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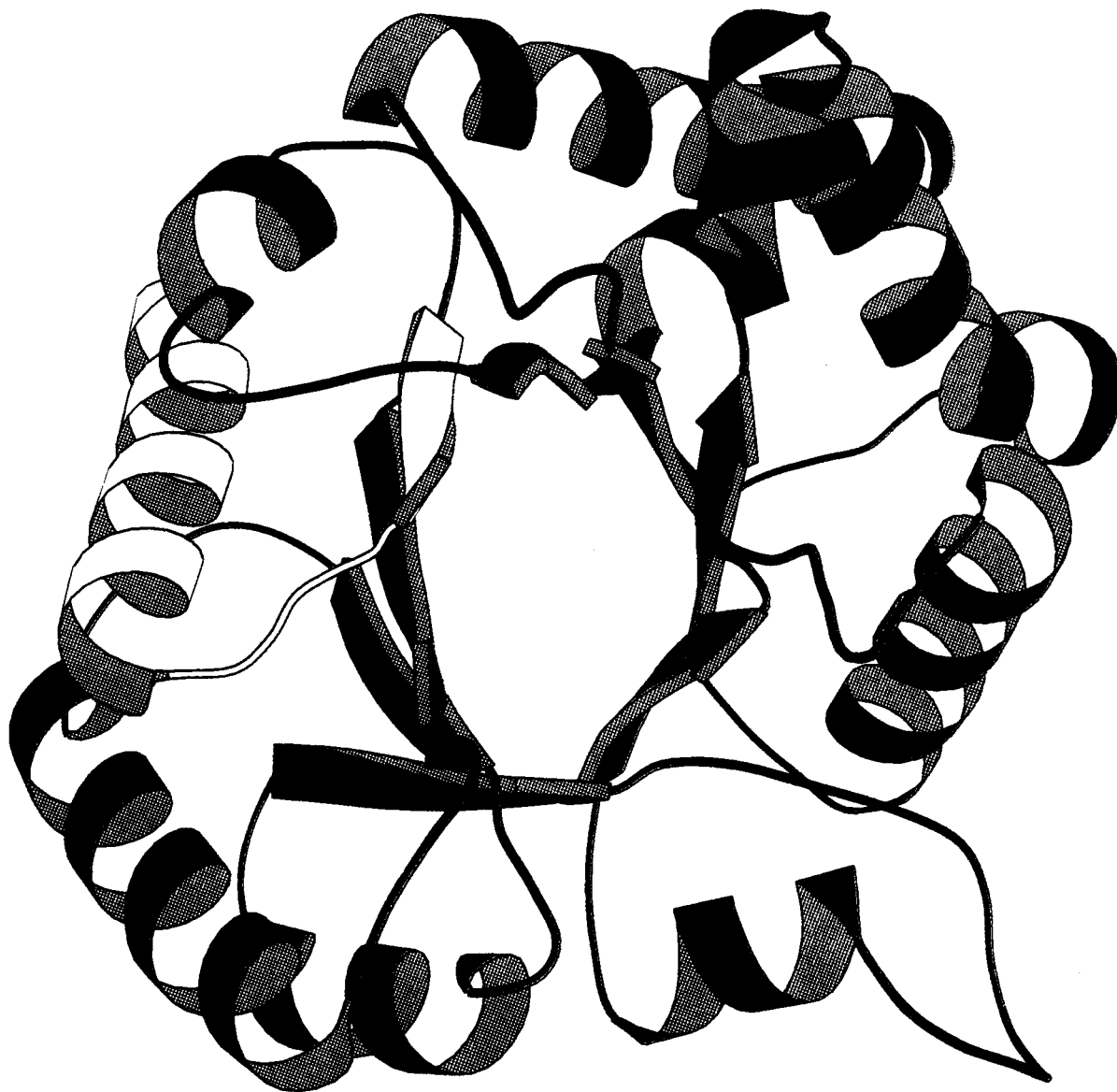
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An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability

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The effects of linker length on binding affinity and degree of aggregation have been examined in the anti fluorescein 4-4-20 and anticarcinoma CC49 single-chain Fvs. Longer linkers in the anti fluorescein sFvs have higher affinities for fluorescein and aggregate less. A proteolytically susceptible site between Lys8 and Ser9, in the previously reported 212 linker has been identified. A new linker sequence, 218 (GSTSGSGKPGSGEGSTKG) was designed in which a proline was placed at the C-terminal side of the proteolytic clip site in the 212 linker. The CC49 sFv containing the 218 linker showed reduced aggregation and was found to be more stable to proteolysis *in vitro*, when compared to the CC49/212 sFv. The CC49 sFv with the longer 218 linker had higher affinity than CC49/212 sFv. An aggregated CC49/212 sFv sample had higher affinity than CC49/218 sFv. The CC49/218 and CC49/212 sFvs had similar blood clearances in mice, while the aggregated CC49/212 sFv remained in circulation significantly longer. In mice bearing LS-174T human colon carcinoma xenografts, the CC49/218 sFv showed higher tumor uptake than the CC49/212 sFv and lower tumor uptake than the aggregated CC49/212 sFv. The higher tumor uptake of the CC49/218 is most likely a result of its higher resistance to proteolysis. The higher affinity and higher tumor uptake of the aggregated CC49/212 sFv are most likely due to the repetitive nature of the TAG-72 antigen and the higher avidity of multivalent aggregates. When the sFvs were radiolabeled with a lutetium-chelate the CC49/218 sFv showed a lower accumulation in the liver and spleen compared to the aggregated CC49/212 sFv.

Key words: aggregation/*in vivo* tumor targeting/linker design/proteolytic stability/single-chain Fv

Introduction

The variable portion (Fv) of a monoclonal antibody (MAb), comprising the variable heavy- and light chains (V_H and V_L), is the smallest portion of the molecule that consistently maintains the binding specificity and affinity of the whole antibody. The production of Fv fragments by proteolytic digestion of antibodies is difficult. However, recombinant techniques have made the

production of a Fv fragment possible (Skerra and Pluckthun, 1988). Although Fv fragments have limited utility *in vivo* due to the dissociation of the V_H and V_L , this problem has been overcome by genetically linking the V_H and V_L with a polypeptide linker to form a single-chain Fv (sFv) (Bird *et al.*, 1988; Huston *et al.*, 1988). [Single-chain Fvs have been called a variety of names including single-chain antigen-binding protein (SCATM protein), single-chain antibodies and biosynthetic antibody binding site (BABSTM).]

A number of studies have examined the effects of different linker designs on the properties of sFvs. Pantoliano *et al.* (1991) examined the effects of three different linkers on the affinity and stability of the anti fluorescein 4-4-20 sFv. Their results showed that both the stability and affinity of the 4-4-20 sFv increase with increasing linker length. However, some questions remain due to the different types of linkers used in these studies. Two of the three linkers in this study were thought to be similar, in that they were designed to be extended polypeptides with an underlying, alternating Gly-Ser sequence. The third was designed to be helical, with four repeats of the pentapeptide sequence Asp-Asp-X-Lys-Lys.

Batra *et al.* (1990) compared four different sFv immunotoxin constructions. All four had the truncated *Pseudomonas* exotoxin (PE40) as the C-terminal domain and an N-terminal anti-Tac sFv domain. The anti-Tac antibody binds to the p55 subunit of the interleukin-2 (IL-2) receptor. These investigators compared three V_L -linker- V_H -PE40 immunotoxins and a V_H -linker- V_L -PE40 immunotoxin for their ability to inhibit [³H]leucine incorporation (protein synthesis) in a variety of cell lines. All of the sFv immunotoxins demonstrated similar activities. These experiments would suggest that the choice of linker has little effect on the *in vivo* performance of an sFv.

In addition to single-chain Fvs, other single-chain proteins have been produced by linking two domains of a multidomain protein. Traunecker *et al.* (1991) have joined the first two N-terminal CD4 domains and an antihuman CD3 sFv, to produce a bispecific molecule that will bind HIV infected cells and human T cells. Novotny *et al.* (1991) and Soo Hoo *et al.* (1992) have constructed a single-chain T-cell receptor from the variable regions of the α - and β -chains encoding the anti fluorescein RFL3.8 and the cytotoxic T-lymphocyte clone 2C respectively. Some of the design criteria developed for single-chain Fvs will likely apply to the design of other single-chain proteins.

The CC49 MAb reacts with the TAG-72 antigen (Muraro *et al.*, 1988) which is found on a number of adenocarcinomas (colon, gastric and pancreatic). It is also found in carcinomas of the breast, lung (non-small cell), ovary and endometrium. Current clinical trials using ¹³¹I-labeled CC49 IgG have demonstrated efficient tumor targeting (Gallinger *et al.*, 1993). Due to its rapid pharmacokinetics, it is interesting to use radiolabeled CC49 sFv in diagnostic procedures or in conjunction with an intraoperative hand-held probe (Martin *et al.*, 1988). CC49 sFv also has potential as a therapeutic agent when conjugated with toxins, cytokines, drugs or pro-drug activating enzymes.

In our attempt to prepare a single-chain Fv of the anticancer

monoclonal antibody CC49 for clinical trials, we became aware of two problems with our current generation of linkers, namely proteolytic stability and degree of aggregation. In addition to the affinity and thermodynamic stability, the proteolytic stability and degree of aggregation may affect the *in vivo* performance and shelf-life of an sFv. We have examined the proteolytic stability and the effects of linker length, on the level of aggregation of the antitumor CC49 sFv. Finally, we have compared the *in vivo* biodistribution patterns in tumor-bearing mice of the radiolabel CC49 sFvs, using both radioiodine and ^{177}Lu as our radiolabels.

Materials and methods

Cloning and genetic constructions

The cloning of the 4-4-20 variable domains has previously been described by Bedzyk *et al.* (1989). The sequences of the variable domains of the CC49 have been reported in Mezes *et al.* (1989). The genetic construction of the 4-4-20/202' (Bedzyk *et al.*, 1990), 4-4-20/212 (Pantoliano *et al.*, 1991) and CC49/212 (Milenic *et al.*, 1991) sFvs have been previously described. (The following single-chain Fv nomenclature will be used throughout this paper: the name of the parent MAb from which a particular sFv is derived is followed by a slash and the name of the linker, for example, the sFv derived from the CC49 MAb with the 212 linker is called CC49/212.)

The 4-4-20/212 and CC49/212 sFv genes were converted to the 4-4-20/216 and CC49/218 sFv genetic constructions respectively, by oligonucleotide-directed mutagenesis. Single-stranded M13 templates, with the 212 linker versions of the sFv genes, were annealed to synthetic oligomers which encoded the desired insertions. The selection of the desired mutant genes was carried out by the thionucleotide substitution method (Nakamaye and Eckstein, 1986), as previously described (Whitlow and Filpula, 1991). The *Aat*II–*Bam*HI gene fragments were excised and ligated into an *Escherichia coli* expression vector containing hybrid λ phage promoter O_L/P_R (Scandella *et al.*, 1985) and an *ompA* signal sequence (Movva *et al.*, 1980). A final DNA sequence confirmation of the successful constructions was performed by the dideoxy method (Biggin *et al.*, 1983). To produce the final expression strains, the completed sFv expression vectors were transformed into *E. coli* host strain GX6712 which has the mutant temperature-sensitive repressor gene cl^{857} (Gottesman *et al.*, 1980) integrated into the chromosome. Fermentations of the sFv-producing *E. coli* strains were performed at 32°C using a casein digest–glucose salts medium. At an OD of 15–20 at 600 nm, the sFv expression was induced by a 42°C temperature shock for 1 h, as previously described (Whitlow and Filpula, 1991).

Purification

The purification of the sFvs has been previously described by Pantoliano *et al.* (1991) and Whitlow and Filpula (1991). Most of the sFv proteins were purified with a minor procedural modification, in which the initial cation exchange HPLC step, using the RCM Waters Accell Plus CM ion exchange column, was omitted.

Affinities of the anti fluorescein sFvs

The affinities were determined at 23.5°C using the fluorescence quenching assay described by Herron and Voss (1984) with a Perkin-Elmer LS-5 fluorescence spectrophotometer. After separating the monomer anti fluorescein sFv from its aggregates, using PolyCAT A cation-exchange HPLC described in Whitlow and Filpula (1991), the monomers were stabilized with a 1:1

molar ratio of fluorescein to sFv. The dissociation rates were determined at 23.5°C by adding 0.8 ml of a 5×10^{-8} M sFv–fluorescein solution and 0.2 ml of 1×10^{-5} M fluoresceinamine and recording the fluorescences at 1 min intervals after the addition (Herron, 1984). The affinity constants were determined using an association rate of $4.0 \times 10^6/(\text{M s})$.

Gel filtration HPLC chromatography

Gel filtration HPLC chromatography was used to quantitate the degree of aggregation and to separate monomeric sFv from the larger aggregates. Samples of 10–50 μl were injected onto a Waters HPLC system equipped with a 7.8×300 mm TSK G3000SW column (Toso Haas, Tokyo, Japan). The column had been previously equilibrated and the samples were eluted using 50 mM MOPS, 100 mM NaCl buffer, pH 7.5, at a flow rate of 0.5 or 1.0 ml/min. The data were collected on a MacIntosh SE (Apple Computer, Cupertino, CA) running the Dynamax software package (Rainin Instrument Co., Woburn, MA).

A GF-250 HPLC (DuPont, Wilmington, DE) was used to quantitate aggregates in the radiolabeled sFv preparations. The mobile phase was 0.25 M sodium acetate, 10% acetonitrile, pH 6.0, at a flow rate of 1.0 ml/min.

Proteolysis studies

CC49/212 and CC49/218 sFvs ($1.0 \pm 0.1 \times 10^{-5}$ M) were treated with $2.6 \pm 0.3 \times 10^{-9}$ M subtilisin BPN' (Type XXVII protease, Sigma, St Louis, MO) or 7.7×10^{-9} M trypsin at 37°C. At various times the proteolytic digestion was stopped by the addition of 2 μl of 0.1 M PMSF solution to a 200 μl sample. The percent intact sFv remaining was monitored by reverse phase HPLC at 60°C. A non-linear gradient of 5–70% acetonitrile, 0.1% TFA was run on a Waters HPLC system with a PLRP-S column (Polymer Labs, Church Stretton, UK) in a heating unit (Timberline Instruments, Boulder, CO), following the procedures of Nugent (1990). The data were collected on a MacIntosh SE as described above. The half-life ($t_{1/2}$) was determined from a semi-log plot of the fraction of intact sFv remaining versus time (see Figure 1) from the mean of three experiments using SigmaPlot 4.1 (Jandel Scientific, San Rafael, CA).

Radiolabeling of proteins

MAb CC49 and CC49 sFvs were labeled with Na^{125}I using Iodo-Gen (Pierce Chemical Co., Rockford, IL) as previously reported (Milenic *et al.*, 1991).

The CC49 sFvs were labeled with the lutetium complex of the macrocyclic bifunctional coordinator PA-DOTA (Cheng *et al.*, 1990). $^{177}\text{Lu}(\text{NO}_3)_3$ in 0.05 N HCl was obtained from the University of Missouri Research Reactor (Columbia, MO), as a 1 mM solution. Twenty microliters of a 1 mM solution of SCN-PA-DOTA in water was mixed with equal volumes of the ^{177}Lu solution and 1.0 M HEPES buffer, pH 7.0, and left at room temperature for 5 min. The reaction mixture was processed over a PRP-1 reverse-phase cartridge (Hamilton Co., Reno, NV) which had been equilibrated with 10% acetonitrile in 20 mM sodium carbonate, pH 9.5. ^{177}Lu -SCN-PA-DOTA was eluted with 1:2 acetonitrile–carbonate buffer and the 60 μl fraction containing the radioactive chelate was used for sFv labeling studies.

For radiolabeling with ^{177}Lu , 1 mg of each of the CC49 sFvs was buffer-exchanged with 20 mM sodium carbonate buffer, pH 9.5, then adjusted to 980 μl with the same buffer. The sample was mixed with 20 μl of the ^{177}Lu -SCN-PA-DOTA solution and left for 3 h at 37°C, followed by isolation of the radiolabeled sFv on a PD-10 column (Pharmacia, Piscataway, NJ) in PBS.

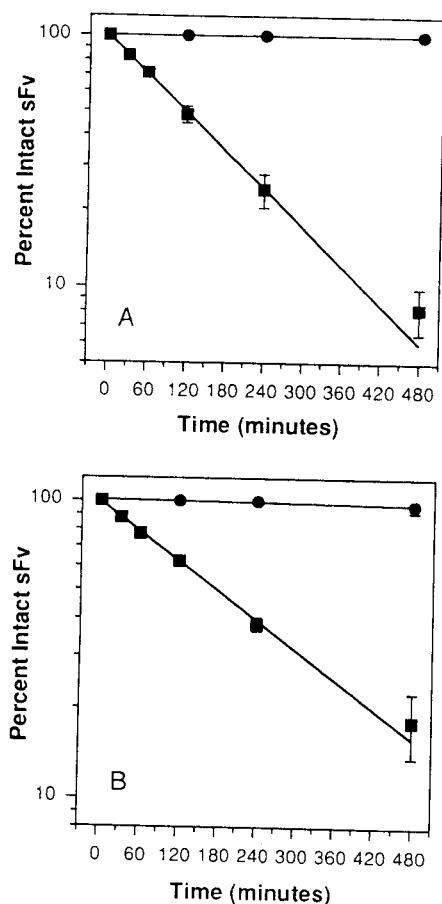


Fig. 1. Proteolytic susceptibility of the CC49/212 and CC49/218 sFv proteins in the presence of (A) subtilisin BPN' and (B) trypsin. The percent intact sFv remaining was determined by reverse phase HPLC at various times. The CC49/212 sFv is shown in closed squares and the CC49/218 sFv is shown in closed circles. The error bars represent one standard deviation and are often obscured by symbols.

Both the iodination and ^{177}Lu -labeling procedures resulted in >90% trichloroacetic acid precipitable counts and SDS-PAGE/autoradiography was used to confirm product integrity.

Competition radioimmunoassay

The immunoreactivities of the CC49 sFv preparations were assessed using a modification of a competition radioimmunoassay (RIA) that has been previously reported (Milenic *et al.*, 1991). Ten nanograms of bovine submaxillary mucin (BSM; Sigma, St Louis, MO) in 50 μl of PBS was adsorbed overnight at 4°C to each well of a microtiter plate. The wells were aspirated, then 50 μl of 5% bovine serum albumin (BSA) in PBS was added to each well. Following a 1 h incubation at 37°C, the wells were aspirated and serial dilutions of the competing molecule were added to the wells (in 25 μl of 1% BSA in PBS) along with ^{125}I -CC49 IgG (50 000 c.p.m. in 25 μl). Following an overnight incubation at 4°C, the plates were washed and the wells counted in a γ -scintillation counter (LKB-Pharmacia, Uppsala, Sweden). The percentage of inhibition was calculated.

Direct binding assays were carried out as described previously (Schott *et al.*, 1992) using BSM coated beads.

Biodistribution studies

Female athymic nude mice (nu/nu), obtained from Charles River (Wilmington, MA) at 4–6 weeks of age, were injected subcutaneously on the back with 1×10^6 LS-174T human colon carcinoma cells, under an NIH-approved protocol (Tom *et al.*,

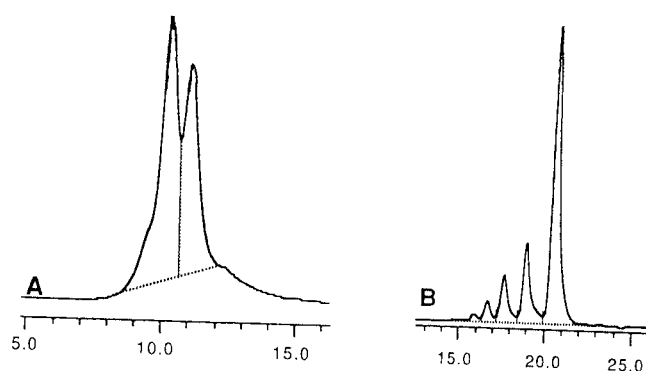


Fig. 2. HPLC size-exclusion chromatographs of the 4-4-20/212 and CC49/212 sFvs. (A) 4-4-20/212 sFv analyzed on a Waters Protein-Pak 300SW column with a flow rate of 1.0 ml/min. Two molecular populations are present, corresponding to mol. wts of 54 kD (dimers, left peak, eluting at 10.20 min) and 27 kDa (monomers, right peak, eluting at 10.98 min). (B) CC49/212 sFv analyzed on a Waters Protein-Pak 300SW column with a flow rate of 0.5 ml/min. The mixture of aggregates: a monomeric peak is visible (20.55 min, far right peak), as well as substantial amounts of what are believed to be dimeric (18.95 min, largest peak), trimeric (17.66 min) and higher multimeric species.

1976). Animals were used in the biodistribution studies when the tumors measured 0.5–0.8 cm in diameter, ~2 weeks after injection. Dual-label studies were performed with tumor-bearing mice injected, via the tail vein, with ~2–7.5 μCi of each ^{177}Lu -labeled CC49 sFv or 7.5 μCi of the radioiodinated CC49 sFv. Mice (three to four/data point were used in the ^{177}Lu studies; five/data point in the radioiodine studies) were killed at various times by exsanguination. The blood, tumor and all major organs were collected, wet-weighed and counted in a γ -scintillation counter. The percent injected dose per gram (%ID/g) was determined for each tissue.

Pharmacokinetics

The pharmacokinetic studies were performed by obtaining blood samples via tail bleeds at various times after administration of 7.5 μCi of the radioiodinated CC49 sFv. The values represent a mean of five mice.

Results

The overall yields for the 4-4-20 and CC49 sFvs were 25 and 8 mg/l fermentation broth respectively. All of the sFv preparations were judged to be at least 95% pure, based on SDS-PAGE analysis.

We designed, constructed, purified and assayed a 4-4-20 sFv with an 18-residue linker, 4-4-20/216 sFv. The affinity of this sFv for fluorescein was $1.24 \times 10^9/\text{M}$, using the fluorescence quenching assay. In this same assay the 4-4-20 Fab has an affinity of $1.99 \times 10^9/\text{M}$ (Pantoliano *et al.*, 1991). The dissociation rates of the 4-4-20/202', 4-4-20/212 and 4-4-20/216 sFvs were $8.2 \pm 1.4 \times 10^{-3}$, $4.86 \pm 0.58 \times 10^{-3}$ and $3.27 \pm 0.22 \times 10^{-3}/\text{s}$ respectively. The calculated affinities from these dissociation rates using an association rate of $4.0 \times 10^6/(\text{M s})$ are $0.49 \pm 0.07 \times 10^9$, $0.82 \pm 0.08 \times 10^9$ and $1.22 \pm 0.08 \times 10^9/\text{M}$ for the 4-4-20/202', 4-4-20/212 and 4-4-20/216 sFvs respectively. These compare well with the previously reported affinities of 0.49×10^9 and $1.07 \times 10^9/\text{M}$ for the 4-4-20/202' and 4-4-20/212 sFv respectively (Pantoliano *et al.*, 1991).

While attempting to crystallize various 4-4-20 sFvs, we concentrated each of the sFvs to over 5 mg/ml and noticed the existence of aggregates under a wide variety of conditions, as judged by size-exclusion HPLC chromatography (see Figure 2).

Table I. Single-chain Fv designs

Single-chain Fv	V _L	Linker	V _H	Linker name
4-4-20/202'	-KLEIE	GKSSGSGSES	TQKLD-202'	
4-4-20/212	-KLEIK	GSTSGSGKSSEGK	EVKLD-212	
4-4-20/216	-KLEIK	GSTSGSGKSSEGSGSTG	EVKLD-216	
CC49/212	-KLVK	GSTSGSGKSSEGK	QVQLQ-212	
CC49/218	-KLVK	GSTSGSGKPGSGEGSTG	QVQLQ-218	

Table II. Aggregation rates of the CC49/212 and CC49/218 sFvs

Protein	Concentration	Rate of aggregation	
	(mg/ml)	(%/h)	(%/day)
CC49/212	1.89	0.732	17.56
	0.49	0.120	2.88
CC49/218	1.49	0.0092	0.221
	0.62	0.00008	0.0018

At 5 mg/ml the levels of aggregation of the 4-4-20/202', 4-4-20/212 and 4-4-20/216 sFvs were 53, 34 and 10% respectively. The 4-4-20 sFv aggregation was easily reduced by simply diluting the samples.

A second discovery we made in trying to crystallize the 4-4-20/212 sFv was that the 212 linker was proteolytically susceptible. We thought we had crystallized the 4-4-20/212 sFv, but upon examination of the crystals it was found that the 4-4-20/212 sFv had been degraded to two 14 kDa polypeptides, as determined by SDS-PAGE analysis. Both the polypeptides and the crystals could be reproduced by treating the 4-4-20/212 sFv with subtilisin BPN' at a 5000:1 molar ratio. N-terminal sequencing of the sFv sample showed that the 4-4-20/212 sFv had been clipped in the linker between Lys8 and Ser9 (see Table I).

The proteolytic susceptibilities of the CC49/212 and CC49/218 sFvs were examined by determining the half-life ($t_{1/2}$) of the two sFvs in the presence of the proteases subtilisin BPN' and trypsin at 37°C. Molar ratios of 3000:1 and 1500:1 sFv to subtilisin BPN' and sFv to trypsin respectively were used. Under these conditions the half-life of the CC49/212 sFv is 118 min in the presence of subtilisin BPN' and 183 min in the presence of trypsin (see Figure 1). In the 8 h incubation period of these experiments, we were unable to detect any degradation of the CC49/218 sFv by subtilisin and only a 2.8% loss of material by trypsin. After 48 h incubations with subtilisin and trypsin the CC49/218 sFv showed 5.0 and 10.7% loss of material respectively.

The rates of aggregation of the CC49/212 and CC49/218 sFvs were determined at room temperature (22°C), various times and concentrations using size-exclusion chromatography. The CC49/212 sFv showed 80-fold faster accumulation of aggregates than did the CC49/218 sFv, at concentrations of ~1.5 mg/ml (see Table II). At 0.5 mg/ml this difference increased to 1600-fold. The highest level of aggregation observed with the CC49/212 sFv was 38%, whereas the highest level seen for the CC49/218 was only 2%.

The binding affinities for the TAG-72 antigen of the CC49/218, 'aggregate-free' CC49/212 and aggregated CC49/212 samples containing 32% aggregates were assessed using a competition radioimmunoassay (RIA). The binding affinities of the aggregated CC49/212, CC49/218 and aggregate-free CC49/212 sFvs were 15-, 125- and 1500-fold lower affinity than the CC49 IgG

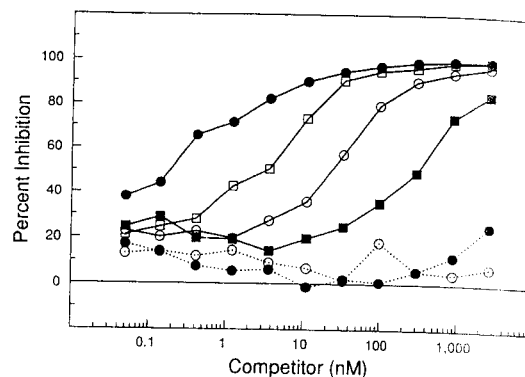


Fig. 3. Competition radioimmunoassay (RIA) in which unlabeled CC49 IgG (closed circles), CC49/212 sFv (closed squares), 32% aggregated CC49/212 sFv (open squares), CC49/218 sFv (open triangles), 4-4-20/212 sFv (open circles and dotted line) or BL-3 IgG (closed circles and dotted line) competed against a [¹²⁵I]CC49 IgG binding to bovine submaxillary mucin. 4-4-20/212 sFv and BL-3 IgG are non-specific controls.

Table III. Comparative biodistribution of the CC49/212, aggregated CC49/212 and CC49/218 sFvs radiolabeled with iodine^a

Organ	Linker	%ID/g			
		1 h	6 h	24 h	48 h
Tumor	212	4.5	2.7	0.9	0.8
	212-A ^b	6.8	4.5	3.1	3.6
	218	6.0	3.4	1.9	1.5
Blood	212	3.5	0.5	<0.1	<0.1
	212-A	5.5	1.0	0.1	<0.1
	218	4.4	0.9	<0.1	<0.1
Liver	212	1.6	0.2	<0.1	<0.1
	212-A	5.1	1.9	0.9	0.4
	218	2.0	0.5	0.1	<0.1
Spleen	212	1.8	0.3	<0.1	<0.1
	212-A	5.6	1.8	0.8	0.4
	218	2.6	0.6	0.2	<0.1
Kidney	212	12.3	0.7	0.1	<0.1
	212-A	10.2	1.0	0.2	<0.1
	218	13.3	0.9	0.2	0.1
Lung	212	2.7	0.4	<0.1	<0.1
	212-A	3.7	0.9	0.2	<0.1
	218	3.6	0.6	<0.1	<0.1

^a[¹²⁵I]CC49/218 and [¹³¹I]CC49/212 or [¹²⁵I]CC49/218 and aggregated [¹³¹I]CC49/212 (7.5 μCi each) were injected into athymic mice (five/group) bearing LS-174T tumor xenografts. The mice were killed at the indicated times and the %ID/g of the tumor and selected normal tissues were determined. The values of the [¹²⁵I]CC49/218 are a mean of both studies. ^b212-A: 31% aggregated CC49/212 sFv.

respectively (see Figure 3). All of the iodinated CC49 sFvs had immunoreactivity >90% using antigen-coated beads.

Dual-label studies were undertaken in which [¹³¹I]CC49/212 and [¹²⁵I]CC49/218 sFvs were compared in athymic nude mice bearing the 2-week-old LS-174T human colon carcinoma xenografts. A second experiment was conducted in which aggregated [¹³¹I]CC49/212 sFv was coinjected with [¹²⁵I]CC49/218 sFv. In all tissues (Table III), with the exception of the kidneys at 1 h, the aggregated CC49/212 sFv demonstrated a higher accumulation (%ID/g). The CC49/218 sFv yielded a %ID/g intermediate to the two conditions of the CC49/212 sFvs, which again was observed throughout the study in each of the tissues.

The plasma retention times of the aggregate-free CC49/212

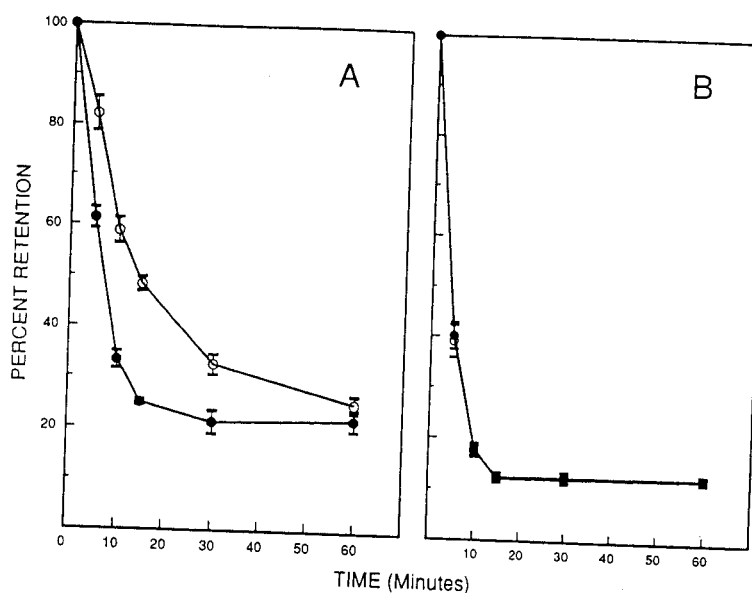


Fig. 4. Pharmacokinetics of CC49/212, CC49/218 and 32% aggregated CC49/212. Plasma retention of $[^{125}\text{I}]\text{CC49/218}$ (closed circles), aggregated $[^{125}\text{I}]\text{CC49/212}$ (panel A, open circles) and $[^{125}\text{I}]\text{CC49/212}$ (panel B, open circles) were performed in athymic mice (five mice) bearing LS-174T tumor xenografts.

sFv and the CC49/218 sFv were superimposable (see Figure 4B), both with apparent $t_{1/2}$ of 7.5 min. In contrast, the aggregated CC49/212 sFv demonstrated a 2-fold increase in the plasma retention time, with an apparent $t_{1/2}$ of 14 min (see Figure 4A).

The biodistributions of the ^{177}Lu -labeled CC49/212 (31% aggregated) and CC49/218 sFvs were determined in athymic mice bearing tumor xenografts. Of the six tissues examined, three tissues showed significant differences between the aggregated CC49/212 and CC49/218 sFvs when labeled with ^{177}Lu (see Table IV). The spleen and the liver showed 3- to 4-fold higher accumulations of the CC49/212 sFv compared to the CC49/218 sFv. At the 24 and 48 h time points, the aggregated CC49/212 sFv showed a 60% higher accumulation at the tumor.

Discussion

All of the sFv linkers were designed to span the ~ 35 Å between the C-terminus of the V_L and the N-terminus of the V_H . To provide flexibility, the linkers have an underlying sequence of alternating Gly and Ser residues. This rationale is similar to the $(\text{Gly}_4\text{-Ser})_n$ linkers first described by Huston *et al.* (1988). Unlike the $(\text{Gly}_4\text{-Ser})_n$ linkers, we have included three charged residues to enhance the solubility of a linker and its associated sFv; two positively charged residues (Lys) and one negatively charged residue (Glu). One of the Lys residues is placed close to the N-terminus of V_H , to replace the positive charge lost when forming the peptide bond between the linker and the V_H .

We previously reported on the stability and affinity of three anti-fluorescein single-chain Fvs (Pantoliano *et al.*, 1991). The data in these studies suggested that the affinity of the 4-4-20 sFvs for fluorescein increased with longer linkers. The data were not completely conclusive, since the longest linker, 205C, was thought to adopt a helical conformation. Thus, we designed, constructed, purified and assayed a 4-4-20 sFv with an 18-residue linker, which is four residues longer than the 212 linker and six residues longer than the 202' linker (see Table I). This new linker was designated 216. The anti-fluorescein sFvs 4-4-20/202', 4-4-20/212 and 4-4-20/216 had affinities of $0.49 \pm 0.07 \times 10^9$, $0.82 \pm 0.08 \times 10^9$ and $1.22 \pm 0.08 \times 10^9/\text{M}$ respectively,

Table IV. Comparative biodistribution of the CC49/212 and CC49/218 sFvs radiolabeled with ^{177}Lu

Organ	Linker	%ID/g			
		1 h	6 h	24 h	48 h
Tumor	212 ^a	2.4	2.0	2.2	1.6
	218	2.6	1.9	1.4	1.0
	212/218 ratio	0.9	1.0	1.6	1.6
Blood	212	1.8	0.2	<0.1	<0.1
	218	0.9	0.2	<0.1	<0.1
	212/218 ratio	2.0	1.0	—	—
Liver	212	7.4	9.4	5.5	4.0
	218	3.1	2.3	1.8	1.1
	212/218 ratio	2.4	4.1	3.1	3.6
Spleen	212	9.6	7.0	7.2	6.8
	218	3.1	2.1	1.9	1.6
	212/218 ratio	3.1	3.3	3.8	4.2
Kidney	212	241.1	219.1	197.6	156.1
	218	303.9	266.0	222.9	161.5
	212/218 ratio	0.8	0.8	0.9	1.0
Lung	212	1.7	0.8	0.7	0.5
	218	1.3	1.0	0.6	0.5
	212/218 ratio	1.3	0.8	1.2	1.0

^aThirty-one percent aggregated CC49/212 sFv.

based on their dissociation rates. The data demonstrate that, for the anti-fluorescein 4-4-20 sFv series, longer linkers correlate with enhanced binding affinity, although the overall difference in binding affinity is only a factor of 2.5.

While attempting to crystallize various 4-4-20 sFvs, we concentrated each of the sFvs to over 5 mg/ml and noticed the existence of aggregates under a wide variety of conditions, as judged by size-exclusion HPLC chromatography. We have made the following observations about the 4-4-20 sFv aggregates.

- The aggregates were easily reduced by simply diluting the 4-4-20 sFv samples.
- We have found an inverse relationship between degree of

aggregation and linker length. The 4-4-20 sFvs with shorter linkers showed higher degrees of aggregation. At 5 mg/ml the 4-4-20/202' sFv sample contained 53% aggregate, whereas the 4-4-20/212 and 4-4-20/216 samples showed 34 and 10% aggregation respectively.

- (iii) The aggregates can be stabilized by the addition of the antigen fluorescein.
- (iv) The aggregation was linker dependent. If the 212 linker in 4-4-20/212 is clipped with subtilisin BPN', the resulting active Fv no longer forms aggregates.

N-terminal sequencing of the proteolytically clipped 4-4-20/212 sFv showed that it had been clipped in the linker between Lys8 and Ser9 (see Table I), effectively converting the sFv to an Fv. This discovery explained the two 14 kDa bands we had observed on SDS-PAGE in the late stages of our standard sFv preparations.

The aggregation of the antitumor CC49/212 sFv was significantly different from that of the 4-4-20 sFvs. Higher aggregates were seen with the CC49/212 sFv than were seen with the 4-4-20/212 sFv (see Figure 2). Like the 4-4-20 sFvs, the CC49 sFvs aggregation was concentration dependent, yet the rate at which the CC49 sFv formed these aggregates was significantly slower. This made it possible to isolate a dimeric CC49 sFv, which was stable for a week or more and was found to have a 10-fold higher binding affinity than the monomeric CC49/212 sFv (M. Whitlow and D. Milenic, unpublished results). We have crystallized the CC49/212 sFv dimer (Essig *et al.*, 1993). A better understanding of the 4-4-20 and CC49 sFv aggregations are the subject of future investigations.

We had two objectives in designing a new linker: first, we wanted to reduce the proteolytic susceptibility of the linker and, second, we wanted to reduce aggregation of the sFvs. In order to reduce the proteolytic susceptibility of the sFvs we sought to protect the peptide bond between Lys8 and Ser9 in a new linker. Most proteases are unable to cleave peptide bonds at the N-terminal side of a proline. Since polypeptides containing prolines have one less degree of freedom (loss of rotation around the proline's ϕ torsion angle) due to the five-membered ring, they are prohibited from adopting an extended conformation characteristic of peptides bound in protease active sites. This probably accounts for the reduced proteolytic susceptibility of polypeptides containing prolines. In order to reduce aggregation we decided to use a long 18-residue linker, similar to the 216 linker described above. The first 11 residues of the 216 linker are identical to the 212 linker, including the proteolytically susceptible peptide bond between Lys8 and Ser9. Therefore, a new linker was designed that places a proline at position 9, adjacent to Lys8, in an 18-residue linker similar to 216. Other minor sequence changes were made between residues 10 and 13, in order to maintain the alternating Gly-Ser sequence and to more evenly distribute the charged amino acids. This linker was designated 218 (see Table I).

The 212 and 218 linkers were then compared in CC49 sFvs. The CC49 MAb binds to the TAG-72 antigen found in most human carcinomas. The 218 linker has significantly improved protease resistance; in the 8 h incubation period of these experiments, we were unable to detect any degradation of the CC49/218 sFv by subtilisin and only a 2.8% loss of material by trypsin. After 48 h incubation with subtilisin and trypsin the CC49/218 sFv showed 5.0 and 10.7% loss of material respectively. In contrast, 50% of the CC49/212 sFv was degraded in ~2 h (118 min) in the presence of subtilisin BPN' and in ~3 h (183 min) in the presence of trypsin (see Figure 1).

The rates of aggregation of the CC49/212 sFv showed 50-fold faster accumulation of aggregates than with the CC49/218 sFv, at concentrations of ~1.5 mg/ml (see Table II). At 0.5 mg/ml this difference increased to 1600-fold. While the reproducibility of these experiments was poor, no matter what experimental conditions were tried, the CC49/218 sFv consistently showed less aggregation than did the CC49/212 sFv. The highest level of aggregation observed with the CC49/212 sFv was 38% (see Figure 2B), whereas the highest level seen for the CC49/218 sFv was only 2%. The aggregation of both proteins showed a concentration dependence, in that higher levels of aggregation were seen at higher sFv concentrations.

The CC49/218 sFv showed an ~12-fold higher binding affinity for the TAG-72 antigen than did the CC49/212 sFv, which was confirmed to be aggregate-free and a 125-fold lower affinity than the CC49 IgG (see Figure 3). To demonstrate the effect of aggregation on the binding affinity of the CC49/212 sFv for TAG-72, a sample containing 32% aggregates was included in the competition RIA. The binding affinity of the aggregated CC49/212 sFv was closer to that of the intact CC49 IgG and was ~100-fold greater than the aggregate-free CC49/212 sFv preparation. This is consistent with our previous studies in which we demonstrated that the bivalent immunoglobulin forms of CC49 [IgG and F(ab')₂] compete with a 10-fold higher avidity than do the monovalent forms (Fab and sFv) (Milenic *et al.*, 1991). Since aggregates are multivalent, it seems likely that they would have higher affinity. Two reasons may account for the quantitative differences between this study and the previous study: (i) the previous study may have been performed with aggregated CC49/212 sFv and (ii) bovine submaxillary mucin was used in this study while a TAG-72-positive extract of human colon carcinoma xenografts was used in the previous study. The use of bovine submaxillary mucin seems to result in a more sensitive competition RIA.

Both the observation that longer linkers result in less aggregation and that linkers could be proteolytically susceptible, have possible implications for the *in vivo* and therapeutic applications of sFvs. Aggregation *in vivo* could result in accumulation of sFvs in non-target organs such as the spleen and liver, due to clearance of aggregates by the reticulo-endothelial system. However, the proteolysis of an sFv to an Fv is likely to result in a loss of affinity. In order to examine the behavior of CC49/212 and CC49/218 sFvs *in vivo* we chose to look at these effects in a human tumor model system, namely LS-174T tumor xenografts in athymic mice. In all tissues (Table III), with the exception of the kidneys at 1 h, the aggregated CC49/212 sFv demonstrated a higher accumulation (%ID/g). The CC49/218 sFv yielded a %ID/g between those of the CC49/212 sFvs (aggregated and aggregate-free), which again was observed throughout the study in each of the tissues.

The longer retention of the aggregated CC49/212 sFv was also observed in its pharmacokinetic behavior (see Figure 4). The plasma pharmacokinetics of the CC49/218 and aggregate-free CC49/212 sFvs were superimposable and similar to the one we previously reported (Milenic *et al.*, 1991). In contrast, the aggregated CC49/212 sFv (Figure 4A) demonstrated a 2-fold increase in the apparent $t_{1/2}$ of the CC49 sFv. In fact its plasma retention is similar to that of the CC49 Fab'. We have previously shown that the plasma retention is correlated with the size of the molecule in circulation (Milenic *et al.*, 1991). The increased plasma retention time of the aggregated CC49/212 sFv is likely due to its increased size and is likely the cause of its higher %ID/g in all tissues. This does not explain the higher %ID/g of the

CC49/218 sFv. The higher levels of the CC49/218 sFv observed in the tumor, as well as normal tissues, might be the result of its higher resistance to proteolysis.

Three tissues, of the six tissues examined, showed significant differences between the 31% aggregated CC49/212 and CC49/218 sFvs in the ^{177}Lu biodistribution study (see Table IV). The spleen and the liver showed 3- to 4-fold higher accumulations of the CC49/212 sFv compared to the CC49/218 sFv. At the 24 and 48 h time points, the aggregated CC49/212 sFv showed a 60% higher accumulation at the tumor. We suspect that the higher level of CC49/212 sFv accumulation in the spleen and liver is due to the higher degree of aggregation of the injected sample. Both the spleen and liver metabolize the sFvs. The accretion of CC49 sFv in the liver and spleen appears to be antigen-mediated (Yokota *et al.*, 1993). The presence of antigen in these organs could be due to (i) the uptake and processing of TAG-72 by macrophages resulting in the presentation of the CC49 epitope on the cell surface; (ii) the formation of CC49 sFv complexes with circulating TAG-72 which are deposited in the liver and spleen or (iii) the formation of CC49 sFv-TAG-72 complexes at the tumor, which are then shed and deposited in the liver and spleen. The higher levels of CC49/212 sFv seen in the liver and spleen, as well as in the tumor at later times, may be due either to the increased avidity and/or the longer circulation time of the aggregates. The very high levels of accumulation of both sFvs in the kidneys, probably reflects the catabolism of the protein by the kidneys, with subsequent retention of the ^{177}Lu (Schott *et al.*, 1992).

Conclusions

We have confirmed the observation of others that the linker length in an sFv affects binding affinity. Within the 12- to 18-residue linker range, sFvs with longer linkers tend to have higher affinities. We have shown that the longer linkers correlate with decreased aggregation and have identified a proteolytically susceptible site in the previously utilized 212 linker.

We have designed a new linker sequence, 218, which is less susceptible to proteolysis due to the placement of a proline on the C-terminal side of the proteolytic clip site in the 212 linker. The new 218 linker, containing 18 amino acids, is four residues longer than our previously reported 212 linker. The CC49 sFv containing the 218 linker shows reduced aggregation when compared to the CC49/212 sFv, along with a lower accumulation in the liver and spleen *in vivo*. The CC49/218 sFv shows a similar plasma clearance to the CC49/212 sFv, yet higher levels of CC49/218 sFv were found in all the tissues examined. This might be the result of the higher resistance of the 218 linker to proteolysis. Hence, the 218 linker may have general utility in a variety of sFvs.

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