

# Penetratin Improves Tumor Retention of Single-Chain Antibodies: A Novel Step toward Optimization of Radioimmunotherapy of Solid Tumors

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## Abstract

Single-chain Fv (scFv) antibody fragments exhibit improved pharmacokinetics and biodistribution compared with intact IgG. The tumor uptake of scFvs is rapid, and the serum half-life is shorter than IgG. However, scFvs exhibit lower net dose deposition in the tumor due to a shorter residence time that limits their use in radioimmunotherapy. To improve the tumor uptake and retention of scFvs, we investigated the utility of cell-penetrating peptides, penetratin and transactivator of transcription (TAT). Biodistribution studies were done in LS174T tumor-bearing mice with divalent scFv derived from anti-tumor-associated glycoprotein 72 monoclonal antibody (mAb) CC49. Penetratin increased the tumor retention of scFvs without affecting the peak dose accumulation. The percentage of doses retained in tumors at 24 hours postadministration with a control (no peptide), penetratin, and TAT were 27.25%, 79.84%, and 48.55%, respectively, of that accumulated at 8 hours postinjection. The tumor-to-blood ratios at 24 hours postadministration were 7.14, 19.53, and 16.48 with control, penetratin, and TAT treatment, respectively, whereas the pharmacokinetics were unaltered. Coinjection with TAT, however, resulted in increased uptake of the radioconjugate by the lungs. Autoradiography of the excised tumors indicated a more homogenous distribution of the radiolabeled scFv with both penetratin and TAT in comparison with the control treatment. Real-time whole-body imaging of the live animals confirmed improved tumor localization with penetratin without any increase in the uptake by normal tissues. In conclusion, a significant improvement in the tumor retention of sc(Fv)<sub>2</sub> was achieved by administration of penetratin. Therefore, the combination of penetratin and scFvs has the potential of improving the utility of mAb-based radiopharmaceuticals. (Cancer Res 2005; 65(17): 7840-6)

## Introduction

Radioimmunotherapy is a rapidly developing therapeutic modality that combines the advantages of specific tumor targeting of anticancer monoclonal antibodies (mAb) with cytotoxic properties of therapeutic radionuclides. Currently, mAbs directed against

tumor antigens, such as carcinoembryonic antigen (CEA), tumor-associated glycoprotein 72 (TAG-72), and prostate-specific antigen, are being used in clinics for tumor imaging, whereas radiolabeled antibodies, such as Bexxar and Zevalin (directed against CD20), have been approved by the Food and Drug Administration for the therapy of non-Hodgkin's lymphoma (1-4). Unfortunately, only modest success has been achieved with tumor-antigen targeted intact antibodies as vehicles to deliver diagnostic and therapeutic agents to solid tumors (5). The major impediments include long circulation times and poor penetration, both attributable to the higher molecular weight of intact IgG.

Genetically engineered single-chain Fvs (scFv) have emerged as powerful alternatives to intact IgG. scFvs exhibit a shorter serum half-life and a rapid peak dose deposition due to their smaller size (6). Recently, patient trials have been done for an engineered anti-CEA minibody cT88.66, where <sup>123</sup>I-labeled minibody successfully targeted colorectal cancer and exhibited faster blood clearance than intact antibody (7). We have previously generated and evaluated monovalent, divalent, and tetravalent scFv constructs of anti-TAG-72 mAb CC49 (8-10). The divalent [sc(Fv)<sub>2</sub>] and tetravalent ([sc(Fv)<sub>2</sub>)<sub>2</sub>] constructs are of particular interest due to the pharmacologic advantage of these molecules over larger IgG and smaller monovalent scFv fragments. These scFvs seem to be the molecules of choice for effective therapeutic dose delivery with minimal side effects (6, 8). However, due to their rapid elimination from circulation, the net dose deposition of scFvs is lower than the intact antibodies, and their lower affinity results in short tumor residence times, thus limiting their clinical potential.

Cell-penetrating peptides (CPP) have been successfully used as "Trojan horses" for intracellular delivery of cargo molecules, such as peptides, DNA, proteins, and liposomes, across cell membranes in a receptor- and energy-independent manner (11, 12). Despite early doubts about the upper molecular weight limit for their cargoes, it has been shown that CPPs can deliver proteins as big as green fluorescent protein (30 kDa), avidin (63 kDa), β-galactosidase (120 kDa), and even IgG (13-16). Transactivator of transcription (TAT) and penetratin, the two most studied CPPs, are basic peptide sequences derived from the HIV TAT protein (amino acids 49-57) and homeodomain of *Drosophila* Antennapedia protein (amino acids 43-58), respectively. Although most of the studies using CPPs have been done *in vitro*, TAT and penetratin have been shown to enhance the delivery of proteins and viruses *in vivo* (16, 17). The utility of CPPs to improve the biodistribution of radiolabeled antibodies in animal models has not been well researched.

We hypothesized that CPPs, if used in conjunction with single-chain antibodies, will result in an increased penetration of radiolabeled scFvs into the tumor, thereby enhancing the tumor

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doi:10.1158/0008-5472.CAN-05-0662

uptake. Additionally, most of the target antigens for solid tumor radioimmunotherapy are cell surface molecules; therefore, increased cellular penetration will render the tumor-deposited antibody resistant to clearing and thus prolong the tumor residence of the therapeutic molecule. In the present study, we investigated the effect of CPPs penetratin and TAT on the biodistribution of a  $^{125}\text{I}$ -labeled, divalent  $[\text{sc}(\text{Fv})_2]$  derivative of mAb CC49 in LS174T colon carcinoma xenograft-bearing mice. Coadministration of penetratin resulted in improved retention and homogenous distribution of scFvs in tumors without alteration in pharmacokinetics and uptake by nontarget tissues.

## Materials and Methods

**Protein expression and purification.** For protein production and purification, the previously described protocol was followed (9). Briefly, *Pichia pastoris* cells transfected with the scFv construct were grown at  $30^\circ\text{C}$  ( $A_{600\text{ nm}}$ , 2-6) in buffered glycerol complex medium containing 100  $\mu\text{g}/\text{mL}$  zeocin. The cultures were induced by the addition of 0.5% methanol every 24 hours. At 96 hours postinduction, the culture was harvested by centrifugation and the supernatant containing the scFv was subjected to immobilized metal affinity chromatography using the nickel-nitrilotriacetic acid (Superflow, Qiagen, Inc., Valencia, CA). Bound fragments were eluted in 50 mmol/L sodium phosphate buffer (pH 7.2) containing 300 mmol/L NaCl and 250 mmol/L imidazole. Tetravalent and divalent scFvs were separated from aggregated and breakdown product using size exclusion chromatography by a Superdex 200 column. The purity of protein was confirmed by SDS-PAGE and the immunoreactivity was ascertained by solid-phase competition ELISA.

**Radiolabeling of CC49 sc(Fv)<sub>2</sub> and quality control assessment.** The scFvs were labeled with  $\text{Na}^{125}\text{I}$  using 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycoluril (Iodo-Gen; Pierce Chemical, Rockford, IL) as the oxidant (18). Briefly, protein (100  $\mu\text{g}$ ) in 0.1 mol/L sodium phosphate buffer (pH 7.2) was transferred into a  $12 \times 75$  mm glass tube coated with 20  $\mu\text{g}$  Iodo-Gen. The protein was incubated for 3 minutes with 100  $\mu\text{Ci}$   $\text{Na}^{125/131}\text{I}$  (NEN, Boston, MA) followed by gel filtration to remove free radiolabel. The amount of free radioactivity associated with protein was determined by instant TLC (ITLC).

SDS-PAGE was done as described by Laemmli (19) to analyze the radiolabeled protein under reducing and nonreducing conditions. Dried gels were exposed to Rad PADD and imaged using a Kodak (Rochester, NY) Image Station 2000MM.

Immunoreactivity of radiolabeled CC49 scFv forms was tested by RIA, where the bovine submaxillary mucin (BSM) or bovine serum albumin (BSA; positive and negative controls, respectively) were attached to a solid-phase matrix (Reacti-Gel HW-65F; Pierce Chemical; ref. 8). Binding was allowed to proceed for 1 hour at room temperature in either absence or presence of penetratin or TAT peptide. The unbound radiolabeled protein was removed by repeated washing with PBS containing 1% BSA and 0.1% Tween 20 followed by centrifugation. The pellet and wash supernatant were measured for radioactivity in a gamma scintillation counter [Perkin-Elmer (Shelton, CT) Wizard 3" 1480], and the total percentage bound and free counts were calculated.

Gel filtration on high-performance liquid chromatography (HPLC) was also used to analyze any degradation or aggregation of the radiolabeled scFvs (8). Samples were injected onto TSK G2000SW and TSK G3000SW (Toso Haas, Tokyo, Japan) connected in a series with 67 mmol/L sodium phosphate buffer (pH 6.8), 0.1 mol/L KCl as the mobile phase. The columns were calibrated using the Gel Filtration Calibration kit (Bio-Rad, Hercules, CA). The elution was monitored by an in-line UV detector at 280 nm, and the radioactivity was determined in a gamma scintillation counter.

**Biodistribution and pharmacokinetics studies.** All the animal studies were done in accordance with protocol no. 00-084-09 obtained from Institutional Animal Care and Use Committee. For biodistribution studies,  $4 \times 10^6$  LS174T cells, a human colon carcinoma cell line, were implanted s.c. in female athymic mice (*nu/nu*; 4-6 weeks old; Charles River, Wilmington, MA). The mice were used 10 to 14 days following implanta-

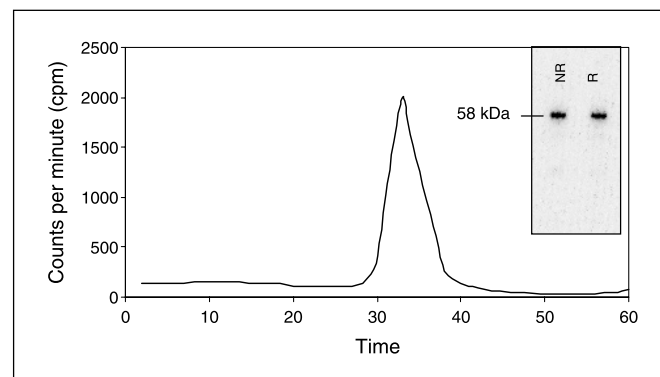
tion (tumor volume,  $\sim 200\text{-}300\text{ mm}^3$ ). Biodistribution studies were done after an injection of  $[\text{I}^{125}]\text{sc}(\text{Fv})_2$  (5  $\mu\text{Ci}/\text{animal}$ ) via the tail vein either alone or in conjunction with 200  $\mu\text{L}$  of 12.5  $\mu\text{mol}/\text{L}$  penetratin (RQIKIWFQNRRMKWKK) or TAT (YGRKKRRQRR) peptides (Anaspec, San Jose, CA). Experiments were also done where the penetratin and TAT were administered either 30 minutes before or 2 hours after the injection of radiolabeled scFvs. At designated times, groups ( $n = 5$ ) were euthanized, and the tumor, blood, and major organs were removed, weighed, and counted in a gamma scintillation counter to determine the  $\% \text{ID}/\text{g}$  for radiolabeled protein.

**Real-time whole-body imaging.** For the whole-body imaging studies, mice bearing the LS174T xenografts (three per group) were injected via the tail vein with 20  $\mu\text{Ci}$  radiolabeled scFv either alone or in combination with 10 nmol penetratin or TAT. At indicated time points, animals were anesthetized with an i.p. injection of 350  $\mu\text{L}$  mixture (4:1) of ketamine (100 mg/mL) and xylazine (20 mg/mL) diluted 10 times in sterile water. The mice were placed on Rad PADD and the images were acquired for 3 minutes (3 hours postadministration) and 6 minutes (24 hours postadministration) using a Kodak Image Station 2000MM. Images were analyzed by Kodak 1D software (version 3.6). Regions of interest (ROI) were defined for liver and tumors, and the mean ROI intensities were determined. The values were corrected for background and exposure time.

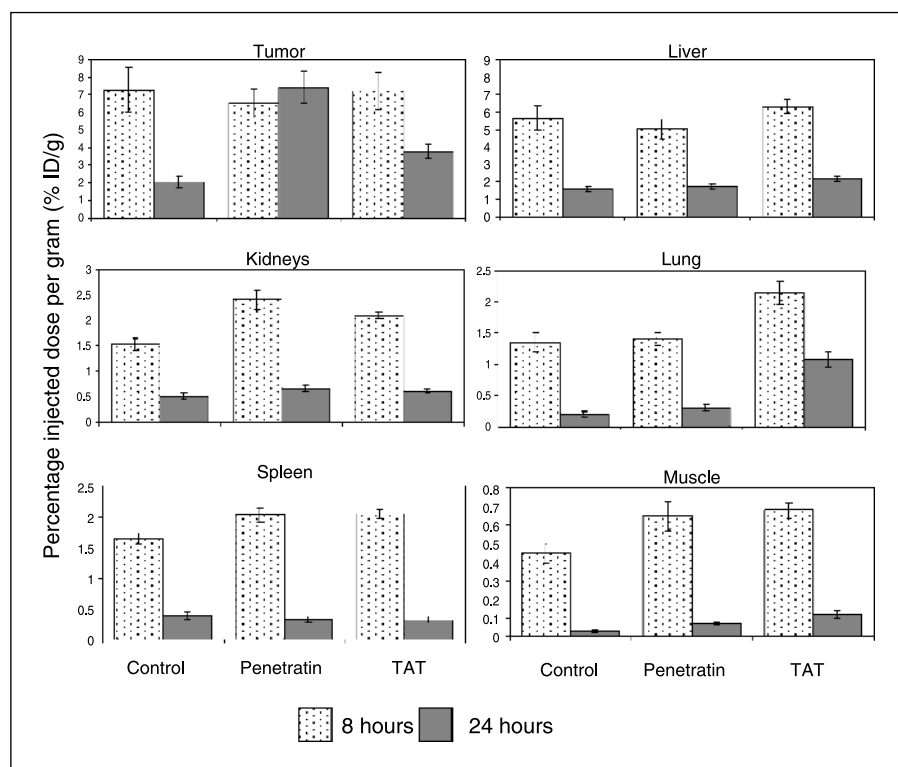
**autoradiography.** Tumors excised during biodistribution studies were counted immediately for radioactivity measurements in the gamma counter and snap frozen. Blocks were prepared on dry ice using Tissue-Tek OCT compound (Sakura, Tokyo, Japan) and 10  $\mu\text{m}$  thick cryosections were cut using CM 1850 cryostat (Leica, Nussloch, Germany). The cryosections were transferred to Superfrost Plus slides and exposed to Kodak BioMax MR Film for 72 hours (20) and analyzed using Kodak 1D software.

## Results

**Radiolabeling and quality control analyses.** Following radiolabeling and desalting,  $>99\%$  of the radioactivity was associated with the  $\text{sc}(\text{Fv})_2$  fraction as determined by ITLC. The SDS-PAGE and HPLC analyses of the radiolabeled protein indicated no apparent degradation or aggregation of  $\text{sc}(\text{Fv})_2$  (Fig. 1). The immunoreactivity of the radiolabeled protein was not affected by radioiodination or the presence of the penetratin and TAT. As determined by RIA using BSA- and BSM-coated beads, the specific immunoreactivity of  $\text{sc}(\text{Fv})_2$  (expressed as percent bound radioactivity) with BSM (antigen) was 76.18, whereas the values in the presence of penetratin and TAT peptides were 76.23 and 75.91, respectively. The nonspecific reactivity of the radiolabeled construct with BSA-coated beads was 4.01, 4.85, and 4.51 for control, penetratin, and TAT, respectively.



**Figure 1.** HPLC profile of  $^{125}\text{I}$ -labeled  $\text{sc}(\text{Fv})_2$ . The radiolabeled protein eluted as a single peak with a retention time of 33 minutes. Autoradiograph of radiolabeled protein following SDS-PAGE (*inset*) indicates no apparent degradation of the protein under reducing (R) or nonreducing (NR) conditions.



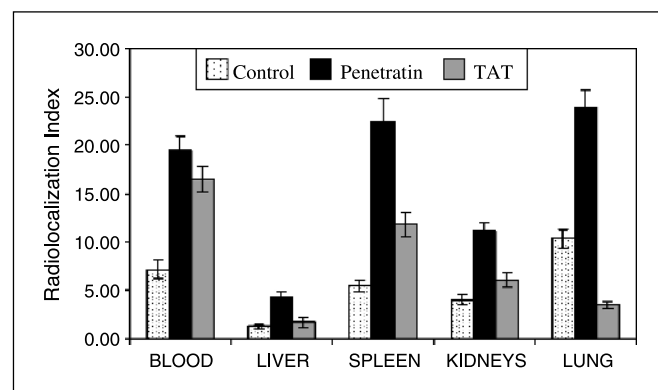
**Figure 2.** Biodistribution of sc(Fv)<sub>2</sub> in LS174T tumor-bearing athymic mice. Animals were injected via the tail vein with sc(Fv)<sub>2</sub>, either alone or in combination with 12.5 μmol/L penetratin or TAT peptide. The animals were sacrificed 8 or 24 hours postadministration, and their organs were removed, weighed, and counted for radioactivity in a gamma counter. Uptake of radioactivity was expressed as %ID/g.

**Biodistribution of sc(Fv)<sub>2</sub> in the presence of cell-penetrating peptides.** There was no change in the biodistribution and tumor retention of the of scFvs when the CPPs were injected either 30 minutes before or 2 hours after the administration of sc(Fv)<sub>2</sub> (data not shown). CPPs altered the biodistribution and tumor retention of radiolabeled sc(Fv)<sub>2</sub> only when they were coadministered with the scFvs. Hence, all the experiments reported here were done by injecting radiolabeled sc(Fv)<sub>2</sub> either alone (control) or in conjunction with penetratin or TAT peptides.

We have determined previously that peak dose accumulation of sc(Fv)<sub>2</sub> occurs at ~8 hours postadministration. Therefore, to examine the total uptake, 8-hour time point was selected and the retention of the peak accumulated dose was studied at 24 hours postadministration. At 8 hours postinjection, the %ID/g values in the tumors were  $7.46 \pm 1.47$ ,  $6.49 \pm 1.00$ , and  $7.19 \pm 1.42$  for the control (no peptide), penetratin, and TAT, respectively, and were not significantly different ( $P > 0.005$ ). At 24 hours postadministration, the values were  $2.07 \pm 0.14$ ,  $7.42 \pm 1.44$ , and  $3.79 \pm 0.39$  for the control, penetratin, and TAT, respectively (Fig. 2). These values were significantly different for control versus penetratin ( $P = 0.0014$ ), for control versus TAT ( $P = 0.0008$ ), and for penetratin versus TAT ( $P = 0.0023$ ). At 24 hours, in comparison with the control treatment, the %ID/g values in tumor were 1.82- and 3.53-fold higher with the coadministration of TAT and penetratin, respectively. Thus, it was evident that both penetratin and TAT peptide treatments resulted in a significant retention of sc(Fv)<sub>2</sub> in the tumor without affecting the peak uptake. Coinjection with penetratin did not affect the dose accumulation in the liver and lungs; however, there was a slight increase in the uptake by kidneys (2.1 with penetratin versus 1.53 with control), spleen (2.03 with penetratin versus 1.64 with control), and muscle (0.38 with penetratin versus 0.24 with control) 8 hours postinjection.

Treatment with the TAT peptide resulted in an increased uptake in nearly all the normal tissues examined in comparison with control and penetratin treatment. The %ID/g values were 6.27, 2.04, 2.40, and 2.14 for the liver, spleen, kidneys, and lungs, respectively, at 8 hours postinjection (Fig. 2). At 24 hours postadministration, the %ID/g values in liver, kidneys, and spleen were comparable for control, penetratin, and TAT. However, with the coinjection of TAT, there was increased retention of sc(Fv)<sub>2</sub> in lungs ( $1.08 \pm 0.12$ ) in comparison with that observed in penetratin ( $0.31 \pm 0.04$ ) and control groups ( $0.2 \pm 0.05$ ).

The radiolocalization index (RI), which is the ratio of %ID/g values of the tumor to normal tissues, was also determined 24 hours postadministration (Fig. 3). RIs for blood were  $7.14 \pm 0.97$ ,  $19.53 \pm 1.38$ , and  $16.48 \pm 1.30$  for the control, penetratin, and

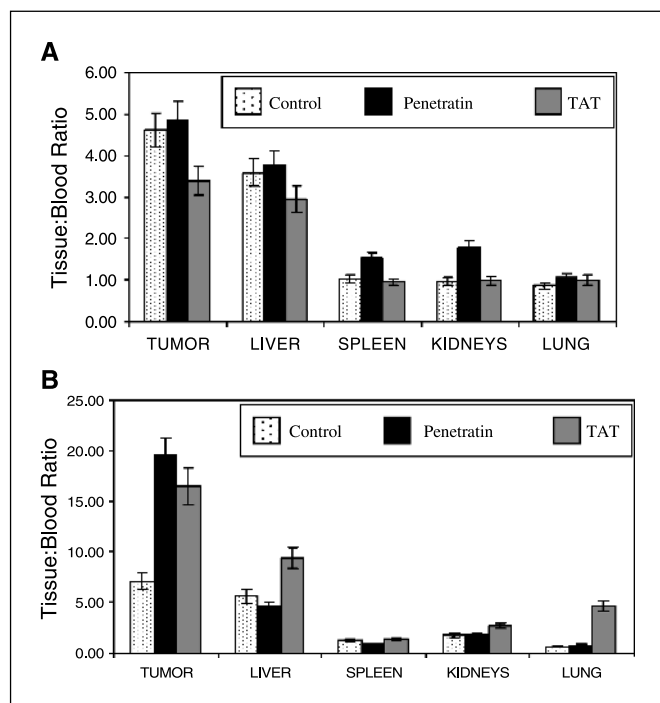


**Figure 3.** RIs (ratio of %ID/g tumor to %ID/g tissue) of sc(Fv)<sub>2</sub> for various tissues at 24 hours postinjection of the radiolabeled protein either alone or in combination with penetratin or TAT. Penetratin treatment improved the RI in all tissues, whereas TAT treatment resulted in the deterioration of RI for the lungs.

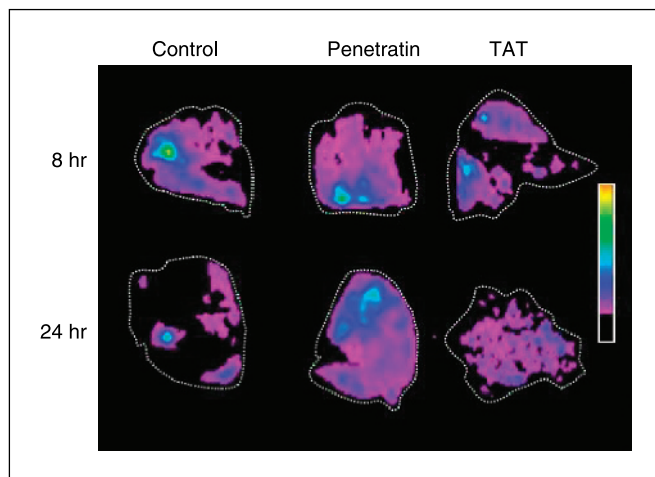
TAT treatment, whereas the corresponding values for the liver were  $1.27 \pm 0.28$ ,  $4.26 \pm 0.57$ , and  $1.76 \pm 0.53$ . In the spleen and kidneys, the penetratin coinjection resulted in a 4.1- and 2.7-fold increase in the RI, respectively, than the  $sc(Fv)_2$  injection alone. The increase due to the TAT treatment was 2.1- and 1.5-fold. TAT treatment resulted in considerable radiolocalization in the lungs (RI,  $3.51 \pm 0.40$ ) at 24 hours postinjection in comparison with the control (RI,  $10.35 \pm 0.93$ ) or penetratin coinjection (RI,  $23.94 \pm 2.31$ ; Fig. 3).

To determine specific uptake in the tumor and nonspecific uptake in normal tissues, tissue-to-blood ratios were calculated at 8 and 24 hours postadministration. At the 8-hour time point, the tumor-to-blood ratios were similar in the control, penetratin, and TAT groups; at 24 hours postinjection, penetratin and TAT treatment significantly improved the tumor-to-blood ratios (7.14, 19.53, and 16.48 for control, penetratin, and TAT, respectively; Fig. 4). Coinjection of TAT with  $sc(Fv)_2$  resulted in considerably higher tissue-to-blood ratios in the lungs, kidneys, and liver when compared with the control and penetratin treatment groups (Fig. 4). The lungs-to-blood ratio with TAT treatment was 6.8- and 5.3-fold higher than the control and penetratin groups, respectively.

Autoradiography with the excised tumor cryosections agrees with the biodistribution data. In addition to a prolonged retention of the  $sc(Fv)_2$  in the tumor, coinjection with penetratin resulted in a more homogeneous distribution than an injection of  $sc(Fv)_2$  alone (Fig. 5). There was uniform distribution of radioactivity within the tumor with TAT treatment also. At 24 hours postinjection; however, the retention was less than that observed with penetratin.



**Figure 4.** Tissue-to-blood ratios of  $sc(Fv)_2$  biodistribution at 8 hours (A) and 24 hours (B) postadministration in LS174T tumor-bearing mice. Animals were injected with  $5 \mu\text{Ci } ^{125}\text{I}$ -labeled  $sc(Fv)_2$  either alone or in combination with penetratin or TAT via tail vein and sacrificed at indicated time points. Their major organs were removed, weighed, and measured for radioactivity to determine tissue-to-blood ratios.

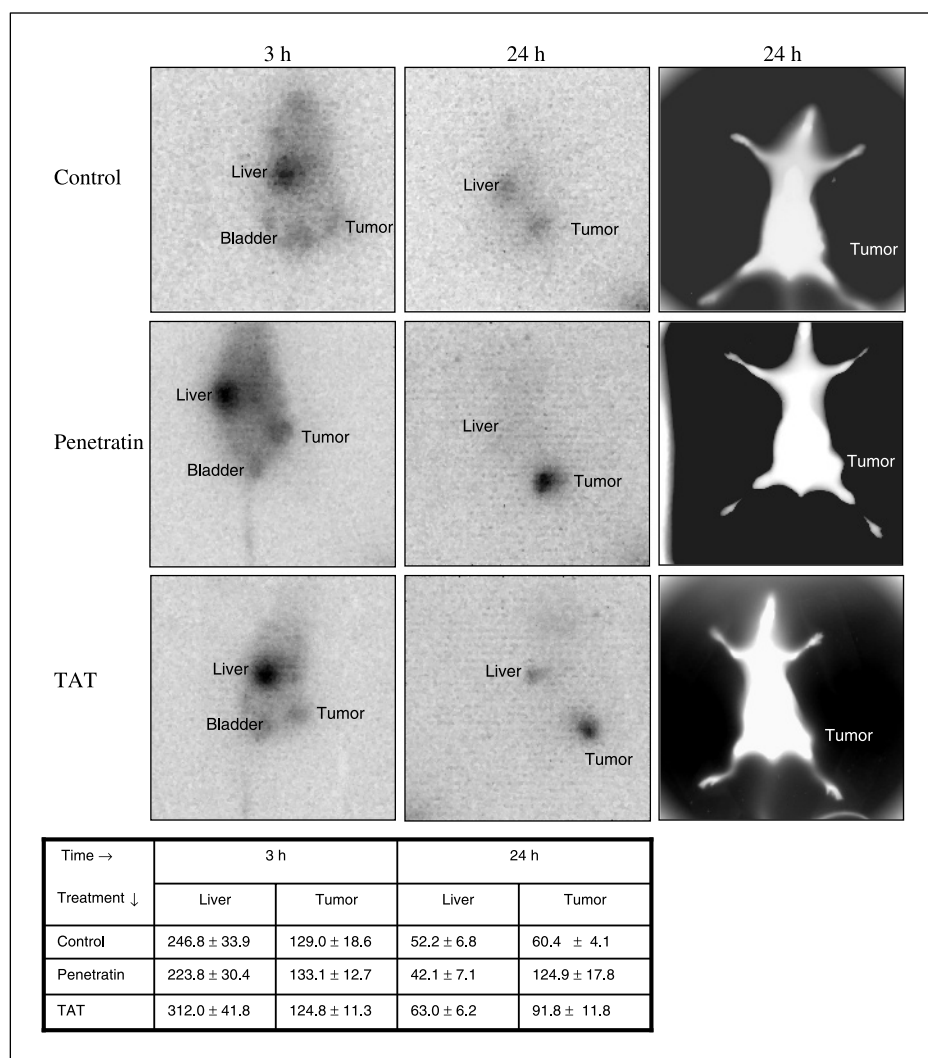


**Figure 5.** Autoradiography of excised tumors following various treatments at 8 and 24 hours postadministration. Following biodistribution experiments, tumors were excised and snap frozen and blocks were prepared. Sections of  $10 \mu\text{m}$  were cut on a cryostat, exposed to X-ray film, and analyzed by Kodak Image Station 2000 MM.

**Pharmacokinetics of  $sc(Fv)_2$  in the presence of penetratin and TAT peptides.** Pharmacokinetic analysis suggested no apparent alteration in the elimination half-life of  $sc(Fv)_2$  in the presence of either penetratin or TAT peptide. The elimination half-life of  $sc(Fv)$  was  $84.2 \pm 3.2$ ,  $83.6 \pm 4.1$ , and  $84.7 \pm 3.5$  minutes in the control, penetratin, and TAT peptide treatments, respectively, whereas the corresponding  $t_{1/2\alpha}$  values were  $10.8 \pm 0.4$ ,  $11.3 \pm 0.6$ , and  $11.8 \pm 0.3$  minutes.

**Whole-body imaging.** Real-time whole-body images were obtained 3 and 24 hours postadministration of  $sc(Fv)_2$  with the CPPs (Fig. 6). At 3 hours postadministration, the mean ROI intensities for liver were higher than that of tumor in control, penetratin, and TAT groups. The mean intensities for tumor at 3 hours were 129, 133.1, and 124.8 for control, penetratin, and TAT, respectively. However, at 24 hours postadministration, the mean ROI intensity in tumor was considerably higher in penetratin-treated animals (124.9) than in control (60.4) or TAT (91.8) groups. Although TAT also improved the relative tumor localization of  $sc(Fv)_2$ , it also caused slightly increased uptake in the liver. No uptake was observed in the brain with either penetratin or TAT.

**Dose response of penetratin on the biodistribution of  $sc(Fv)_2$ .** Encouraged by the positive effects of penetratin coinjection on the biodistribution of  $sc(Fv)_2$ , we investigated the optimal amount of peptide, which can increase tumor uptake without affecting the uptake in normal tissues. Animals were injected with radiolabeled  $sc(Fv)_2$  either alone or in the presence of increasing amounts (0.5, 2.5, and 10 nmol) of penetratin. As indicated in Fig. 7, coinjection with 0.5 and 2.5 nmol (amounting to 5- and 25-fold molar excess of peptide to  $scFv$ , respectively) of penetratin increased the tumor uptake of  $[^{125}\text{I}]sc(Fv)_2$  without much increase in the normal tissues uptake. The tumor uptake increased from  $2.68 \pm 0.11$  with control to  $4.93 \pm 0.53$  and  $7.67 \pm 0.69$  with 0.5 and 2.5 nmol penetratin, respectively. However, no further improvement in tumor localization was observed when penetratin was injected in higher dose of 10 nmol. Additionally, there was a noticeable increase in the uptake in the liver, spleen, and kidneys at high dose of penetratin. Therefore, it seems that for penetratin the optimal peptide-to- $sc(Fv)_2$  molar ratio is 25:1 to improve the tumor retention of  $sc(Fv)_2$ .



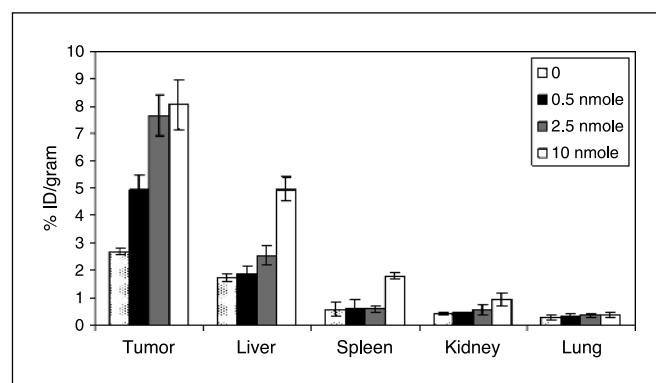
**Figure 6.** Real-time whole-body imaging of live animals injected with sc(Fv)<sub>2</sub> either alone (control) or in combination with penetratin or TAT peptide. Animals were anesthetized as described in Materials and Methods. Images were captured at 3 and 24 hours postadministration. Corresponding bright-field images captured at 24-hour time point are shown on the right, whereas the table depicts the mean ROI intensities for liver and tumor at 3 and 24 hours in various treatment groups.

## Discussion

Single-chain antibodies emerged as attractive alternatives to intact antibodies for specific targeting of imaging and therapeutic molecules due to faster blood clearance time and rapid peak dose deposition in the tumor (6, 21, 22). However, the development of scFvs into clinically viable targeting molecules has been hampered due to low net dose deposition and subsequent rapid clearing from the tumor in comparison with intact antibodies. Efforts to improve the uptake of antibody fragments led to the generation of fusion constructs or chemical conjugation of scFvs with vasoactive agents, such as interleukin (IL)-2, IL-2 permeability-enhancing peptide, and angiotensin (23–26). Fusion constructs of scFvs with biotin mimetic peptide and antigen chelator bispecific scFvs have also been generated to exploit the superior pharmacology of scFvs for a novel approach of pretargeted radioimmunotherapy (27–29).

In the present study, we investigated the effect of coinjection of CPPs, penetratin and TAT, on the biodistribution of a covalent dimeric scFv derivative of anti-TAG-72 mAb CC49. To the best of our knowledge, this is the first report investigating the utility of free CPPs for the improvement of the biodistribution of genetically engineered antibody fragments. Our results indicated that coinjection with penetratin resulted in improved retention of sc(Fv)<sub>2</sub>

in the tumor compared with the TAT peptide and control. The total uptake was not affected because the %ID/g values in the tumor were not significantly different 8 hours postadministration (when peak dose uptake is observed). The percent of retained dose in



**Figure 7.** Effect of increasing the dose of penetratin on the biodistribution of sc(Fv)<sub>2</sub> in tumor-bearing mice. <sup>125</sup>I-labeled sc(Fv)<sub>2</sub> was injected either alone or with  $0.5 \times 10^{-9}$ ,  $2.5 \times 10^{-9}$ , or  $10 \times 10^{-9}$  mol penetratin in 200  $\mu$ L sterile PBS. Animals were euthanized and biodistribution studies were carried out as described previously 24 hours postadministration.



the tumor at 24 hours postadministration with the control (no peptide), penetratin, and TAT was 27.25%, 79.84%, and 48.55%, respectively, of that accumulated at 8 hours postinjection. The increased residence time can have important implications from a therapy standpoint as it will increase the biological half-life of the radiolabel specifically in the tumor, ultimately leading to enhanced dosimetry to the tumor and without any harmful effect on the normal tissues.

Niesner et al. evaluated the biodistribution of the TAT peptide conjugated scFv L-19 directed against the ED-B domain of fibronectin (30). Conjugation of TAT to scFv resulted in a drastic reduction in tumor uptake concomitant to a very high uptake in the liver and spleen. In our studies, we also observed an increased uptake in the liver with the TAT coinjection; however, we observed an increase in the uptake in the lungs as well. Our studies differ from the earlier report using TAT in two major aspects: (a) our studies used a free peptide coinjected with sc(Fv)<sub>2</sub> instead of conjugating the TAT peptide to the antibody and (b) we used a covalent dimeric antibody (58 kDa) instead of the monovalent scFv (30 kDa). Additionally, biodistribution studies with radiolabeled and fluorescently tagged TAT peptide conjugated to chelating agents have indicated increased uptake in the liver and kidneys but not any uptake in the brain (31). There are no reports available describing the biodistribution of the penetratin peptide as such; however, it has been shown that fluorescein-tagged penetratin was not detectable in the liver, kidney, and brain following systemic administration (32). We have examined all the well-vascularized organs (lungs, liver, spleen, and kidneys), heart, muscle, and bone for the nonspecific uptake of a radiolabeled antibody fragment. Although not examined directly, there is some indication that penetratin or TAT treatments did not lead to nonspecific uptake in the brain. Real-time whole-body images of live animals did not suggest any uptake of the radiolabeled scFvs in the brain following coinjection with penetratin or TAT (Fig. 6). Pharmacologic effects of the CPPs might be a concern if these peptides are to be exploited in *in vivo* systems for improved delivery of therapeutic cargoes. Thus far, no serious side effects of CPPs have been observed in either *in vitro* or *in vivo* applications (12). No effect on cell viability was observed with 100 μmol/L of a synthetic CPP Pep-1 (33). Bolton et al. reported that direct injection of 10 μg penetratin in rat brain caused neurotoxic cell death and led to the recruitment of inflammatory cells (32). However, the toxicity decreased remarkably at 1 μg dose. In conventional transduction, penetratin is used at a much lower amount, which is incapable of crossing the blood-brain barrier. Thus, it seems that both penetratin and TAT are safe at the amount administered in our study.

The mechanism of improved tumor retention mediated by penetratin is still not clear. It is known that the binding affinity of scFvs is lower than the parent antibody, which is also true for CC49 sc(Fv)<sub>2</sub> (8). Due to their lower affinity, the scFvs tend to clear rapidly from the tumor. TAG-72, the antigen recognized by the CC49 sc(Fv)<sub>2</sub>, is primarily present in the extracellular matrix (ECM) of the tumor and on the tumor cell surface. The penetratin treatment may have resulted in the penetration of sc(Fv)<sub>2</sub> into the tumor cells from the ECM and cell surface. The internalized antibody then would have been more resistant to clearing by vascular flow than the antibody localized on the cell surface or ECM, thus resulting in an increased residence time. Some evidence of increased tumor penetration was provided from the autoradiography of excised tumors following biodistribution experiments where the penetratin treatment resulted in a more homogeneous

distribution of radioactivity in the tumor than the control (Fig. 5). Most of the studies indicating the enhanced permeability of CPPs have used either chemically or genetically modified (fusion products) cargoes to the CPPs. It may therefore seem intriguing that a mere coinjection of penetratin with sc(Fv)<sub>2</sub> resulted in an improved tumor retention of the radiolabeled antibody fragment. *In vitro* studies with PEP-1, a synthetic CPP, have shown that coincubation of this peptide with peptide and protein cargoes resulted in intracellular delivery in various mammalian cell lines (33). Similarly, Gratton et al. showed that coincubation of penetratin or TAT improved the intracellular uptake of adenoviruses both *in vitro* and *in vivo* (17). Penetratin is a basic peptide with a *pI* of 12.34 and therefore should be positively charged at physiologic pH. The variable fragments in CC49 sc(Fv)<sub>2</sub> are connected through a 205C helical linker, which has a *pI* of 5.31 and thus would be negatively charged under physiologic conditions (10). Considering these facts, there is a possibility of an electrostatic interaction of penetratin with the sc(Fv)<sub>2</sub> molecules through linker regions, which results in the increased penetration of the radiolabeled antibody-penetratin complex. It is also worth mentioning that the injection of penetratin or TAT before or following the administration of sc(Fv)<sub>2</sub> resulted in a similar biodistribution profile as that observed in the control group (data not shown).

In conclusion, the results of our studies indicate that a nonspecific cell-penetrating activity of penetratin (and possibly other CPPs) can be combined with the antigen-specific, tumor-targeting property of scFvs. This effect is observed only at optimal peptide-to-antibody molar ratios (Fig. 7) and does not involve any alteration in the pharmacokinetics of the scFvs. When used in higher amounts, the nonspecific cell-penetrating activity of penetratin predominates over the antigen specificity of the antibody fragment, causing an increased nonspecific uptake of sc(Fv)<sub>2</sub> in normal tissues. Most of the target antigens for radioimmunotherapy are cell surface molecules, and scFv fragments against many of these molecules are available. It will be of future interest to evaluate the effect of penetratin and other cell penetrating peptides in other antigen-antibody systems. The ultimate test for this novel penetratin/sc(Fv)<sub>2</sub> system would be a successful outcome in therapy. It is expected that an effective system would be to engineer a single molecule in which the scFv is tethered to the cell-permeable domain. Nevertheless, the present work for the first time shows that CPPs can improve RI and tumor retention, which are intrinsically required in the successful application for radioimmunotherapy. These studies provide a strong rationale for designing scFv-penetratin fusion proteins by genetic engineering to generate targeting molecules with unaltered antigen specificity, superior pharmacokinetics, and prolonged tumor residence time.

## Acknowledgments

Received 2/25/2005; revised 5/31/2005; accepted 7/1/2005.

**Grant support:** U.S. Department of Energy grant DE-FG0295ER62024.

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We thank Dr. Janina Baranowska-Kortylewicz (University of Nebraska Medical Center), Dr. Pradeep Garg (Wake Forest University), and Dr. Vishal Soni (Harvard Medical School) for helpful discussions and Erik D. Moore for excellent technical support. The CC49 scFv construct was a generous gift from Dr. Jeffrey Schlom (National Cancer Institute Laboratory of Tumor Immunology and Biology) and the Dow Chemical Co.

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