Pentamerisation of a scFv directed against TRAIL receptor 2 increases its antitumour efficacy

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Single-chain variable fragments (scFvs) have been considered as promising therapeutic drugs. However, their low affinity and rapid clearance from the blood have limited their wider application clinically. In this study, we aimed to improve scFv antibodies by multimerising a scFv against the death receptor 5 (DR5, TNFR10B) through fusion with a coiled-coil domain of human cartilage oligomeric matrix protein 48. By forming a pentamer, nick-named a combody, the avidity of the scFv increased 104-fold and combody was more effective than its monomeric counterpart for antitumour activity in both in vitro and in vivo experiments.

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Full-sized antibody drugs are being successfully used for treating cancer clinically. However, because of the low yield and high cost of full-sized antibody production, single-chain variable fragments (scFv) expressed in Escherichia coli may be a good alternative for large-scale production, especially for preclinical study. However, scFv also has the disadvantages of rapid clearance from the blood and a lower affinity for its target that seriously limited its application as a therapeutic drug. Currently, only two scFvs are in active clinical trials (ESBA105 and Efungumab), while the majority of clinical trials involving scFvs have been terminated.1 Evidence has shown that molecules >70 kDa have a relatively prolonged circulation time. Therefore, multimerisation of a scFv seems to be an effective way to extend its half-life in blood while increasing its affinity without altering its binding specificity. There are several methods available to generate multimeric scFvs. For example, two or more scFvs have been assembled as dimers or trimers by altering the length of the linker between the VH and the VL domains,2 and several scFv molecules have been linked together by chemical crosslinking, such as PEGylation.3 In addition, scFv multimers have been formed by fusion to a multimerisation motif, such as the Fc4 or a leucine zipper.5 A recent paper indicated that scFv-Fc-scFv bispecific molecules resulted in antibody-like clearance with a t1/2 of about 460 h.6

As a critical molecule involved in apoptosis, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2 ligand)7 has been exploited as an effective target for cancer therapy. TRAIL is a member of the tumour necrosis factor family. By forming a trimer, TRAIL clusters its receptors, TRAIL receptor 1 (death receptor (DR) 4)8 and/or TRAIL receptor 2 (DR 5),9,10 and triggers apoptosis through the extrinsic pathway by a caspase cascade. TRAIL is superior to tumour necrosis factorz or CD95 in that it shows little or no cytotoxicity against non-transformed cells.11–14 This advantage makes TRAIL an ideal target for cancer therapy. Indeed, TRAIL or monoclonal agonistic antibodies directed against DR4 and DR5 are presently in active phase I/II clinical trials and have shown good prospects for cancer therapy. Promising candidate drugs include Dulanermin (rhuTRAIL),15,16 Apomab,17 Conatumumab18/AMG 655, Tigatuzumab19/CS-1008/TRA-8, Mapatumumab,20,21 Lexitumumab22,23 and chimeric LBY135.24

In this project, we aimed to generate a pentavalent scFv molecule that was directed against the TRAIL receptor 2 (DR5) to significantly increase its affinity, as with the naturally pentavalent immunoglobulin M molecule. The improved affinity of this scFv will make the antibody a drug candidate that is suitable for clinical use. Previously, Zhu et al.25 in our lab established a method to multimerise a single domain antibody to form a pentameric antibody, nick-named combody, by fusion with a COMP domain, and showed that the combody had a much greater avidity. Building on the same concept, we fused a scFv directed against DR5, to form a combody called 8fC, and tested its binding affinity for DR5 and its efficacy in the treatment of colon cancer in an animal model. The 8fC combody, showed a higher affinity for DR5 compared with its monomeric counterpart 8fM; its KD value changed from ~10−7 M (scFv) to 10−11 M (combody). Picomolar concentrations of combody are enough to trigger the apoptosis of tumour cells such as Colo205, A549 and HCT116. The apoptosis induced by the combody was confirmed to be caspase-8- and caspase-3-dependent by western blotting. An
in vivo xenograft tumour model revealed that this combody could significantly suppress the growth of colo205 tumour.

RESULTS
The expression of a scFv and its pentameric version using an E. coli expression system
To improve the avidity of the scFv 8fM, we fused the coiled-coil domain of COMP to the C-terminus of 8fM through a linker to generate a combody 8fC (Figure 1a). Both 8fM (~28 kDa) and 8fC (~35 kDa) were expressed well in bacteria and were purified from the soluble fraction under native conditions (Figure 1b) with yields of ~0.3 mg l⁻¹ (8fM) or ~0.1-0.2 mg l⁻¹ (8fC).

To investigate the oligomerisation status of the proteins under native conditions, 8fM and 8fC were analysed by size exclusion chromatography. Compared with the single dominant peak of the monomeric size (~28 kDa) generated by a non-fused scFv 8fM, the combody 8fC eluted with a major peak at an apparent molecular mass corresponding to the pentamer (~175 kDa) (Figure 1c). Although tiny fractions of higher oligomers and aggregates were observed in the void volumes, these data demonstrate that the scFv 8fM was mainly present in a monomeric form in solution, while combody 8fC was produced as a soluble pentamer.

The ScFv 8fM and the combody 8fC bound specifically to human DR 5
We investigated the influence of Pentamerisation of the scFv 8fM on the binding to DR5. To examine whether the improvement in the avidity of 8fM would cause crossreactivity with DR4, 8fC was added to DR4- and DR5-coated plates at various concentrations. The result (Figure 2a) showed that 8fC bound to DR5 specifically without crossreactivity with DR4. In surface plasmon resonance experiments, an accurate measurement of pentavalent binding kinetics is difficult due to rebinding of the multivalent antibody to neighbouring antigens during the dissociation phase. Thus, even though we determined the binding kinetics of the scFv 8fM with DR5 immobilised on a CM-5 chip, the measured binding parameter of the pentavalent combody 8fC is actually an apparent value due to the affinity effects. The $K_D$ value of the monomeric 8fM ($K_D \approx 10^{-7} \text{ M}$) (Figure 2b) exhibited ~$10^4$-fold lower-binding affinity to DR5 than that of the pentameric 8fC ($K_D \approx 10^{-11} \text{ M}$) (Figure 2c). Please see more details in Table 1. Taken together, these results demonstrate that...
the fusion of a scFv to the N-terminus of COMP48 greatly improves its avidity. As the DR5 antigen could be present in many orientations on the BIAcore chip, the $K_D$ value determined by surface plasmon resonance may not accurately reflect that measured for cell-surface binding to DR5. Therefore, different tumour cell lines were used to test antibodies’ binding ability to DR5 in a natural configuration by using fluorescence cytometry. As shown in Figure 3, both 8fM and 8fC can bind to the TRAIL receptor 2 on the cell-surface. Moreover, 8fC showed a greater shift in the binding assay, suggesting that the pentavalent 8fC might have a higher affinity to DR5 than that of 8fM.

The scFv and the combody activated apoptotic signalling and reduced the viability of human tumour cell lines

We examined the effects of pentamerisation of the scFv 8fM on cell death by incubating the antibodies with various cancer cell lines. As shown in Figure 4a, both the scFv and the combody were unable to induce cell death in non-transformed HEK 293T cells; as a control, TRAIL also did not induce death. Figure 4b indicates that at a low dosage, monovalent 8fM was unable to induce cell apoptosis in Colo205 cells, but, at a much higher concentration, it could induce significant apoptosis in Colo205 cells. In contrast, the pentavalent combody could efficiently induce cell death in A549, Colo205 and HCT116 cells (Figures 4b–d) at a very low concentration. Interestingly, the combody could lead to the apoptosis of A549 cells even though A549 cells were resistant to TRAIL (Figure 4c).

Based on the results of the apoptotic assay revealed by FACS, the Colo205 cell line was chosen for further studies, such as a cell viability assay and the detection of a signalling pathway. To further elucidate how 8fM and 8fC induces cell apoptosis, we detected the activation of molecules involved in apoptotic cell death. The results of the experiments in which Colo205 cells were treated with TRAIL revealed that cell death was initiated by caspase-8, and finally executed by caspase-3. For the scFv 8fM, a dose of 10 mg, but not 2 mg, was able to induce apoptosis. For the combody 8fC, a dose of 1 mg could significantly induce time-dependent cleavage and activation of caspase-8 and caspase-3. Taken together, these results demonstrate that both 8fC and 8fM mediate cell death through an extrinsic signalling pathway (Figures 5a and b).

The data in Figure 5c show that 8fC is more efficient than 8fM in cell cytotoxicity. The IC50 of 8fC is 8 nM (1.4 μg ml−1), far lower than that of 8fM, which is nearly 300 nM (8.9 μg ml−1) (Figure 5c). The scFv and the combody suppressed the growth of colon tumours in a xenograft model

To evaluate the antitumour activity of 8fM and 8fC in vivo, Balb/c nude mice bearing colon (Colo205) tumour xenografts were employed in these experiments. Tumours were inoculated subcutaneously 3 days before the initiation of antibody treatments. On day 3, tumour-bearing mice were administered by tail vein with 8fM, 8fC or irrelevant scFv, as a control, at a dose of 10, 2 and 10 mg kg−1, respectively, with 6 doses at 2-day intervals (Figure 6a). Two weekly treatments of 8fM, at a concentration of 10 mg kg−1, showed
antitumour activity in the xenograft model. As expected, 8fC treatment, at a dose of 2 mg kg$^{-1}$, revealed significant tumour repression, resulting in a greater than twofold reduction in tumour volume and weight at the end of the treatment (day 17) (Figures 6a–c). Statistical analysis shows that 8fC significantly inhibited Colo205 tumour growth. Notably, no signs of systemic toxicity such as body weight loss were observed in the 8fM- and 8fC-treated mice. These results suggest that 8fC is a potent antitumour agent for the suppression of tumour growth in vivo.

DISCUSSION

It is easy for scFvs to penetrate into tumour tissue. Nonetheless, the therapeutic significance of these monovalent scFvs has been limited because of their relatively low affinity, rapid clearance from the blood and short retention time on the target antigen. To improve their efficacy, several techniques in multivalency engineering, such as domain-swapping and natural dimeric and oligomeric protein domains fused to scFvs, have been used to make recombinant antibodies with different valency.

The natural immunoglobulin M antibody provides an excellent example for multivalency engineering. The natural pentameric domain, B-subunit of E. coli verotoxin$^{27}$ and coiled-coil domain of human cartilage oligomeric matrix protein (COMP55, residues 26–80)$^{28}$ were applied successfully. Considering verotoxin’s potential immunogenicity, Zhu et al.$^{25}$ in our lab constructed a combody, a single domain antibody multimerised by a human origen peptide COMP48 (residues 29–76) to minimise the risk. In this study, we followed the same strategy and pentamerised our scFv 8fM to a pentavalent form. Consistent with earlier research that showed that this coiled-coil domain of COMP can be easily produced in E. coli and purified under non-denaturing conditions,$^{29}$ we successfully obtained monovalent 8fM and pentavalent 8fC. Although research had demonstrated that multimerisation domains severely affect production properties of soluble antibody fragments in E. coli, especially p53 and tetraZIP,$^{30}$ it was noted that the combody showed only an ~40% reduction in yield when compared with its monomeric counterpart. As expected, the avidity of 8fC was greatly improved. As demonstrated by surface plasmon resonance, the $K_D$ value of 8fC reaches $10^{-11}$M, which is $10^4$-fold stronger than that of the scFv 8M. The combody’s $K_D$ value is comparable to the values for diabody, tribody, tetrabody,$^2$ Fab$^31$ and natural antibodies, which are usually in the $10^{-9}$ to $10^{-10}$ M range. In practice, the most popular therapeutic or diagnostic antibodies are in the nM range. These data show that our combody strategy is successful in enhancing avidity, comparable to antibodies the LZ-hAY4 (bivalent) and ILZ-hAY4 (trivalent) reported by Lee et al.$^{32}$ which were also targeted to the TRAIL receptor.

The clustering of TRAIL receptors on the cell-surface induces intracellular death signalling pathways that lead to apoptosis. In this report, we examined the bioactivity of 8fM and 8fC. Not surprisingly, 8fC showed potential antitumour activity in vitro and in vivo.

Most research has revealed that cell apoptosis is initiated by caspase-8 and/or caspase-10,$^{33–35}$ thus leading to cleavage and activation of caspase-3. Although there are other signalling pathways,$^{36,37}$ our 8fM and 8fC are also involved with apoptosis signalling via the caspase cascade. In this study, A549 cells were resistant to TRAIL, which is consistent with earlier results. But, interestingly, after treatment with combody, A549 cells were much more sensitive and underwent apoptosis immediately. Chen et al.$^{38}$ examined the sensitivity of five human lung cancer cell lines and found that A549 and small-cell lung cancer showed a moderate sensitivity to AD5–10 (an antibody directed against DR5) and three other cell lines were resistant, while cell line H460 is resistant to AD5–10 despite a high level of cell-surface DR5 expression.

Figure 3 Binding ability of the scFv 8fM and the combody 8fC to bind to different cell lines was analysed by FACS. A total of 5 x 10$^4$ cells were incubated with 0.2 or 1 µg 8fM and 8fC for 1 h. After washing with PBS, the cells were probed with a mouse anti-myc secondary antibody and a goat anti-mouse antibody conjugated with fluorescein isothiocyanate. The isotype control was an anti-CD3 combody, which is part of bispecific combody Zhu et al.$^{25}$ used in their research. Each experiment was repeated three times.
However, the exact mechanism remains unclear. Typically, the tumour necrosis factor superfamily members induce cell death by trimerisation of their receptors. People believe that inefficient clustering of DR4 and/or DR5 may act as the main mechanisms for resistance to TRAIL and agonistic mAbs. A correlation between the valency of 8fC and its proapoptotic ability may be explained by a hypothesis that the multivalency of 8fC could effectively drive dispersed, monomeric DR5 on the cell-surface into an oligomeric state, and thus stimulate the formation of DISC. Furthermore, the higher avidity may stabilise or facilitate the clustering of more receptors in an active conformation, thus allowing more time for cell death signalling mediated by DISC. Another phenomenon that we observed is that high dose monovalent 8fM was able to trigger cell death. This is rare, although a few studies have shown that monovalent anti-DR5 scFv antibodies can directly activate DR5 and induce cell death through autophagy. One explanation is that at a higher concentration, partial crosslinking of the scFv may occur spontaneously. Alternatively, differences in anti-DR5 antibody epitopes may determine their ability to trigger apoptosis of cancer cells.

In conclusion, we have successfully isolated one scFv against DR5 and obtained its corresponding combody. As the combody is a pentameric structure, the affinity and efficacy of the original scFv antibody were improved in the combody. As a DR5 antibody, the combody 8fC is highly promising in various clinical areas, especially in the field of DR-mediated cell death. Compared with conventional antibodies, combodies possess a number of advantages. They can be produced on a large scale at a low cost in E. coli, and they have higher affinities in binding to their targets than a scFv or a full length antibody. Furthermore, combodies were shown to be effective in tumour inhibition in vivo, which makes the strategy a promising method for improving a scFv or similar antibody fragment for cancer therapy.

METHODS

Cell lines and reagents

The human embryo kidney cell line HEK 293T and the tumour cell lines Colo205, A549 and HCT116 were obtained from the American Type Culture...
Figure 5 Tumour cell killing and apoptotic signalling induced in Colo205 by the scFv 8fM and the combbody 8fC in vitro. TRAIL was used as a positive control, while an irrelevant scFv was added as a negative control. (a) Colo205 cells were plated in six-well plates at $\sim 5 \times 10^5$ cells per well, and cultured overnight. Indicated concentrations of 8fM, 8fC and TRAIL were added for 4, 4 and 0.5 h, respectively. Procaspase 8 (57 kDa) was partially cleaved into a $\sim 43$-kDa form. The 17-kDa and 11-kDa cleaved band is the active form of caspase 3. (b) Colo205 cells were treated with 10 ($\mu$g/ml) 8fM or 1 ($\mu$g/ml) 8fC or for 0.5, 1, 2, 4 h respectively. Cells were harvested and divided into three aliquots. The calnexin protein levels are included as a control for protein loading. Procaspase 8 (57 kDa) was partially cleaved into a $\sim 43$-kDa form. The 17- and 11-kDa cleaved band is the active form of caspase 3. (c) Cells were seeded in 96-well plates at $1 \times 10^4$ cell per well and treated with serial diluted doses of 8fM and 8fC for 24 h. Cell viability was assessed by CCK-8 staining, measured at 450 nm. Each experiment was repeated three times.

Figure 6 Repression of the growth of a Colo205 tumour by the scFv 8fM and the combbody 8fC in vivo. BALB/c nude mice were injected subcutaneously at day 0 with $2 \times 10^6$ Colo205 cells. (a) After 3 days, mice were administered irrelevant scFv as a control or were administered 8fM or 8fC by tail vein injection. Seven mice were included in each group, each time point represents the mean value of the tumour size on that day as measured by a calliper ruler (each repeated three times, mean values were taken as measurements), arrows indicate the injection times. Comparing with control, 8fC showed statistically significance in tumor inhibition, while 8fM did not. (b, c) Mice were killed on day 17 and tumours were isolated and weighed ($n = 7$, where *means $0.01 < P < 0.05$, ** means $P < 0.01$, $t$-test).
Collection. Tumour cell lines were cultured and the cell assays were performed in the media recommended by the supplier containing 10% serum.

Reagents sources were as follows: recombinant human TRAIL, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), Peprotech (Rocky Hill, NJ, USA.); Cell Counting Kit-8 (CCK-8), Doinjo Molecular Technologies, Inc. (Kumamoto, Japan.); and rabbit anti-caspase-3 and mouse anti-caspase-8 antibodies, Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA.).

Construction of plasmids
The original 8f scFv against DR5 was in an expression vector pSANG14-3F, with an alkaline phosphatase, 6His and tri-FLAG coding region following it. Briefly, the complementary DNA encoding 8fM was inserted into the expression vector pET26b (+) flanked by the restriction sites NcoI and HindIII with a myc-tag and a His-tag at its C-terminal end. For 8fC, the complementary DNA was also inserted into the pET26b (+) vector using the NcoI and HindIII restriction sites with the coiled-coil domain of human COMP (Asp29-Gln76, COMP48), a myc-tag and a His-tag at its C-terminal end (Figure 1a).

Expression and purification of 8fM and 8fC
The 8fM and 8fC were expressed as soluble forms and purified by immobilised metal affinity chromatography. Briefly, E. coli BL21 gold cells containing the pET26b-8fM or pET26b-8fC plasmid were grown in LB broth at 37°C until the optical density at 600 nm reached 0.6, and then the cells were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 16°C for 16h. The cells were then harvested, suspended in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl buffer and lysed by sonication. The extract was centrifuged at 16000 g for 20 min, and the supernatant was first filtered by a 0.45 μm membrane and then purified using affinity chromatography by loading it onto a Ni-NTA column pre-equilibrated with 20 mM Tris-HCl (pH 7.5), 500 mM NaCl buffer. After washing with 30, 60, and 100 mM imidazole in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl buffer, the protein was finally eluted in 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 200 mM imidazole buffer. The excess imidazole and NaCl were removed from the purified protein by dialysis in phosphate-buffered saline (PBS) buffer.

SDS-polyacrylamide gel electrophoresis and size exclusion chromatography analysis
For electrophoresis analysis, quantified proteins were boiled in loading buffer with or without the reducing reagent dithiothreitol at 100°C for 3 min. Approximately 1 μg of 8fM was run on a 12% SDS-polyacrylamide gel electrophoresis gel, and 1 μg of 8fC was run on a 10% SDS-polyacrylamide gel electrophoresis gel. All proteins in the gels were stained by Coomassie brilliant blue R250. The oligomeric status of purified 8fM and 8fC was further analysed by Superdex 200 10/300 GL (code no.: 28-4038-42, GE Healthcare UK limited) using the AKTA purifier 2000 system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions. A gel filtration calibration kit (code no.: 28-4038-42, GE Healthcare UK limited) was used to calculate their molecular weights. Size exclusion chromatography was carried out in PBS (pH 7.4) at a flow rate of 0.5 ml/min⁻¹.

Surface plasmon resonance
Kinetic measurements of the interactions of the 8fM scFv and the 8fC combody with DR5 were evaluated at 25°C using a BLAcore 3000 biosensor (GE Healthcare). After immobilisation of DR5 onto the carboxymethylated dextran surface of a CM-5 sensor chip at a density of ~2000 response units, the serially diluted monovalent 8fM (0.7, 1.4, 7, 14 and 70 nM) was injected into the flow cell at a flow rate of 20 μl/min⁻¹ for 2 min. However, only three concentrations of the pentavalent 8fC (0.7, 1.4 and 7 nM) were injected at the same rate because there was not an appropriate model for the BLAevaluation software to calculate the kinetics of multivalent analytes.

Enzyme-linked immunosorbsent assay
To determine the specific binding of 8fC to DR5, the TRAIL receptor extracellular domain fusion proteins (DR4, DR5) were immobilised onto 96-well plates at a concentration of 1μg/ml⁻¹, and various concentrations of 8fC (0.11–28 nM) were added. After incubation for 2 h at room temperature, the wells were washed with PBS. Mouse anti-myc antibody and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G were added sequentially. Finally, the 3’,5’-tetramethylbenzidine substrate was added, and the absorbance was read at a wavelength of 450 nm.

Flow cytometry
To determine whether the scFv and the combody could recognise TRAIL receptors expressed on the cell-surface, HEK 293T, A549, Colo205 and HCT116 cells were washed with PBS and resuspended at 1 × 10⁶ cells per 100 μl. The cells were incubated with 1 μg scFv or 0.5 μg combody on ice for 1 h. After washing three times with PBS, the cells were incubated with mouse anti-myc secondary antibody, followed by incubation with fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin G using the same procedure. Fluorescence was read by a Guava Easycyte mini (Guava Technologies, Hayward, CA, USA.).

For the detection and quantification of apoptosis, cells treated with 8fM and 8fC were stained with Annexin-V-fluorescein isothiocyanate and propidium iodide and analysed by flow cytometry. For the assay, 1 × 10⁵ cells were pelleted at 800 g for 5 min and washed with 1 ml PBS. Then, the cells were resuspended in 400 μl binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 5 mM CaCl₂) containing 5 μl Annexin-V-fluorescein isothiocyanate and 100 ng propidium iodide. After a 15-min incubation at 4°C in the dark, the cells were immediately analysed by flow cytometry. Fluorescence was analysed with Flowjo 7.2.5 software (Tree star Inc., Ashland, OR, USA.).

Cell viability assay
The viability of Colo205 cells was analysed by using a CCK-8 kit, which is similar to the MTT assay. It produces a water-soluble formazan dye upon reduction, and thus is much more convenient than the MTT assay. Experiments were repeated at least three times, and the cell viability was recorded as a percentage of viable cells compared with untreated control cells. Briefly, Colo205 cells were seeded in RPMI 1640 culture medium onto 96-well tissue culture plates at a density of 1 × 10⁴ the day before the assay and were grown overnight at 37°C and 5% CO₂. After centrifuging the cells at 350 g for 5 min, serially diluted 8fM or 8fC in 100 μl RPMI 1640 were added, and the cells were then incubated at 37°C and 5% CO₂. After 24 h, 10 μl CCK-8 was added into each well, and cells were incubated in the dark. Two hours later, the absorbance was read at 450 nm on a SUNRIZE microplate reader (Tecan Trading AG, Männedorf, Switzerland).

Western blotting analysis
Colo205 cells were seeded in culture medium onto six-well tissue culture plates the day before the assay at a density of 5 × 10⁴ cells per well and grown overnight at 37°C and 5% CO₂. For positive controls, 0.25 μg ml⁻¹ TRAIL were added for 0.5 h. The 8fM scFv was added to the tumour cells at doses of 2 or 10 μg ml⁻¹ for 4 h, while the 8fC combody was added at 0.5 or 1 μg ml⁻¹ for 4 h. Additionally, 8fM (10 μg ml⁻¹) and 8fC (1 μg ml⁻¹) were added into other six-well tissue culture plates, with a time-course of 0.5, 1, 2 and 4 h incubation at 37°C and 5% CO₂. An irrelevant scFv was added as a negative control. Tumour cells were harvested and lysed with NP40 plus protease inhibition agents. Samples were boiled and loaded onto a 15% polyacrylamide gel. The gels were electropherated and stained with Coomassie brilliant blue R250. The protein expression levels of caspase-3, caspase-8, caspase-9, and rabbit anti-caspase-3 and mouse anti-caspase-8 antibodies, Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA.) were determined using a densitometry software and expressed as a percentage of the control. Cell viability was recorded as a percentage of viable cells compared with untreated control cells. The experimental results were repeated at least three times, and the cell viability was recorded as the average of three experiments ± standard deviation.
pentobarbital sodium with a dose of 60 µg·kg⁻¹·h⁻¹, then, Colo205 cells in 0.1 ml of serum-free medium were implanted into the subcutaneous space of the left flank of the mice at a density of 2 × 10⁶ cells·ml⁻¹. Starting on day 3, mice were treated with irrelevant scFv, 8Fm or 8Fc that was administered by tail vein in PBS, every other day for a total of six doses. On day 17, the mice were sacrificed, and the tumours were isolated for weighing. Tumour size was measured using a calliper, and the formula for calculating tumour volume was

\[ V = \frac{1}{2} \times \frac{l \times d^2}{W} \]

(\(l\); length; \(W\); width). The dosages were calculated according to their IC₅₀. The amounts of irrelevant fcFv, 8fC and 8fM were sacrificed, and the tumours were isolated for weighing. Tumour size was measured using a calliper, and the formula for calculating tumour volume was

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All animals were cared for and maintained under the supervision and guidelines of the Institutional Ethics Committee of the Institute of Microbiology, Chinese Academy of Sciences (permit number CASP0101). All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimise suffering.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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