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Optimization of in vivo tumor targeting in SCID mice with divalent forms of 741F8 Anti-c-erbB-2 single-chain Fv: effects of dose escalation and repeated i.v. administration

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Abstract Single-chain Fv molecules in monovalent (sFv) and divalent [$(sFv')_2$] forms exhibit highly specific tumor targeting in mice as a result of their small size and rapid systemic clearance. As a consequence, there is a rapid reversal of the sFv blood/tumor gradient, resulting in diminished retention of sFv species in tumors. In this report we investigate two distinct strategies, dose escalation and repetitive intravenous (i.v.) dosing, aiming to increase the absolute selective retention of radiolabeled anti-c-erbB-2 ^{125}I-741F8 $(sFv')_2$ in c-erbB-2-overexpressing SK-OV-3 tumors in mice with severe combined immunodeficiency (SCID). A dose-escalation strategy was applied to single i.v. injections of ^{125}I-741F8 $(sFv')_2$. Doses from 50 μg to 1000 μg were administered without a significant decrease in tumor targeting or specificity. High doses resulted in large increases in the absolute retention of ^{125}I-741F8 $(sFv')_2$. For example, raising the administered dose from 50 μg to 1000 μg increased the tumor retention 24 h after injection from 0.46 μg/g to 9.5 μg/g, and resulted in a net increase of greater than 9 μg/g. Over the same dose range, the liver retention rose from 0.06 μg/g to 1 μg/g, and resulted in a net increase of less than 1 μg/g. The retention of 9.5 μg/g in tumor 24 h following the 1000-μg dose of $(sFv')_2$ was comparable to that seen 24 h after a 50-μg dose of ^{125}I-741F8 IgG, indicating that the use of large doses of $(sFv')_2$ may partially offset their rapid clearance. When two doses were administered by i.v. injection 24 h apart, the specificity of delivery to tumor observed after the first dose was maintained following the second injection. Tumor retention of ^{125}I-741F8 $(sFv')_2$ was 0.32 μg/g at 24 h and 0.22 μg/g at 48 h following a single injection of 20 μg, while 0.04 μg/ml and 0.03 μg/ml were retained in blood at the same assay times. After a second 20-μg injection at the 24-h assay time, tumor retention increased to 0.49 μg/g, and blood retention was 0.06 μg/ml, at the 48-h point. These results suggest that multiple high-dose administrations of radiolabeled 741F8 $(sFv')_2$ may lead to the selective tumor localization of therapeutic radiation doses.

Key words Single-chain Fv molecules · Dose · Immunotargeting · c-erbB-2 · Specificity

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Introduction

Successful radioimmunotherapy of solid malignancies will depend upon the highly selective tumor localization of radiolabeled molecules, with an efficient and uniform diffusion through solid tumor, and the retention of sufficient radionuclide to deliver a tumoricidal dose of radiation. The development of single-chain Fv (sFv) molecules has resulted in the production of a new generation of reagents with immunotherapeutic potential [5, 10]. Monovalent and divalent forms of sFv molecules display highly specific tumor targeting in murine xenograft models [3, 7, 15]. Their small size of about 26 kDa (52 kDa for the dimer) allows for

penetration and diffusion into solid tumors that is far superior to that of IgG [23]. Radiolabeled sFv molecules exhibit rapid serum and whole-body clearances from tumor-bearing mice, resulting in highly specific localization of sFv in tumor [3, 7, 15]. Clearance of the labeled sFv from tumor was significantly slower than from normal organs but occurred rapidly after a lag period, possibly reflecting a reversal of the blood/tumor gradient. Improvements in sFv engineering will be needed to develop molecules with avidity or affinity characteristics that decrease the rapidity of dissociation of these molecules from their target antigens. New dosing and scheduling strategies may be useful adjuncts for improving the tumor retention of systemically administered sFv-based molecules.

We have previously determined the pharmacokinetics and biodistribution of radioiodinated 741F8 $(sFv')_2$, specific for c-erbB-2 overexpressed on ovarian carcinoma xenografts, and compared these results with those achieved with an irrelevant control $(sFv')_2$ in the same model [3]. This study conclusively demonstrated that the tumor localization of these $(sFv')_2$ molecules is mediated by their antigen specificity. We also have observed that the administration of $(sFv')_2$ molecules by continuous infusion leads to a decreased specificity and reduced $(sFv')_2$ delivery to tumor, as compared to those levels achieved following an i.v. bolus injection [19]. In the present report, we examine alternative methods of increasing the localization, without decreasing the specificity of delivery, of intravenously administered radioiodinated anti-c-erB-2 $(sFv')_2$ dimers in a mouse with severe combined immunodeficiency (SCID), using a subcutaneous human tumor xenograft model.

Materials and methods

Preparation of $(sFv')_2$ molecules

The sFv species utilized in these experiments were produced at Creative BioMolecules Inc. (Hopkinton, Mass.) as described elsewhere [3, 13] (M-S. Tai et al. and J. E. McCartney et al., manuscripts in preparation). The 741F8 $(sFv')_2$ used in the dose-escalation study, designated 741F8-4 $(sFv')_2$, was produced without a leader sequence as a C-terminal disulfide dimer, constructed as $(V_L$-GSSGGGGSGGGGSMA-V_HGGGGC$)_2$. The 741F8 $(sFv')_2$ utilized in the dual-dose study, designated 741F8-2 $(sFv')_2$, had the following orientation: $(V_H$SSSSGSSSSGSSSS-V_LGGGGC$)_2$ with a nine-residue N-terminal extension (ADNKFNKDP) on each heavy chain to promote high expression levels. Both $(sFv')_2$ molecules were produced in *E. coil* as a sFv′ monomers. Refolding of the 27-kDa sFv′ analogues involved a slight modification of the 3 M urea/glutathione redox refolding procedure of Tai et al. [18]. The 741F8 sFv′ species were refolded to yield stable monomers with the C-terminal cysteine in a mixed disulfide with glutathione. Blocked 741F8 sFv′ was purified by anion/cation exchange followed by size-exclusion chromatography and then converted to $(sFv')_2$ homodimers. Monovalent sFv′ was deblocked with mild reduction and $(sFv')_2$ dimers were formed through disulfide bonds by oxidation as described [13].

Characterization of $(sFv')_2$ molecules

The association constants of both $(sFv')_2$ species were determined using the BIAcore plasmon resonance instrument, by immobilizing the $(sFv')_2$ directly on the chip and passing various concentrations of c-erbB-2 extracellular domain (ECD) over the chip, as previously described [3]. The rate of association of both $(sFv')_2$ species was close to that of the parent antibody ($K_a = 5.0 \times 10^7$). However, the dissociation kinetics were biphasic, resulting in a faster rate of dissociation and resulting in a K_a of about 1.55×10^7 for the process associated with the slowest dissociation. Demonstration of divalent binding of soluble c-erbB-2 ECD by both 741F8 $(sFv')_2$ species was achieved through sedimentation analysis on an analytical ultracentrifuge as previously described [3]. Sedimentation equilibrium experiments with 741F8 $(sFv')_2$, ECD, or 741F8 $(sFv')_2$ and excess ECD, showed that the stoichiometry of association was one molecule of 741F8 $(sFv')_2$ to two molecules of ECD.

Labeling

Both $(sFv')_2$ molecules were labeled with radioiodine using the chloramine-T method [9] and 1.0–2.0 mg of $(sFv')_2$ was combined with ^{125}I(14–17 mCi/μg; Amersham, Arlington Heights, Ill.) at an iodine-to-protein ratio of 1:10 in a 12 × 75-mm plastic test-tube. A 10-μl (1 mg/ml) sample of chloramine-T (Sigma, St. Louis, Mo.)/100 μg protein was added and allowed to incubate for 3 min at room temperature. The unincorporated ^{125}I was separated from the labeled sFv by the G-50-80 centrifuged-column method [14]. The specific activities of the ^{125}I-labeled products were 0.5 mCi/mg and 0.4 mCi/mg for the dual-dose and dose-escalation experiments respectively.

Quality control

The quality of the radiopharmaceuticals was evaluated using high-performance liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE) and a live-cell binding assay. The HPLC analysis was performed using a Spherogel TSK-3000 molecular-sieving column (Beckman, San Ramon, Calif.). Samples of 1 μl were assayed at a flow rate of 1 ml/min, and 0.3-ml fractions were collected and counted in a gamma well counter (Beckman) [1]. In each case, the elution profile demonstrated that more than 99% of the radioactivity was associated with the protein peak.

The size of the ^{125}I-741F8 $(sFv')_2$ products was evaluated by sodium dodecyl sulfate (SDS)/PAGE. Reduced and non-reduced forms were run on 12% gels (10 cm × 12 cm) with 3% stacking gels [12] and migration of the labeled proteins was detected by autoradiography (Kodak X-ray film with Kodak X-Omatic regular intensifying screens) at −70 °C. More than 98% of all nonreduced ^{125}I-741F8 $(sFv')_2$ preparations migrated on SDS-PAGE as approximately 50-kDa bands with the remaining activity migrating as monomer, while 100% of all reduced ^{125}I-741F8 $(sFv')_2$ migrated as a monomer. Immunoreactivity of the radiopharmaceuticals was determined in a live-cell binding assay utilizing c-erbB-2-positive SK-OV-3 cells (HTB 77; American Type Culture Collection, Rockville, Md.) and c-erbB-2-negative CEM cells (119; American Type Culture Collection) [4]. SK-OV-3 cells expressed c-erbB-2 on the cell surface when grown in SCID mice [21]. Either 1 ng or 10 ng labeled $(sFv')_2$ in 100 μl phosphate-buffered saline (PBS: 10.154 M NaCl, 10 mM phosphate, pH 7.2) was added in triplicate to 5×10^6 SK-OV-3 or CEM cells in 15-ml polypropylene centrifuge tubes. After an incubation of 30 min at room temperature, the cells were washed with 2.0 ml PBS and centrifuged for 5 min at 500 *g*. The supernatants were separated from the cell pellets, both were transferred to 12 × 75-mm counting tubes and the percentage of activity associated with the cell pellet was determined. Both ^{125}I-741F8

$(sFv')_2$ species consistently showed 70%–80% of the activity associated with the positive cell pellet and less than 3% bound to the negative control cells.

Mice

C.B17/Icr SCID mice, 4–6 weeks old, were obtained from the Fox Chase Cancer Center Laboratory Animal Facility, and 2.5×10^6 SK-OV-3 cells in log phase were implanted s.c. on the abdomens of the mice. After 5 weeks, when the tumors had achieved sizes of 100–200 mg, Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Three days later, the mice were used in biodistribution studies.

Biodistribution studies

^{125}I-741F8-2 $(sFv')_2$ was diluted in PBS (pH 7.2) to a concentration of 0.2–1.0 mg/ml for the dual-dose study and ^{125}I-741F8-4 $(sFv')_2$ was diluted to concentrations ranging from 0.5 mg/ml–4.0 mg/ml for the dose-escalation study. The radiopharmaceuticals were administered to each mouse by tail-vein injection, with the mice in the dose-escalation study receiving 100–250 µl (50 µg–1 mg) and the mice in the dual-dose study receiving 100 µl (20 µg). The total injected dose was determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model 2007, Canberra, Meridian, Conn.) Blood samples and whole-body counts of the mice were obtained at regular intervals. Groups of three or four mice were sacrificed at various times after injection and the tumors and organs were removed, weighed and counted in a gamma counter to determine the percentage of the injected dose per gram of tissue (%ID/g) and the mass (µg) of labeled $(sFv')_2$/g retained [2, 8]. The mean and standard error of the mean (SEM) for each group of data were calculated, and tumor : organ (T : O) ratios were determined. Significance levels were determined using Student's *t*-test.

Results

Dose-escalation study

The effect of escalating doses of ^{125}I-741F8-4 $(sFv')_2$, an sFv dimer utilizing a V_LV_H orientation of its subunits, was examined in SCID mice bearing SK-OV-3 tumors. The biodistributions of 50, 100, 369, and 1000 µg i.v. administered ^{125}I-741F8-4 $(sFv')_2$ were examined 24 h after injection. While the percentage of the injected dose that localized per gram of tumor and tissue remained constant, the absolute quantity of ^{125}I-741F8-4 $(sFv')_2$ localized in tumors increased dramatically with escalating doses as compared to the amounts seen in normal organs (Fig. 1 and Table 1). For instance, 0.46 µg/g and 9.5 µg/g localized in tumor following 50-µg and 1000-µg doses respectively, resulting in a net increase of 9.04 µg/g retained in tumor. By comparison, the liver retention rose from 0.06 µg/g to 1 µg/g over the same dose range, resulting in a net increased retention of only 0.94 µg/g (nearly ten times less than that seen in the tumor). the biodistributions of 100-µg and 1000-µg doses were further examined 4, 24 and 48 h after injection to compare the effects of escalating dose

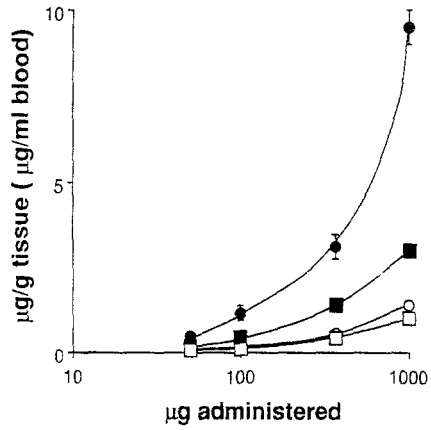


Fig. 1 Effects of dose on the biodistribution of ^{125}I-741F8-4 $(sFv')_2$. Doses of 50, 100, 369 or 1000 µg ^{125}I-741F8-4 $(sFv')_2$ were injected i.v. into SCID mice bearing SK-OV-3 tumors. Groups of three or four mice were sacrificed 24 h after injection and the biodistribution (µg/g or µg/ml) was determined. The values for tumor (●), blood (○), liver (□) and kidney (■) are plotted. *Error bars* SEM

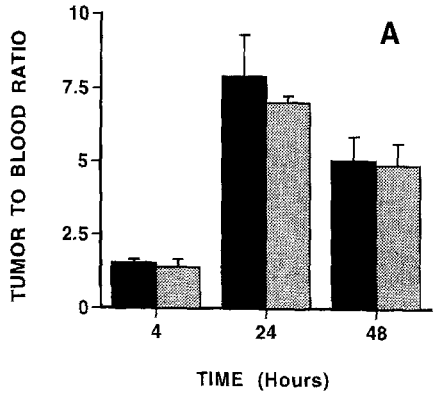
Table 1 Effects of escalated dose on the biodistribution of ^{125}I-741F8-4 $(SFv')_2$. Doses of 50, 100, 369 or 1000 µg ^{125}I-741F8-4 $(sFv')_2$ were injected i.v. into SCID mice bearing SK-OV-3 tumors. Groups of three or four mice were sacrificed 4, 24 or 48 h after injection and the percentage of the injected dose per gram of tissue (%DI/g) and SEM ($\leqslant$ 30% of the value unless indicated) was determined

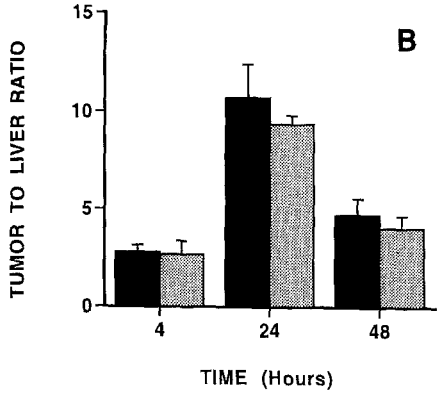
	^{125}I-741F8-4 $(sFv')_2$ retained (%ID/g)							
	50 µg	100 µg			369 µg	1000 µg		
organ	24 h	4 h	24 h	48 h	24 h	4 h	24 h	48 h
Tumor	0.92	4.26	1.17	0.37	0.85	2.62	0.95	0.25
Blood[a]	0.15	2.83	0.17	0.08	0.15	1.92	0.14	0.05
Bone	0.03	0.70	0.05	0.04	0.05	0.49	0.05	0.03
Heart	0.07	1.68	0.07	0.04[c]	0.09	1.08	0.08	0.03
Intestine	0.08[b]	1.14	0.07	0.04	0.13[b]	0.65	0.04	0.02
Kidney	0.31[c]	2.20	0.43	0.28	0.37	1.76	0.30	0.19
Liver	0.12	1.65	0.11	0.08	0.13	1.03	0.10	0.06
Lung	0.14	2.03	0.19	0.09	0.17	1.53	0.23[b]	0.07
Muscle	0.02	0.91	0.03[b]	0.01	0.04[c]	0.35	0.02	0.01
Spleen	1.13	2.31	0.14[c]	0.14	0.32[c]	1.92[c]	0.22	0.14[c]
Stomach	0.14	4.99	0.33[c]	0.09	0.16[c]	3.43	0.12	0.03[b]

[a] Represented as %ID/ml
[b] SEM $\leqslant$ 40% of associated value
[c] SEM > 40% of associated value

over time. The specificity of tumor targeting, as demonstrated by the tumor-to-organ ratios, was maintained through this wide dose range at all times studied (Fig. 2). Additionally, the normal organ reservoirs of the labeled 741F8-4 $(sFv')_2$ did not vary significantly with increasing doses. Similar results were observed when escalating doses of monomeric 741F8 sFv' were used in place of the divalent 741F8-4 $(sFv')_2$ (data not shown).

Significantly, the mass of radiopharmaceutical localized per gram of tumor 24 h following the administration of the 1000-µg dose was very similar to that





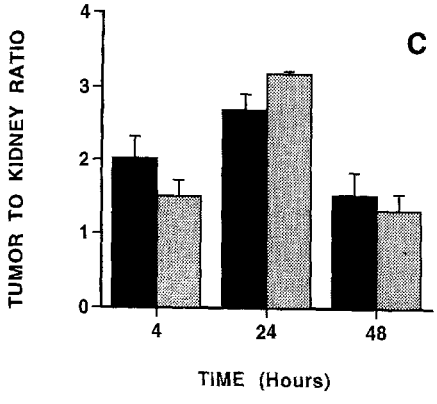


Fig. 2A–C The tumor-to-organ ratios of escalated doses of sFv dimer. Doses of 100 μg (*grey*) or 1000 μg (*black*) ^{125}I-741F8-4 $(sFv')_2$ were injected into SCID mice bearing SK-OV-3 tumors. Mice were sacrificed 4, 24 or 48 h after injection and the tumor-to-organ ratios were determined. Values are presented for blood (**A**), liver (**B**) and kidney (**C**). The standard error of the mean is indicated

resulting from a 50-μg dose of 741F8 IgG (Fig. 3). For instance, 9.5 μg/g of ^{125}I-741F8-4 $(sFv')_2$ was present in subcutaneous tumor xenografts in SCID mice 24 h after the administration of a 1-mg dose, while 9.8 μg/g was retained in tumor 24 h following a 50-μg dose of ^{125}I-741F8 IgG. At the same assay time the improved targeting specificity of the $(sFv')_2$ was revealed in the normal organ retentions, with liver, blood and kidney retention being 1.0 μg/g, 1.4 μg/ml and 3.0 μg/g respectively for ^{125}I-741F8-4 $(sFv')_2$ and 4.2 μg/g, 13.4 μg/ml, and 3.6 μg/g respectively for ^{125}I-741F8 IgG (Table 2).

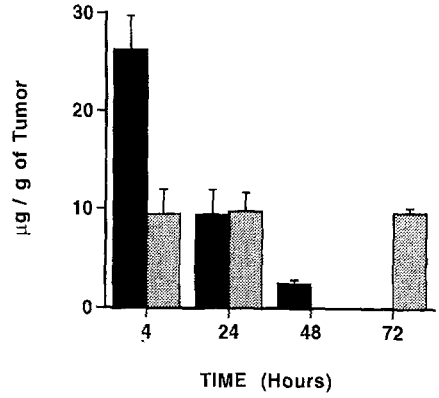


Fig. 3 Comparison of tumor localization of 741F8 IgG and $(sFv')_2$ dimer administered in a high dose. Following the i.v administration of 1000 μg ^{125}I-741F8-4 $(sFv')_2$ (*black*) or 50 μg of ^{125}I-741F8 IgG (*grey*) to SCID mice bearing subcutaneous SK-OV-3 ovarian carcinoma xenografts, approximately 10 μg IgG was retained/tumor between 4 h and 72 h after injection. By comparison, the tumor retention of the ^{125}I-741F8-4 $(sFv')_2$ was greater than that achieved with IgG at 4 h and rapidly decreased, resulting in equal amounts of both reagents retained in tumor at 24 h. By 48 h after injection, $(sFv')_2$ values were significantly lower than the steady-state IgG value. The mean retention of $(sFv')_2$ in tumor between 4 h and 48 h after injection was 12.7 μg/g and was very similar to that estimated for IgG (9.7 μg/g) based upon the steady-state retention for 72 h after injection

Table 2 Comparative 24-h biodistributions of ^{125}I-741F8-4 $(sFv')_2$ and ^{125}I-741F8 IgG. The superior targeting specificity of 741F8-4 $(sFv')_2$ over the parent IgG molecule is demonstrated in this study performed in SK-OV-3 tumor-bearing SCID mice. Administered doses of 1 mg for the 741F8-4 $(sFv')_2$ and 50 ng for the 741F8 IgG, were selected to yield similar 24-h tumor retention of both reagents. Results are expressed as per gram tissue or per milliliter blood; unless indicated the SEM is $\leqslant 30\%$ of the associated value. Tumor: organ ratios are presented in parentheses

	Biodistribution (μg/g)	
Organ	$(sFv')_2$	IgG
Tumor	9.5	9.8
Blood[a]	1.4 (6.8)	15.5 (0.6)
Bone	0.5 (19.0)	1.4 (6.9)
Heart	0.8 (11.9)	3.0 (3.3)
Intestine	0.4 (23.8)	1.6 (6.1)
Kidney	3.0 (3.2)	3.6 (2.7)
Liver	1.0 (9.5)	4.2 (2.4)
Lung	2.3[b] (4.1)	6.4 (1.5)
Muscle	0.2 (47.5)	1.2 (8.5)
Spleen	2.2 (4.3)	6.5 (1.5)
Stomach	1.2 (7.9)	1.8 (5.4)

[a] Represented as μg/ml
[b] SEM $\leqslant 40\%$ of associated value

Dual dose biodistribution studies

The biodistribution of a single 20-μg i.v. bolus of ^{125}I-741F8-2 $(sFv')_2$, in SCID mice bearing SK-OV-3 tumors is presented in Table 3. This divalent radiopharmaceutical, produced from sFv subunits with

Table 3 Biodistribution of a single dose of ^{125}I-741F8-2 $(sFv')_2$. Doses of 20 μg ^{125}I-741F8-2 $(sFv')_2$ (specific for c-erbB-2) were injected i.v. into SCID mice bearing SK-OV-3 tumors over-expressing c-erbB-2. Three mice were sacrificed at each assay time and the average and SEM (⩽ 27% of the value unless indicated) were determined

Organ	^{125}I-741F8-2 $(sFv')_2$ retained (%ID/g)									
	1 h	4 h	8 h	16 h	24 h	25 h	28 h	32 h	40 h	48 h
Tumor	4.97	5.18	2.52	1.01	1.59	1.31	1.35	1.39	0.84	0.60
Blood[a]	17.00	9.18	2.09	0.65	0.38	0.42	0.30	0.24	0.15	0.12
Bone	2.31	1.78	0.39	0.11	0.10	0.11	0.07	0.06	0.06	0.06
Heart	7.42	5.00	0.76	0.27	0.16	0.23	0.18	0.11	0.06	0.07
Intestine	2.87	3.27	0.39	0.19	0.06	0.11	0.07	0.07[b]	0.03	0.04
Kidney	10.70	6.58	1.82	0.88	0.61	0.68	0.47	0.35	0.36	0.34
Liver	7.72	4.48	1.04	0.44	0.30	0.36	0.25	0.21	0.14	0.16
Lung	14.20	7.04	1.91	0.56	0.54[b]	0.39	0.29	0.23	0.15	0.18[b]
Muscle	1.50[c]	1.45	0.17	0.05	0.03	0.04	0.04	0.02	0.01	0.01
Spleen	7.63	6.31	1.38	0.64	0.40	0.50	0.32	0.24	0.30	0.31[c]
Stomach	11.80	23.20	0.90	0.57	0.23	0.32	0.15	0.08	0.06	0.07

[a] Represented as %ID/ml
[b] SEM ⩽ 40% of the associated value
[c] SEM < 45% of the associated value

a V_HV_L orientation, was selectively retained in tumor and rapidly cleared from circulation and all normal tissues, resulting in favorable tumor:organ ratios as early as 8 h after injection. By 24 h after injection, 1.59%ID/g was present in tumor with blood, liver and muscle retaining 0.38, 0.30 and 0.03 %ID/g respectively. However, while the specificity of localization in tumor persisted at least for 48 h after injection, the quantitative tumor retention of radiolabel continuously decreased. To examine the value of using serial i.v. bolus injections to maintain relatively high tumor concentrations of radioiodinated 741F8-2 $(sFv')_2$, tumor-bearing SCID mice were given two 20-μg i.v boluses 24 h apart. When biodistributions performed following single and dual doses of this $(sFv')_2$ were compared, both doses were cleared from the circulation in a similar manner (Fig. 4). The tumor retentions also were similar (0.32 μg/g and 0.37 μg/g respectively) 24 h following both the first and second doses. The normal organ retention of radiolabel over the same assay times demonstrated a lack of nonspecific accumulation with the addition of a second i.v. dose. This resulted in similar T:O profiles from both the single- and double-dose groups (Fig. 5). For example, 0.06 μg/g and 0.08 μg/g were retained in the liver 24 h following the first and second doses respectively, resulting in tumor:liver ratios of 5.33 and 4.63. Similar results were seen at other times, with tumor to liver ratios of 1.16 4 h following a single dose and 1.33 4 h following the second dose (28 h after the first of the two doses). In a second dual-dose biodistribution study, the retention of specificity through both doses was maintained and an additive increase in retention of the radiolabel was observed in tumor and normal organs following the second dose (Table 4). Tumor retentions of 0.32 μg/g and 0.22 μg/g of ^{125}I-741F8-2 $(sFv')_2$ were observed at 24 h and 48 h respectively, following a single 20-μg injection, while 0.48 μg/g was present 24 h following the second i.v. injection (48 h following the first injection).

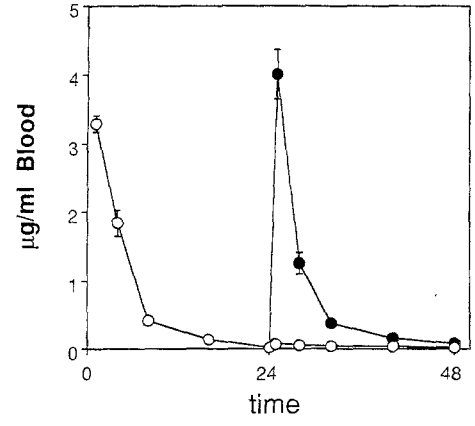


Fig. 4 Comparison of the biodistribution of single and double doses of ^{125}I-741F8-2 $(sFv')_2$. SCID mice, bearing SK-OV-3 tumors over-expression c-erbB-2, were given one (○) or two (●) i.v injections of 20 μg ^{125}I-741F8-2 $(sFv')_2$ (specific for c-erbB-2) 24 h apart. Groups of three to five mice were sacrificed at each indicated time and the average distribution (%μg/ml) in blood and the associated SEM were plotted

However, the tumor retention values observed 24 h following a single dose and 24 h following the second injection of a double dose approached but did not reach statistical significance ($P = 0.0748$). Again, no loss of specificity was observed with sequential dosing in this study.

Discussion

This report demonstrates that divalent single-chain Fv molecules can be administered to tumor-bearing SCID mice in escalating single doses and by multiple i.v. boluses without altering the specificity of their delivery to tumor. We observed that escalations in $(sFv')_2$ dosage of up to 1 mg, in a 20 to 25-g SCID mouse, do not

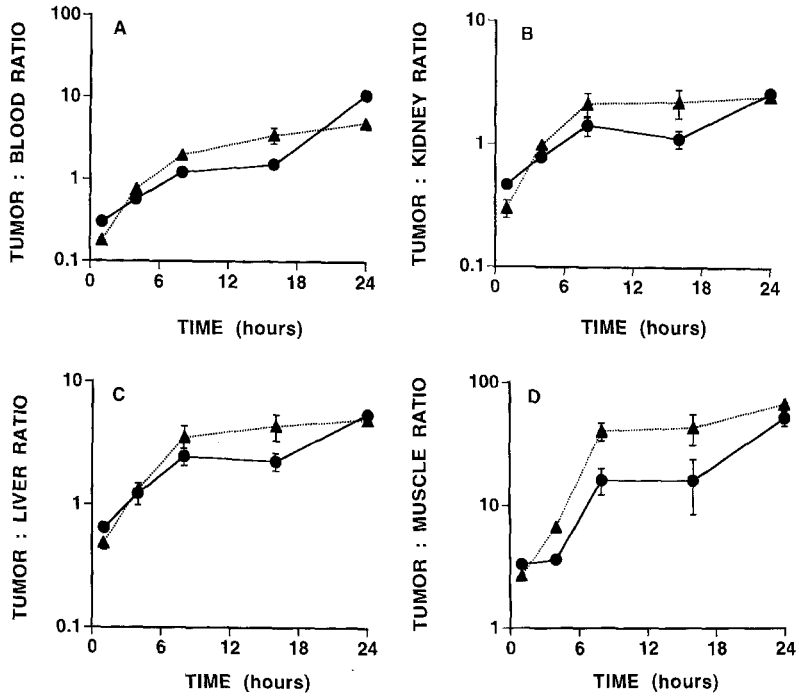


Fig. 5A–D Tumor-to-organ ratios following single or double doses of ^{125}I-741F8-2 $(sFv')_2$. SCID mice bearing subcutaneous SK-OV-3 tumors were given a single 20-μg dose, or two 20-μg doses 24 h apart, of ^{125}I-741F8-2 $(sFv')_2$ by i.v. injection. Groups of three to five mice were sacrificed at various times and the tumor-to-organ ratios were determined. The values for the 24-h period following a single dose (●) are plotted along with those for the 24 h immediately following the second of two doses administered 24 h apart (▲) for the tumor-to-blood (**A**), tumor-to-kidney (**B**), tumor-to-liver (**C**) and tumor-to-muscle (**D**) ratios. The standard error of the mean is indicated for all values

result in a significant loss of specificity of tumor targeting. The retention of targeting specificity over a wide range of doses resulted in the ability of large increases in administered dose to amplify small differences in the resulting percentage of the injected dose retained in tumor and normal organs. For example, raising the administered dose from 50 μg to 1000 μg increased the tumor retention 24 h after injection from 0.46 μg/g to 9.5 μg/g, a net increase of more than 9 μg/g. Over the same dose range, the liver retention rose from 0.06 μg/g to 1 μg/g, representing a net increase of less than 1 μg/g. The tumor retention 24 h after injection, resulting from a 1-mg dose of ^{125}I-741F8-4 $(sFv')_2$, on a mass per unit mass of tumor basis, was comparable to that acheived with a 50-μg dose of ^{125}I-741F8 IgG. Accordingly, the use of large doses of $(sFv')_2$ may offset the rapid clearance of these small molecules, without sacrificing their advantage in specificity over that observed with IgG. When a single i.v. bolus of 20 μg was compared to two 20-μg injections given 24 h apart, the predominant factor in the degree of localization in tumor and normal organs appeared to be the amount of time elapsed between the final (or single) injection and sacrifice. In contrast, when the interval between injections was reduced to only 4 h, a significant loss in tumor specificity was observed (results not shown). At the dosage employed in the current study, there was little evidence of a significant depot effect in which the activity retained in tissue would have included residual activity from the first injection. We have previously demonstrated that the observed tumor retention of 741F8 sFv-derived molecules is antigen-specific, as 26-10 sFv (specific for digoxin but irrelevant in this model) is not actively retained in tumor [3]. Taken together, these results suggest that highly specific, but not additive, localization can be maintained with multiple i.v. bolus injections administered as frequently as 24 h apart. These data suggest that therapeutic tumor levels of radiolabeled $(sFv')_2$ may be achieved through multiple, high-dose administrations.

Modifications of dose and dosing strategies are being employed to overcome the rapid clearance and resulting low retention of labeled sFv from tumors. While therapeutic results have been observed using a continuous infusion of an sFv-based immunotoxin [20], continuous infusions do not provide advantages in quantitative delivery and tumor specificity [19]. By utilizing repeated i.v. bolus strategies such as those described here, it is likely that a greater quantity of sFv could be specifically delivered to tumor resulting in increased therapeutic potential.

We have found that dose-escalation studies performed with 741F8 sFv′ yield the same results as those with $(sFv')_2$ (data not shown). Furthermore, the biodistributions and pharmacokinetics of all radiolabeled antitumor sFv molecules (dimer or monomer) described to date in the literature are very similar [3, 7, 15]. Pending substantial improvements in the

Table 4 Comparison at 24 h and 48 h of single- and double-dose injections of ^{125}I-741F8-2 $(sFv')_2$. SCID mice, bearing SK-OV-3 tumors over-expressing c-erbB-2, were given one (*S*) or two (*D*) i.v. injections of 20 μg ^{125}I-741F8-2 $(sFv')_2$ (specific for c-erbB-2) 24 h apart. Groups of five or six mice were sacrificed at each indicated time and the average biodistribution (μg/g) and SEM (⩽ 18% of the value unless indicated) was determined. Tumor-to-organ ratios are presented in parenthesis

	Biodistribution following single or double doses of ^{125}I-741F8-1 $(sFv')_2$ (μg/g organ)					
	1 h	24 h	25 h		48 h	
Organ	*S*	*S*	*S*	*D*	*S*	*D*
Tumor	0.57	0.32	0.32	1.23	0.22[b]	0.49
Blood[a]	2.12 (0.3)	0.04 (9.0)	0.06 (7.6)	2.85 (0.4)	0.03 (8.2)	0.06 (8.0)
Kidney	1.56 (0.7)	0.06 (5.8)	0.06 (5.2)	2.19 (0.6)	0.05 (4.6)	0.12 (4.1)
Liver	0.81 (0.7)	0.02 (13.9)	0.03 (12.2)	0.93 (1.3)	0.02 (11.6)	0.05 (12.1)
Lung	1.76 (0.3)	0.04 (7.1)	0.04 (8.1)	2.96 (0.4)	0.04 (3.7)	0.07 (7.1)
Muscle	0.18 (3.1)	0.01 (61.9)	0.02[b] (33.2)	0.19 (7.6)	<0.01 (67.1)	0.01[b] (48.3)
Spleen	0.83 (0.7)	0.03 (11.8)	0.05 (6.4)	1.01 (1.2)	0.03 (9.0)	0.06 (7.7)

[a] Represented as μg/ml
[b] SEM ⩽ 31% of the associated value

affinity or avidity of such structures, it is likely that the results from the studies presented in the current report may be applied to this whole class of molecules [11]. Indeed, the differing orientations of the 741F8-2 and 741F8-4 $(sFv')_2$ species have only small effects on targeting properties, which are largely overcome by the use of high $(sFv')_2$ doses.

A number of sFv-based structures with potential as tumor-targeting agents have been prepared and tested. Pack and Pluckthun have described the production of sFv dimers using amphiphilic helices [16]. Other novel constructs, such as bispecific sFv dimers and fusion molecules composed of antitumor sFv and cytokines, are currently being pursued [17]. Additionally, human sFv molecules, now readily available from human hybridomas or through phage display techniques, may prevent the onset of anti-sFv antibody responses in future clinical trials [22]. Therefore, it is likely that the dosing strategies described here may be appropriate for a wide variety of applications.

While we have determined that large quantities of radiolabeled $(sFv')_2$ can be delivered to tumor with a high degree of specificity during their terminal distribution phase, their therapeutic potential must still be determined. Furthermore, the initial (1-h) values for blood, kidneys and lung were high (17.0%ID/ml, 10.7%ID/g and 14.2%ID/g respectively) as compared with about 5%ID/g in tumor. Accordingly the T:O ratios do not exceed 1:1 for these organs until 8 h after injection. This indicated that static %ID/g values are unlikely to provide accurate predictions of the therapeutic potential of these reagents. T:O ratios based upon area-under-the curve (AUC) analysis of the data may be a preferential method for the evaluation of therapeutic potential. In particular, the degree of toxicity that large transient doses of radiolabeled $(sFv')_2$ will impart to the marrow and kidneys will be a critical factor, especially when radiometal-chelated sFv are employed, because of their increased retention in some non-targeted organs (i.e. the kidneys). Studies are currently in progress to determine the early biodistribution characteristics of radioiodinated $(sFv')_2$ in sufficient detail to allow for the performance of formal AUC and predictive human dosimetry based upon the Medical Internal Radiation Dose (MIRD) formulations. However, difficulties have been encountered when applying the MIRD formulations to biodistribution data generated in the mouse [6]. Accordingly, therapy trials are currently underway to determine the potential therapeutic efficacy and toxicity of ^{131}I-741F8 $(sFv')_2$ in the tumor-bearing SCID mouse model.

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