were combined with  $^{125}\text{I}$  [14–17 mCi/mg] (Amersham, Arlington Heights, IL) or  $^{131}\text{I}$  [9.25 mCi/mg] (DuPont NEN, Wilmington, DE) at an iodine to protein ratio of 1:10. Ten micrograms chloramine-T (Sigma, St. Louis, MO) were added per 100 μg protein and the resulting mixture was incubated for 3 min at room temperature. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method (19). The final specific activity of the chloramine-T labeling was 1.4 mCi/mg for the  $^{131}\text{I}$ -741F8-1 (sFv')<sub>2</sub> and typically about 1.0 mCi/mg for the  $^{125}\text{I}$ -741F8-1 (sFv')<sub>2</sub>. The PIB labeling was performed as previously described (17,18). Briefly, PIB was dissolved in 5% HoAC/MeOH,  $^{125}\text{I}$  or  $^{131}\text{I}$  was added at a PIB:iodine ratio of 1:1 (pH 5) and incubated at room temperature for 10 min. The reaction was quenched with an equal amount of sodium metabisulfite and the mixture was dried under a gentle stream of nitrogen. Antibody 741F8-1 (sFv')<sub>2</sub> in 1.0 M sodium carbonate buffer (pH 9.3) was added to the dried \*I-PIB and the mixture was incubated for 10 min at room temperature (pH 8.5). The reaction volume was increased to 100 μl and the free iodine and unconjugated \*I-PIB were removed from the labeled (sFv')<sub>2</sub> by gel filtration as previ-

ously described. The final specific activity *p*-iodobenzoate labeling was 0.7 mCi/mg for  $^{131}\text{I}$ -741F8-1 (sFv')<sub>2</sub> and was typically 0.2 mCi/mg for  $^{125}\text{I}$ -741F8-1 (sFv')<sub>2</sub>.

### Quality Control

Radiopharmaceutical quality was evaluated by high-performance liquid chromatography (HPLC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a live-cell binding assay. The HPLC analysis was performed using a Spherogel TSK-3000 molecular sieving column (Beckman, San Ramon, CA). Eluted fractions were collected and counted in a gamma well counter (Beckman) (9). The elution profiles consistently demonstrated that greater than 99% of the radioactivity was associated with the protein peak. Radioiodinated 741F8-1 (sFv')<sub>2</sub> was evaluated by SDS-PAGE. Reduced and nonreduced SDS-PAGE were run using 12% gels (10 × 12 cm) with 3% stacking gels (9,20). Migration of the (sFv')<sub>2</sub> was detected by autoradiography at –70°C using x-ray film with regular intensifying screens. Greater than 98% of the nonreduced  $^{125}\text{I}$ -741F8-1 (sFv')<sub>2</sub> preparations migrated on SDS-PAGE as approximately 55 kDa proteins, with the remaining activity migrating as monomer. The immunoreactivity of the radiopharmaceuticals was determined in a live-cell binding assay utilizing c-erbB-2 positive SK-OV-3 cells and c-erbB-2 negative CEM cells (9). One or 10 ng labeled (sFv')<sub>2</sub> in 100 μl PBS (0.154 M NaCl, 10 mM sodium phosphate, pH 7.2) were added in triplicate to  $5 \times 10^6$  SK-OV-3 or CEM cells in 15 ml polypropylene centrifuge tubes. The cells were incubated for 30 min at room temperature, washed with 2.0 ml PBS and centrifuged for 5 min at 500 × g. Supernatants were separated from the cell pellets, both were transferred to 12 × 75 counting tubes and the percentage of activity associated with the cell pellet was determined. Typically, live-cell binding assays revealed >70% of the activity associated with the positive cell pellet and less than 3% bound to the negative control cells; these results are equivalent to those seen with parental 741F8 IgG.

### Biodistribution Studies

Four to 6-wk-old C.B17/Icr-*scid* mice were studied. The  $2.5 \times 10^6$  SK-OV-3 cells in the log phase were implanted subcutaneously on the abdomens of the mice. After about 5 wk, the tumors had achieved sizes of 100–200 mg and Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Three days later, biodistribution studies were initiated.

Iodine-125-741F8 (sFv')<sub>2</sub>, labeled by the chloramine-T or *p*-iodobenzoate method, was diluted in PBS to a concentration of 0.2 mg/ml and each mouse was given 100 μl, containing 20 μg radiopharmaceutical by tail vein injection. Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (Canberra, Meriden, CT). Blood samples and whole-body counts of the mice were obtained at regular intervals. Groups of eight mice were killed at various times after injection and the tumors and organs were removed, weighed and counted in a gamma counter to determine the %ID/g (9,21). The mean and standard error of the mean (s.e.m.) for each group of data were calculated, and T:O ratios were determined. Significance levels were determined using Student's t-test.

### Radioimmunoimaging Studies

Injections were performed following the protocol described above for the biodistribution studies. Tumor-bearing *scid* mice were injected intravenously with 100 μg (100 μl)  $^{131}\text{I}$ -741F8-1 (sFv')<sub>2</sub> labeled by the chloramine-T or *p*-iodobenzoate methods. At 21 hr after injection, the mice were euthanized by asphyxiation

TABLE 1

Comparative 24-Hour Biodistribution Studies of Anti-c-erbB-2 Iodine-125-741F8-1 (sFv')<sub>2</sub> Labeled by the *p*-iodobenzoate and Chloramine-T Methods

Organ	<i>p</i> -iodobenzoate	T:O	Chloramine-T	T:O	p value
	(%ID/g, mean ± s.e.m.)		(%ID/g, mean ± s.e.m.)		
Tumor	2.77 ± 0.17	—	1.78 ± 0.14	—	0.001
Bone	0.04 ± 0.01	82.2	0.10 ± 0.03	24.2	0.098
Heart	0.05 ± 0.01	84.7	0.08 ± 0.01	21.4	0.013
Intestine	0.08 ± 0.01	44.7	0.13 ± 0.04	21.8	0.263
Kidney	0.51 ± 0.04	5.7	0.55 ± 0.03	3.2	0.441
Liver	0.12 ± 0.03	49.4	0.19 ± 0.01	9.8	0.057
Lung	0.24 ± 0.10	21.8	0.27 ± 0.03	7.0	0.800
Muscle	0.01 ± <0.01	288.8	0.02 ± <0.01	99.4	0.114
Bladder	0.27 ± 0.07	48.0	0.13 ± 0.02	14.8	0.088
Spleen	0.08 ± 0.01	55.1	0.27 ± <0.01	6.6	<0.001
Stomach	0.05 ± 0.01	82.7	0.19 ± 0.03	10.1	0.003
Blood	0.10 ± 0.01	29.2	0.26 ± 0.01	6.8	<0.001

Each study was performed in CB.17 *lcr scid* mice bearing s.c. SK-OV-3 tumors (*p*-iodobenzoate, *n* = 16; chloramine-T, *n* = 8). The values are given for the mean, s.e.m. and the tumor: organ ratio (T:O). Significance levels (p values) were determined by Student's *t*-test.

%ID = percent injected dose per gram of blood activity that was measured as the percent injected dose per milliliter.

with CO<sub>2</sub> and images were acquired on a gamma camera with a high-energy collimator. Preset acquisitions of 100,000 counts were obtained.

**In Vitro and In Vivo Stability Studies.** The degree of dehalogenation of <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub>, labeled by the chloramine-T and *p*-iodobenzoate methods, was determined following both in vitro and in vivo incubations. The in vitro studies were performed by adding 1 μg of either radiopharmaceutical to 100 μl of fresh *scid* mouse plasma, urine or PBS. Samples were incubated for various periods of time at 37°C and the amount of protein-bound radioiodine was determined. Plasma and urine samples for the in vivo stability studies were collected at various times following intravenous administration of 100 μg of the dimer radioiodinated by either the chloramine-T or *p*-iodobenzoate methods. The amount of protein-bound radioactivity present in each sample was determined by trichloroacetic acid (TCA) precipitation (22). Briefly, 5–30 μl of sample were added to 100 μl of a 0.1% bovine serum albumin solution in a plastic 12 × 75 mm test tube at 4°C. One milliliter of a 10% solution of TCA (4°C) was added to each tube, the samples were mixed, counted on a gamma counter (to determine the total counts per sample) and incubated on ice for 30 min. The samples were then applied dropwise to premoistened glass fiber filters under a gentle vacuum. The filters were washed twice with 2 ml of 10% TCA (4°C), dried and counted on a gamma counter. The amount of protein-bound radioactivity was determined by dividing the filter-bound counts by the total number of counts in the sample. All samples were assayed in triplicate and the mean and s.e.m. were calculated for each group of data.

## RESULTS

### Biodistribution Studies

Groups of 8 *scid* mice bearing 200 to 300 mg subcutaneous SK-OV-3 tumors were killed 24 hr following intravenous administration of <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub>. The results of an experiment representative of 12 such studies, performed with 741F8-1 (sFv')<sub>2</sub> radioiodinated by the chloramine-T method, were compared with representative biodistributions performed with the same reagent labeled by the

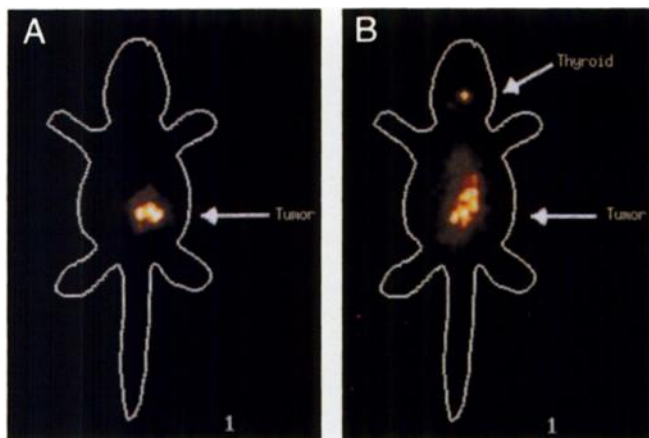
*p*-iodobenzoate method (Table 1). Significantly greater tumor localization was observed with labeled <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> than with chloramine-T labeled <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> (2.77 %ID/g versus 1.78 %ID/g, respectively, *p* = 0.0012). The normal organ retention of both preparations also favored *p*-iodobenzoate labeled <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub>. The largest differences were observed in the blood, spleen and stomach with values of 0.10 %ID/ml, 0.08 %ID/g and 0.05 %ID/g, respectively, for *p*-iodobenzoate labeled <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> and 0.25 %ID/ml, 0.27 %ID/g and 0.19 %ID/g for the chloramine-T labeled <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub> (*p* = 0.00002, *p* = 0.000008 and *p* = 0.0025, respectively). Collectively, this resulted in significantly greater tumor-to-organ ratios for *p*-iodobenzoate-labeled dimer for all tissues sampled (*p* < 0.005 for all organs, Table 1).

### Radioimmunoimaging

The 21-hr images acquired from *scid* mice bearing subcutaneous SK-OV-3 tumors revealed better imaging with *p*-iodobenzoate labeled <sup>131</sup>I-741F8-1 (sFv')<sub>2</sub> as compared with chloramine-T labeled <sup>131</sup>I-741F8-1 (sFv')<sub>2</sub> (Fig. 1). Significantly less residual activity was visible in the abdominal cavity and the thyroids of the mice that received *p*-iodobenzoate labeled <sup>131</sup>I-741F8-1 (sFv')<sub>2</sub>.

### In Vitro and In Vivo Stability

Plasma and urine samples were collected from *scid* mice at various times following intravenous administration of either tracer. The degree of dehalogenation of the <sup>125</sup>I-sFv dimer conjugate was determined in TCA precipitation assays performed in triplicate. In the mice given *p*-iodobenzoate labeled <sup>125</sup>I-741F8-1 (sFv')<sub>2</sub>, 100% of the radioiodine present in the circulation at 2 hr after injection was protein-bound. By 6 hr, the protein-associated counts accounted for 96.9% ± 3.6% of the radioactivity present in the circulation. The susceptibility to dehalogenation of proteins radioiodinated by the chloramine-T method was evident in the



**FIGURE 1.** Radioimmunoimaging of subcutaneous SK-OV-3 tumors in C.B17/ICR-*scid* mice by  $^{131}\text{I}$ -741F8-1 (sFv) $_2$  labeled by the *p*-iodobenzoate or chloramine-T method. Gamma camera images were obtained 21 hr after intravenous administration of 100  $\mu\text{g}$  (140  $\mu\text{Ci}$ ) *p*-iodobenzoate labeled  $^{131}\text{I}$ -741F8-1 (sFv) $_2$  (A) or 100  $\mu\text{g}$  (70  $\mu\text{Ci}$ ) chloramine-T labeled  $^{131}\text{I}$ -741F8-1 (sFv) $_2$  (B). Image acquisition was terminated when 100,000 counts were acquired.

mice given chloramine-T labeled  $^{125}\text{I}$ -741F8-1 (sFv) $_2$ , in which  $89.2\% \pm 1.5\%$  and  $61.2\% \pm 1.8\%$  of the activity were protein-associated at 2 and 6 hr postinjection, respectively. In urine, the method of iodination did not affect the amount of protein-bound radioactivity retained over time. At all times sampled, only about 5%–10% of the  $^{125}\text{I}$  in the urine was protein-associated, irrespective of the labeling method used. In contrast with the *in vivo* results, samples of *p*-iodobenzoate labeled  $^{125}\text{I}$ -741F8-1 (sFv) $_2$  or chloramine-T labeled  $^{125}\text{I}$ -741F8-1 (sFv) $_2$  incubated in plasma or urine *in vitro* displayed a high degree of stability, with approximately 90% of the radioiodine remaining protein-bound in all samples after incubations at 37°C for 1 and 4 hr, respectively.

## DISCUSSION

In the present study, we have demonstrated that significantly greater quantities of  $^{125}\text{I}$ -741F8-1 (sFv) $_2$  are localized in tumor when the radioiodination is performed using a stable iodine-protein linkage (*p*-iodobenzoate) rather than with direct labeling as in the chloramine-T method. The PIB method involves the coupling of the radioiodine to a nonphenolic aromatic ring of the PIB reagent. The  $^{125}\text{I}$ -PIB complex is then conjugated to lysine residues on the sFv through the formation of an amide bond (17,18,23). This reportedly yields a radiopharmaceutical which exhibits significantly more resistance to dehalogenation *in vivo* than that achieved by direct coupling of the radioiodine to phenolic tyrosine residues with the chloramine-T procedure (17,18). Radioiodination of sFv by the Bolton-Hunter method, which also utilizes an acylating reagent (24), results in a product with an intermediate stability between that achieved with the chloramine-T and *p*-iodobenzoate methods (Hylarides M, *personal communication*, 1994).

In contrast with the unacceptably high kidney, liver and

spleen retention reported when radiometals were chelated to CC49-sFv (13), we observed the same or less retention of the stably iodinated sFv, as compared with directly iodinated sFv, in all normal organs. In particular, the kidneys, which act as the route of clearance and a possible site of metabolism of sFv molecules, displayed the same localization for both the chloramine-T and the *p*-iodobenzoate-labeled preparations, whereas liver retention was lower and spleen retention was significantly lower than that seen with the chloramine-T labeled dimer. *In vivo* stability studies revealed that essentially all of the radioactivity present in the circulation of *scid* mice was TCA precipitable at least through 6 hr after the intravenous injection of *p*-iodobenzoate labeled  $^{125}\text{I}$ -741F8-1 (sFv) $_2$ , whereas the TCA precipitable activity associated with the chloramine-T  $^{125}\text{I}$ -741F8-1 (sFv) $_2$  steadily decreased over the same period. In contrast with the observation of a greater stability of the *p*-iodobenzoate labeled  $^{125}\text{I}$ -741F8-1 (sFv) $_2$  in the circulation, the majority of the radioactivity present in the urine from both the *p*-iodobenzoate and chloramine-T labeled sFv dimers was not protein-bound. When TCA precipitation assays were performed on samples incubated *in vitro* in plasma at 37°C, the labeling method did not effect the stability of either preparation, possibly indicating a role for a metabolic organ in the dehalogenation process observed *in vivo* with chloramine-T labeled proteins.

The selective tumor biodistribution of sFv-based reagents during their terminal distribution phases indicates their potential application as radioimmunoimaging agents. This is apparent when the tumor-to-blood ratios are considered. Radioiodinated IgG molecules rarely achieve tumor-to-blood ratios of more than 1.5:1 by 24 hr after their administration (8). In contrast, radioiodinated sFv molecules frequently achieve tumor-to-blood ratios of up to 10-fold greater than the IgG values after the same period of time (7–9). This increase in specificity of tumor targeting with sFv over that observed with IgG results in a clear advantage for the use of sFv in diagnostic imaging (8). In our study, we found that the stabilization of the radioiodine-sFv bond further enhances this potential. As shown in Figure 1, the acquisition of the same number of counts in the *p*-iodobenzoate and chloramine-T images resulted in significantly greater specificity of tumor localization and an associated reduction of normal tissue activity with the preparation labeled by the *p*-iodobenzoate method. In particular, the absence of detectable thyroid uptake in the mice treated with  $^{131}\text{I}$ -741F8 (sFv) $_2$  labeled by the *p*-iodobenzoate method is of interest. Such increases in targeting specificity will likely result in the ability to detect smaller tumor masses in the clinical setting.

The 741F8 (sFv) $_2$  antibody is specific for the extracellular domain of the human c-erbB-2 protein product of the HER2/neu oncogene. c-erbB-2 has been found to be overexpressed in a number of cancers, including breast and ovarian carcinoma (25–28), gastric tumors and colon ade-

nocarcinomas (29). In breast cancer, overexpression of c-erbB-2 has been correlated with a poorer prognosis (30,31). In immunohistochemical studies of normal human tissues, c-erbB-2 was not found to be widely expressed. Only the proximal kidney tubules, the gastrointestinal tract mucosal epithelium and the squamous epithelium in skin revealed significant reactivity with antibodies directed against c-erbB-2 (32–35), indicating that this is an attractive target for antibody-based imaging and therapy.

The specificity of tumor targeting that we (9) and others (7,8) have observed with sFv-based reagents is indicative of the potential of this class of molecules. Optimal therapeutic applications of sFv will require that their low degree of retention in tumor be further addressed. Whereas the dimers utilized in these studies have been shown to be capable of binding two c-erbB-2 ECD molecules in solution, improved dimeric constructs with greater flexibility may display an increased functional avidity, which allows for divalent binding of cell surface antigen and results in greater tumor retention (9). The generation of sFv molecules with a higher affinity for target antigen also may be required to prolong the tumor retention of these agents. The molecules we used in this study were derived from the gene sequence of the parent IgG, 741F8 and thus are limited by its original qualities. The newly developed phage display methodologies for the production of sFv and Fab molecules, however, have no such limitations (36). Furthermore, the ability to alter the affinity of sFv molecules produced in this manner, through chain shuffling (37,38) or point mutations (39), makes the production of higher affinity sFv molecules likely in the near future.

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