An enediyne-energized single-domain antibody-containing fusion protein shows potent antitumor activity

Qing-fang Miao, Xiao-yun Liu, Bo-yang Shang, Zhi-gang Ouyang and Yong-su Zhen

Single-domain antibodies are attractive as tumor-targeting vehicles because of their much smaller size than intact antibody molecules. Lidamycin is a macromolecular antitumor antibiotic, which consists of a labile enediyne chromophore (AE) and a noncovalently bound apoprotein (LDP). An enediyne-energized fusion protein VH-LDP-AE composed of single-domain antibody directed against type IV collagenase and lidamycin was prepared by a novel two-step method including DNA recombination and molecular reconstitution. VH-LDP-AE demonstrated extremely potent cytotoxicity to cancer cells and marked antiangiogenic activity *in vitro*. In the mouse hepatoma 22 model, drugs were administered intravenously as a single dose on day 1 with maximal tolerated doses. VH-LDP-AE (0.25 mg/kg) suppressed the tumor growth by 95.9%, whereas lidamycin (0.05 mg/kg) and mitomycin (1 mg/kg) by 79.6 and 51.1%, respectively. In the HT-1080 xenograft model in nude mice, drugs were given intravenously as a single dose on day 4 after tumor implantation. VH-LDP-AE at 0.25 mg/kg suppressed tumor growth by 76% ($P<0.05$) compared with that of lidamycin at 0.05 mg/kg (53%) on day 18. No obvious toxic effects were observed in all groups during treatments. The results showed that energized fusion protein VH-LDP-AE was more effective than lidamycin and mitomycin. These properties, together with its much smaller size than conventional antibody-based agents, suggested that VH-LDP-AE would be a promising candidate for cancer-targeting therapy. In addition, the two-step approach could serve as a new technology platform for making a series of highly potent engineered antibody-based drugs for a variety of cancers. *Anti-Cancer Drugs* 18:127–137 © 2007 Lippincott Williams & Wilkins.

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Introduction

Monoclonal antibodies (mAbs) could target cancer cells selectively and deliver target-aiming payloads (e.g. radioisotopes, drugs or toxins) to kill tumor cells. Since 1997, eight antibody-based products have been approved for the treatment of cancer [1]. Although these are promising developments, mAb-based immunotherapy of solid tumors still has been hindered to some extent by the physical characteristics of the mAbs. With a molecular weight of approximately 150 kDa, antibodies of IgG type exhibit both a slow penetration into tumors and a slow elimination from circulation. The former property leads to heterogeneous delivery into tumors, whereas the latter property results in dose-limiting myelotoxicity [2]. Thus, there is an increasing enthusiasm in recent years for hunting the smallest antibody fragments still capable of binding to antigens and the progress has come from full antibody molecules to recombinant antibody fragments [e.g. classic monovalent antibody fragments (Fab) and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies)]. These smaller antibody fragments are now emerging as credible alternatives for intact antibodies; therein, single-domain antibodies are promising candidates to target payloads (e.g. toxins, drug and cytokines) to kill cancer cells [3]. The concept of single-domain antibodies was first introduced by Ward *et al.* [4], who successfully expressed and isolated heavy chain variable domain (VH) fragments from the spleen genomic DNA of immunized mice with good antigen binding affinities. Single-domain fragments have a low molecular weight (around 14 kDa) and are thus better expressed in prokaryotes. They show lower potential immunogenicity, faster blood clearance, higher thermodynamic stability and better penetration into the target cells as compared with the complete antibodies and scFvs [5]. Decreased or even lost antigen-binding activity (owing to the absence of the partner domain in the incomplete construct of the active site) can be restored by genetic manipulations [6]. Due to the loss of the Fc moiety and its inability to trigger human effector functions, a single-domain antibody can be used as a targeting carrier to combine with a bioactive 'warhead'

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molecule such as toxin, cytokine or radionuclide to prepare effective fusion proteins or immunoconjugates.

Engineered fusion proteins composed of an antibody fragment and an effector molecule represent a new class of highly active antibody-based therapeutics that is now being developed for immunotherapy of malignant tumors. This approach might be safer and more effective than current nonspecific chemotherapeutic agents [7]. Besides the targeting vehicles, it is also important to select appropriate effector agents, i.e. 'warheads', which must be highly potent to kill tumor cells. Lidamycin (LDM), also called C-1027, is produced by a streptomyces strain isolated from a soil sample collected in China [8]. LDM is an attractive candidate as a 'warhead' molecule in antibody-based therapy of cancer. Being a member of the enediyne-containing antitumor antibiotics family, LDM shows extremely potent cytotoxicity, antiangiogenic activity and marked growth inhibition of transplantable tumors in mice [9–11]. In terms of IC_{50} values, the cytotoxicity of LDM was 10 000-fold more potent than that of mitomycin (MMC) and doxorubicin [9,10,12]. As reported, LDM exerted its biological actions through the induction of cellular DNA damage [13–16]. Apoptosis or mitotic cell death in different cancer cells can be induced by LDM [17–19]. LDM is now in phase I clinical trials. Notably, the biosynthesis of LDM (C-1027) and another enediyne antibiotic calicheamicin has drawn much attention [20–22]. The potential clinical value of enediyne antibiotics is also highlighted by the approval of Mylotarg, an immunoconjugate consisting of calicheamicin and anti-CD33 antibody for use in refractory acute myeloid leukemia [23]. In the previous work of our laboratory, the immunoconjugates composed of LDM and monoclonal antibody or its Fab′ fragments have shown remarkable inhibitory effects on the growth of transplantable tumors in mice [24,25]. All of these showed that LDM could serve as a promising 'warhead' compound for preparing antibody-based therapeutics. LDM contains an enediyne chromophore (843 Da) responsible for the extremely potent bioactivity and a noncovalently bound apoprotein LDP (10 500 Da), which formed a hydrophobic pocket for protecting the chromophore [26,27]. The apoprotein and chromophore can be dissociated and reconstituted, and the biological activity of the rebuilt molecule is comparable to that of natural LDM [28].

Matrix metalloproteinases (MMPs) are a family of enzymes involved in degradation of the extracellular matrix, and some of them are key for metastasis and angiogenesis. Of the MMPs, type IV collagenase, including gelatinase A (MMP-2, 72 kDa) and gelatinase B (MMP-9, 92 kDa), is an important member. It has been suggested that type IV collagenase degrades type IV collagen, a main component of the basement membrane, and thus it plays a pivotal role in tumor invasion, metastasis and angiogenesis through the destruction of extracellular matrix and the basement membrane. Expression and activation of type IV collagenase are elevated in proliferating endothelial cells and in a number of malignant tumors, especially in those cancer cells with a high potential of metastasis. As reported, the inhibition of this enzyme may prevent angiogenesis, tumor growth, invasion and metastasis. In addition, there is sufficient evidence that type IV collagenase is a potential target for cancer therapy. In recent years, synthetic MMP inhibitors were designed to target these enzymes for preventing tumor growth and metastases [29–31]. In our laboratory, the prepared monoclonal antibody 3G11 directed against type IV collagenase has shown specific binding to the target enzyme and can neutralize its activity. Conjugates of mAb 3G11 or Fab′ fragment to drug displayed therapeutic efficacy in mouse tumor models [24,25].

In the present study, we developed a 2-step approach to construct a single-domain-based energized fusion protein VH-LDP-AE composed of LDM and a VH derived from 3G11. First, the fusion protein VH-LDP that is composed of the apoprotein LDP of LDM and the VH domain of mAb 3G11 was prepared by recombinant DNA techniques. Second, the energized fusion protein VH-LDP-AE was prepared by molecule reconstitution, i.e. putting the active enediyne chromophore (AE) into VH-LDP. The energized fusion protein VH-LDP-AE displayed immunoreactivity with type IV collagenase, extremely potent cytotoxicity, marked antiangiogenic activity and antitumor efficacy *in vivo*.

Materials and methods

Cell culture

Mouse hepatoma 22 cells, human hepatoma SMMC-7721 cells and Bel-7402 cells, human fibrosarcoma HT-1080, and human colon carcinoma HT-29 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator. All cell lines were passaged every 3 days and maintained in exponential growth to approximately 80% confluence for experiments.

Human umbilical vein endothelial cells (HUVECs) were detached from human umbilical veins by type I collagenase and cultured in Dulbecco's modified Eagle's medium medium (low glucose) supplemented with 20% fetal bovine serum and 20 μg/ml endothelial cell growth factor, and cultured at 37°C in an atmosphere of 5% CO_2. HUVECs were passaged and the cells from passages 3–5 were used.

Animals

Kunming mice and nude mice (BALB/c, *nu/nu*), 16–18-week-old, were obtained from the Institute of Experi-

mental Animals, Chinese Academy of Medical Sciences & Peking Union Medical College.

Construction of expression vector pET-VH-LDP

The vector pKFv1027 carried the gene for the scFv (3G11) in the format VH-$(G_4S)_3$-VL. The pET-VH-LDP plasmid encodes for the isolated VH domain of the mouse monoclonal antibody 3G11 to type IV collagenase. The VH domain-encoding fragment was amplified by polymerase chain reaction from the pKFv1027 plasmid DNA using a direct primer (5′-GATACATATGCAGGTGAAGCTGCAGCAGTCT-3′) including the *Nde*I restriction site and a reverse primer (5′-CATAGGATCCGCCACCGCCTGAGGAGACGGTGACCGTGGT-3′) to add the *Bam*HI restriction site and spacer sequence of G_4S residues to the C-terminus of the VH domain. The LDP-encoding fragment was amplified by polymerase chain reaction from pIJ1027-GRGDS plasmid DNA encoding for LDP gene of LDM using a direct primer (5′-GATAGGATCCGCGCCCGCCTTCTCCGTCAGT-3′) including the *Bam*HI restriction site and a reverse primer (5′-GATAGGATCCGCGCCCGCCTTCTCCGTCAGT-3′) to add the *Xho*I restriction site. The synthesized gene of the VH domain and LDP fragment were digested by *Nde*I/*Bam*HI and *Bam*HI/*Xho*I, respectively, and then both ligated into a *Nde*I/*Xho*I-cleaved pET-30a(+) to give plasmid pET-VH-LDP. Sequence analyses confirmed the expected DNA sequence.

Expression, cellular location of fusion protein VH-LDP and Western blot analysis

The procedures for growth of the *Escherichia coli* strain BL21*star* transformed with pET-VH-LDP and induction of VH-LDP were conducted according to a standard protocol. After induction, the four fractions, including medium, periplasmic, soluble cytoplasmic and insoluble sample, were obtained according to the *pET system manual* (9^{th} ed; Novagen, Heidelberg, Germany). Then 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis were performed as described. For Western blotting, the polyvinylidine difluoride membranes containing transferred proteins were probed with a 1 : 2000 dilution of anti-His-Tag monoclonal antibody (Novagen), and the bound antibody was detected using a 1 : 3000 dilution of goat anti-mouse horseradish peroxidase-conjugated antibody and the enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology, Santa Cruz, California, USA).

Purification and refolding of fusion protein VH-LDP

The bacterial cell pellets were resuspended in binding buffer (5 mmol/l imidazole, 0.5 mol/l NaCl and 20 mmol/l Tris–HCl, pH 7.9) and sonicated, then the cell lysate was centrifuged at 5000 *g* for 15 min at 4°C. This step was repeated and the resulting pellet was resuspended and incubated in binding buffer containing 6 mol/l urea on ice for 1 h. Then the insoluble material was removed by centrifugation at 16 000 *g* for 30 min. The supernatant was filtered through a 0.45-μm membrane and then purified by column chromatography using His Bind metal chelation resins under denaturing conditions according to the manufacturer's protocol (Novagen). The purified proteins were refolded in a way of step-wise dialysis as reported previously [32].

The protein concentration of all fractions was determined by the BCA protein assay kit (Pierce, Rockford, Ilinois, USA) using bovine serum albumin as a standard. Proteins were analyzed throughout by SDS–PAGE. Gels were stained with Coomassive brilliant blue.

Enzyme-linked immunosorbent assays

The cells were grown in 96-well plates till confluence, washed with phosphate-buffered saline (PBS) and fixed for 15 min with 50 μl/well of 0.05% glutaraldehyde/PBS at 4°C. The fixed cells were washed with PBS and nonspecific binding was blocked by incubation with 1% BSA/PBS at 4°C overnight. The wells were emptied, 50 μmol/l of VH-LDP was added in two-fold serial dilutions at concentrations ranging from 50 to 0.1 μmol/l in 1% BSA/PBS and remained for 2 h at 37°C. After removing unbounded VH-LDP, 50 μl/well of 2000-fold diluted mouse anti-His-Tag mAb (Novagen) was used as a primary antibody, followed by 50 μl/well horseradish peroxidase-conjugated goat anti-mouse IgG at a dilution of 1 : 3000 as a secondary antibody in 1% BSA/PBS. Color development was with 100 μl of *o*-phenylenediamine substrate solution and it was stopped after 10 min with 50 μl of 2 mol/l H_2SO_4. Absorbance was measured at 492 nm by the use of a microplate reader. All assays were performed in triplicate.

Immunofluorescent cytochemical staining

Immunofluorescent staining was performed on antigen-positive HT-1080 cells. The cells were grown on slides and fixed with ice-cold acetone for 15 min. Nonspecific binding was blocked with normal goat serum. After washing with PBS, the cells were incubated with VH-LDP or scFv. The cells were then overlaid with mouse anti-His-Tag monoclonal antibodies after being washed with PBS. After a final washing step with PBS, the slides were mounted with fluorescein isothiocyanate-conjugated goat anti-mouse antibody and the fluorescence images were captured by a fluorescence microscope.

Gelatin zymography

Gelatinolytic activity of HT-1080 cells was analyzed according to the method for gelatin zymography. Cells were detached by trypsinization, seeded at 10 000 cells/well in a 24-well plate and cultured overnight. After washing gently with serum-free RPMI 1640 medium twice, the cells were incubated for about 18–24 h with

serum-free RPMI 1640 medium and tested samples. Medium of each tested well was harvested and centrifuged at 3000 r.p.m. to remove cellular debris. The condition medium was collected. Then 20 μl of condition medium was mixed with 10 μl 3 × sample buffer containing 10 mmol/l Tris–HCl (pH 6.8), 20% glycerin, 2% SDS and 0.1% bromophenol blue. Samples (30 μl) were then separated by electrophoresis on 10% SDS–PAGE. Thereafter, gels were washed in the reaction buffer (50 mmol/l Tris–HCl, pH 7.6, 0.15 mol/l NaCl, 10 mmol/l $CaCl_2$, 0.02% NaN_3) containing 2.5% Triton X-100 for 1 h to remove SDS. During this process, progelatinase A and progelatinase B were autocatalytically activated *in situ*. Gels were then incubated for 16 h at 37°C in the reaction buffer. White zones of lysis indicating gelatin-degrading activity were revealed by staining with Coomassive brilliant blue.

Preparation of energized fusion protein VH-LDP-AE

LDM was prepared using a novel improved technique (data shown elsewhere) and applied for molecular reconstitution, which contained high potent activity with more than 80% of AE in total enediyne chromophore. After 10 mg LDM was suspended in 5 ml methanol and whisked for 5 min at 4°C, the mixture was placed at −20°C for 1 h. AE in the supernatant of reaction mixture was obtained by centrifugation of 16 000 *g* for 20 min at 4°C. The above procedure was repeated once to isolate AE completely. For obtaining energized fusion protein VH-LDP-AE, AE in methanol was added to VH-LDP/PBS (10 mmol/l, pH 7.0) with the molecular ratio of 5 : 1 and the volume ratio of 1 : 50, respectively, and then placed at room temperature for 12 h and separated from free AE by using a PD-10 column; purified VH-LDP-AE was prepared finally.

The (4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assays

Cells were detached by trypsinization and seeded at 3000 cells/well in a 96-well plate overnight. Then different concentrations of LDM were added and incubated for an additional 48 h. The effects on cell growth were examined by (4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 4 h. The supernatant was removed and cells were dissolved in 150 μl of dimethylsulfoxide, and then monitored with a microplate reader at a wavelength of 560 nm. Survival ratio was calculated according to the following formula: survival ratio = $(A_{test} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$.

Chick chorioallantoic membrane assay

The surfaces of 7-day-old postfertilization chick eggs (White Leghorn) in a humidified incubator at 37°C were sterilized and the chick chorioallantoic membrane (CAM) was exposed by cutting a window (2 cm^2) on the egg shell using the false air sac technique. After 24 h, basic fibroblast growth factor at a dose of 200 ng/egg as an angiogenic stimulator was added to agar disks with or without the energized fusion protein VH-LDP-AE and then the disks were placed on top of the CAM. After the windows were sealed with transparent tape, the eggs were incubated continuously for 72 h. Then the CAM was examined and photographed with a digital camera.

Tube formation assay

Fifty microliters of Matrigel was placed in each well of 96-well tissue culture plate. One hundred microliters per well of HUVECs (1×10^5 cells/ml) in Dulbecco's modified Eagle's medium containing 0.1, 1 and 10 nmol/l VH-LDP-AE, respectively, was seeded onto the surface of the Matrigel. The cells were incubated for 24 h at 37°C in a CO_2 incubator. Tube formation was observed under a light microscope at × 40 magnification. Microscopic fields were photographed with a digital camera.

In-vivo therapy experiments

The first experiment was performed with 7-week-old female Kunming mice. Hepatoma 22 cells suspended in sterile PBS were inoculated subcutaneously (day 0) at the right axilla of the mouse with 1.5×10^6 cells/0.2 ml/mouse. The mice were divided into six groups, with 10 mice in each group. At day 1, VH-LDP-AE was administered at doses of 0.125 and 0.25 mg/kg of body weight. LDM, MMC and VH-LDP fusion protein were administered at doses of 0.05, 1 and 0.5 mg/kg, respectively. All treatments were administered by intravenous injection into the tail vein in 200 μl of sterile PBS.

To further verify the antitumor efficacy of VH-LDP-AE, a second experiment was performed with 16–18-week-old nude mice. Exponentially growing human fibrosarcoma HT-1080 cells were implanted into two nude mice by the subcutaneous injection of 5×10^6 cells in the right axilla. Tumors resulting after 2 weeks in donor animals were aseptically dissected and mechanically minced. Pieces of tumor tissue (2 mm^3 in size) were transplanted (subcutaneously) by a trocar needle into nude mice. On day 4, tumors were measurable (around 3 mm in diameter), and mice were grouped ($n = 6$) and injected intravenously in the tail vein with VH-LDP-AE at different doses (0.125, 0.1875 and 0.25 mg/kg), and with LDM (0.05 mg/kg), VH-LDP (0.25 mg/kg) and MMC (1 mg/kg), respectively. Control mice were injected with PBS.

In both experiments, mice were monitored and tumor growth was measured with a caliper, and tumor volumes were calculated with the following formula: $V = 0.5a \times b^2$, where a and b are the long and the perpendicular short diameters of the tumor, respectively. The data are presented as the mean ± standard deviation (SD). Tumor growth curves were plotted and the inhibitory rates of

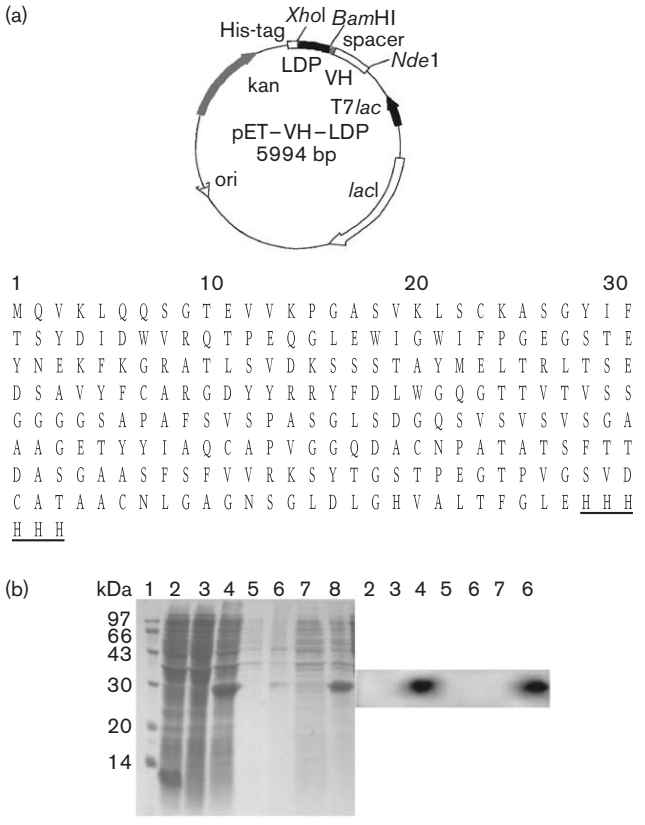
tumor growth were calculated according to the tumor volume. Student's *t*-test was used to determine statistically significant differences. $P < 0.05$ was considered significant.

Results

Construction and expression of fusion protein VH-LDP

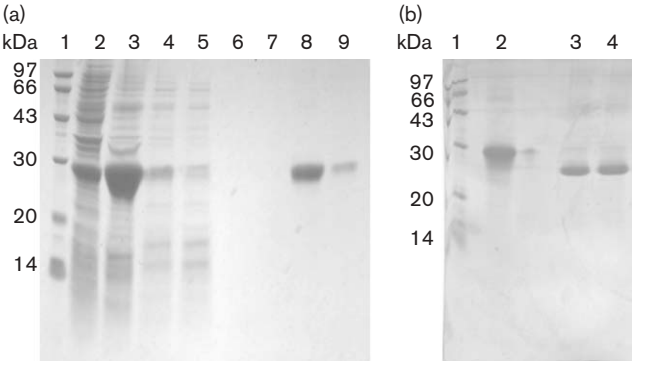
In order to achieve the single-domain-based fusion protein, the DNA sequence encoding for the VH fragment of mAb 3G11 was genetically fused to the LDP gene, which encodes for the apoprotein of LDM, with a five-amino-acid spacer (GGGGS) between the C-terminus of the VH (3G11) and the N-terminus of LDP, and cloned into the *Nde*I/*Xho*I restriction sites of the pET-30a(+) expression vector, leading to plasmid pET-VH-LDP (Fig. 1a). The VH-LDP fusion gene was under the control of the T7 promoter and a $(His)_6$-tag was introduced at the C-terminus of the constructs to facilitate purification by immobilized metal-affinity chromatography. As shown in Fig. 1(a), the fusion protein was composed of 243 amino acids and the theoretical molecular weight was 25.4 kDa.

Fig. 1



Construction and expression of the fusion protein VH-LDP. (a) Construction of the plasmid pET-VH-LDP carrying the gene of fusion protein VH-LDP and its amino acid sequence. kan, the kanamycin-resistance gene; T7*lac*, T7*lac* promotor; VH, sequence encoding for the variable heavy chain domain of the parental monoclonal antibody 3G11; spacer, sequence encoding for the GGGGS spacer peptide; LDP, sequence encoding for apoprotein of LDM. His-tag, C-terminal tag of six histidine residues. (b) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (left) under reducing conditions and Western blot (right) of each fraction of *Ecsherichia coli* BL21*star* (DE3) cells expressing VH-LDP fusion proteins. Lanes: 1, molecular weight marker; 2, total proteins of *E. coli* carrying plasmid pET-30a (+) after isopropylthio-β-D-galactopyranoside (IPTG) induction; 3, total proteins of *E. coli* carrying plasmid pET-VH-LDP without IPTG induction; 4, total proteins of *E. coli* expressing VH-LDP fusion protein; 5, medium sample; 6, periplasmic fraction; 7, cytoplasmic soluble fraction; 8, cytoplasmic insoluble fraction.

Fig. 2



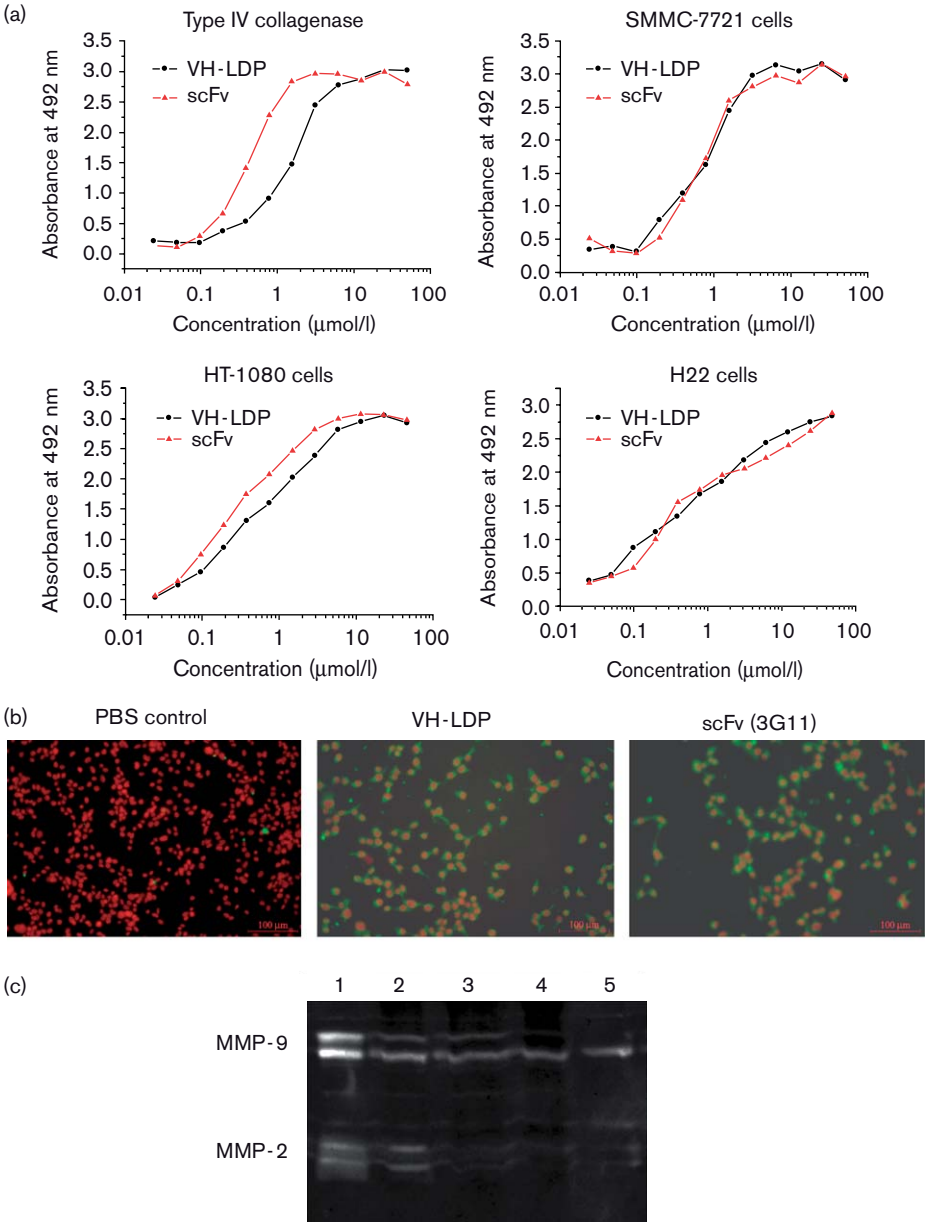
Purification and refolding of the fusion protein VH-LDP. (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis after purification by immobilzed metal-affinity chromatography under reducing conditions. Lanes: 1, molecular weight marker; 2, total cell protein; 3, sample before Ni^{2+} column; 4, contaminating protein not bound to the sorbent; 5 and 6, washed fractions by 1 × binding buffer; 7, washed fractions by 1 × washing buffer; 8 and 9, VH-LDP protein obtained by eluting with 1 × strip buffer. (b) SDS–PAGE analysis of VH-LDP fusion proteins after refolding under reducing (left) and nonreducing (right) conditions. Lanes: 1, molecular weight marker; 2, VH-LDP under reducing conditions; 3 and 4, VH-LDP under nonreducing conditions.

The plasmid vector pET-VH-LDP was transformed into *E. coli* BL21*star* (DE3) and the target protein was induced by the addition of isopropylthio-β-D-galactopyranoside (IPTG). As shown in the Coomassive Blue-stained gel in Fig. 1(b, left), recombinant proteins accumulated in intracellular inclusion bodies and made up about 30% of the total cell protein. The fusion construct was further confirmed by Western blot using an anti-His-Tag antibody (Fig. 1b, right).

Purification and refolding of fusion protein VH-LDP

The fusion protein VH-LDP was purified using immobilized metal-affinity chromatography resin under denaturing conditions and the target proteins of over 90% purity were obtained as shown in Fig. 2(a). After refolding in a stepwise way, 60 mg soluble VH-LDP could be harvested from 1 l fermentation broth, and the protein was further illustrated by SDS–PAGE under reducing and nonreducing conditions (Fig. 2b). Only one single band could be discerned in both conditions, which indicated that no

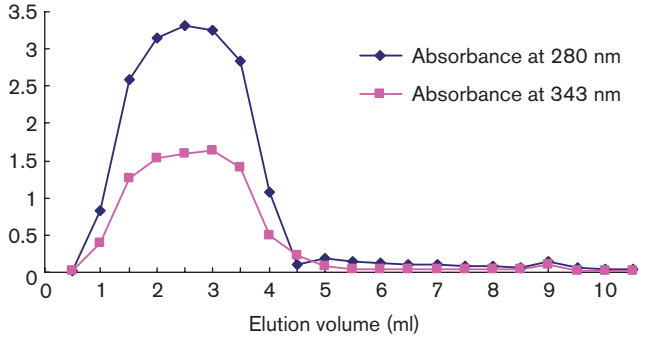
Fig. 3



The immunoactivity analysis of VH-LDP and scFv (3G11). (a) The immunoreactivity to type IV collagenase, SMMC-7721 cells, HT-1080 cells and H22 cells by enzyme-linked immunosorbent assay. (b) Immunofluorescence detection on HT-1080 cells by bivariate fluorescein isothiocyanate–goat antimouse antibody and propidium iodide. Scale bars are 100 μm. (c) Gelatin zymography analysis of VH-LDP using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels in HT-1080 cells. Lanes: 1, control, treated with PBS; 2, treated with 25 μmol/l VH-LDP fusion protein; 3, treated with 50 μmol/l VH-LDP fusion protein; 4, treated with 100 μmol/l VH-LDP fusion protein; 5, treated with 25 μmol/l scFv (3G11). MMP, matrix metalloproteinase; PBS, phosphate-buffered saline.

degradation or aggregation occurred in the process of purification and refolding. In addition, Fig. 2(b) shows that VH-LDP migrated as a roughly 25 kDa band under nonreducing conditions, fully consistent with the M_r of 25.4 kDa calculated from the amino acid sequence (243 amino acid residues), but appeared slightly larger when running under reducing conditions. This may be due to the occurrence of intramolecular disulfides in the LDP molecule that made the protein more stretched under reducing conditions and it migrated slower on a reducing gel. On a nonreducing gel, however, the protein was more compact and then it migrated faster. The VH-LDP protein was still soluble at a concentration of 15 mg/ml.

Fig. 4



Separation of the energized fusion protein VH-LDP-AE from free AE on a PD-10 column (Sephadex G-25; Amersham). Elute: 1 × PBS.

Binding assays

To confirm the correct folding and functional binding of the fusion protein, the ability of VH-LDP to bind to the target antigen type IV collagenase or antigen-relevant tumor cells was examined by enzyme-linked immunosorbent assay (ELISA). The data indicated that VH-LDP bound to type IV collagenase or antigen-related cancer cells including SMMC-7721 cells, HT-1080 cells and H22 cells in a dose-dependent and saturable manner (Fig. 3a). Although the immunoreactivity of VH-LDP to type IV collagenase was slightly lower than the corresponding scFv (3G11), there was still considerable binding ability to antigen for VH-LDP (Fig. 3a).

Determined by ELISA, VH-LDP and scFv (3G11) interacted with type IV collagenase with an association constant of 0.5×10^7 and 6×10^7/mol/l, respectively. The VH-LDP fusion protein preserved the antigen-binding function with a 12-fold decrease vs. the scFv fragment.

The immunoreactivity of VH-LDP was further tested by immuofluorescence detection with HT-1080 cells. As shown in Fig. 3(b), VH-LDP and the positive control scFv (3G11) showed green fluorescence, whereas PBS control showed no fluorescence, and the result further confirmed the immunoreactivity of VH-LDP.

Gelatin zymography assay

Type IV collagenase, also named gelatinase, includes gelatinase A (MMP-2, 72 kDa) and gelatinase B (MMP-9, 92 kDa). Using this technique, the inhibition of the activity of gelatinase, both active and latent species, can be visualized. It showed that the clearance of the gelatin substrate by gelatinases of 72 and 92 kDa was detected as a negative band. As shown in Fig. 3(c), the activity of MMP-2 and MMP-9 secreted by HT-1080 cells was strongly inhibited by VH-LDP in a dose-dependent manner.

Preparation of energized fusion protein VH-LDP-AE

By adding AE, the active enediyne chromophore of LDM, to VH-LDP solution in controlled conditions, the energized fusion protein VH-LDP-AE was prepared. After separating from free AE by the PD-10 (Sephadex G-25) column VH-LDP-AE was purified. As shown in Fig. 4, the correct VH-LDP-AE molecules came into being because they showed strong absorbance at both 343 and 280 nm, which represents the absorbance of the chromophore and protein moiety, respectively.

Cytotoxicity of the energized fusion protein VH-LDP-AE to cancer cells

Using the MTT assay, VH-LDP-AE displayed extremely potent cytotoxicity to HT-29, HT-1080 and Bel-7402 cells with IC_{50} values of 9.22×10^{-12}, 6.13×10^{-12} and 7.01×10^{-11} mol/l; correspondingly, the IC_{50} values of free LDM to these cells were 1.80×10^{-12}, 2.83×10^{-12} and 2.12×10^{-12} mol/l, respectively (Fig. 5a).

Antiangiogenic activity of the energized fusion protein VH-LDP-AE in chick chorioallantoic membrane assay

The antiangiogenic activity of the energized fusion protein VH-LDP-AE was examined in CAM. After 72 h, the change in blood vessel density was assessed in the area of disk placement. As shown in Fig. 5(b), a vessel-free speckle was formed owing to the inhibition of new blood vessel formation by VH-LDP-AE.

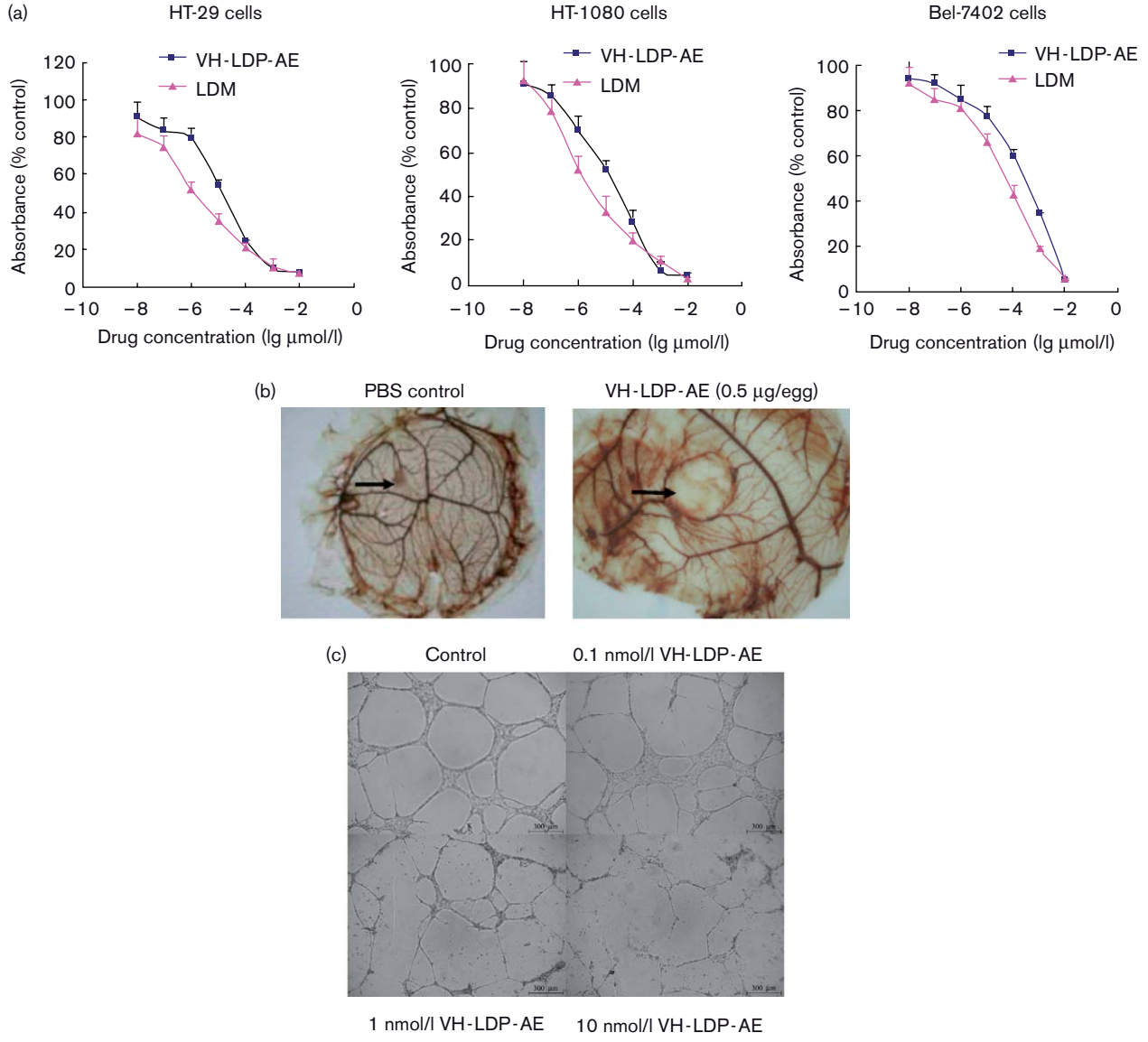
Tube formation assays

The effect of VH-LDP-AE on HUVECs tube formation was examined. HUVECs on Matrigel migrated, attached to each other and formed tube structures (Fig. 5c). Energized fusion protein VH-LDP-AE strongly suppressed HUVEC tube formation at concentrations between 1 and 10 nmol/l. The inhibition efficacy was dose-dependent.

In-vivo therapy with the energized fusion protein VH-LDP-AE

First, the in-vivo therapeutic efficacy of energized fusion protein VH-LDP-AE on the growth of subcutaneous transplanted murine hepatoma 22 in Kunming mice was investigated. The tumor-bearing mice were treated by injection into the tail vein once 24 h after transplantation. The results indicated that VH-LDP-AE significantly inhibited the growth of hepatoma 22 tumor (Fig. 6a). As evaluated on day 14, VH-LDP-AE at 0.25 and 0.125 mg/kg suppressed the tumor growth by 95.9% ($P < 0.05$ vs. LDM) and 88.1% ($P\& < 0.01$ vs. LDM), respectively, whereas free LDM at the tolerated dose of 0.05 mg/kg showed an inhibition rate of 79.6% (Fig. 6a). All treated mice survived at that time. No severe side-effects were observed during the treatment, which implied that the therapeutic efficacy of VH-LDP-AE at a tolerated dose was stronger than that of free LDM.

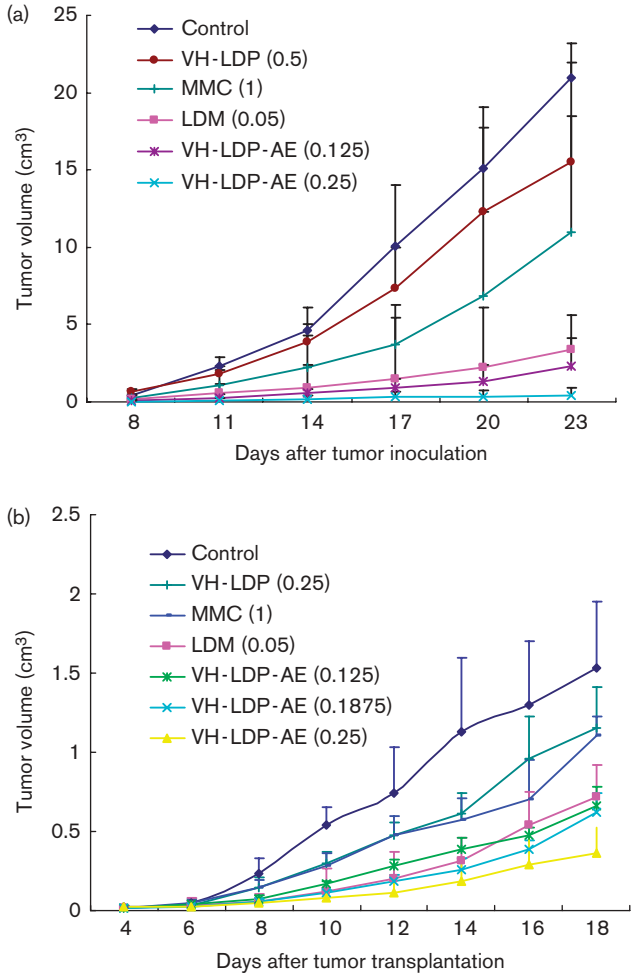
Fig. 5



The antitumor activity analysis of energized fusion protein VH-LDP-AE *in vitro*. (a) Growth inhibition of HT-29 cells, HT-1080 cells and Bel-7402 cells in (4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay. (b) Inhibition of basic fibroblast growth factor-stimulated angiogenesis in chick chorioallantoic membrane assay. (c) Inhibition of human umbilical vein endothelial cell tube formation on Matrigel. Scale bars are 300 μm. The arrows indicate the area of disk placement. PBS, phosphate-buffered saline.

The antitumor activity of VH-LDP-AE was also investigated in nude mice with established HT-1080 human fibrosarcoma xenografts that highly expressed type IV collagenase. Treatments were started intravenously once on day 4 after tumor implantation, when solid tumor nodules started becoming measurable. As shown in Fig. 6(b), HT-1080 xenografts were growing rapidly in mice, and the tumor size in the control group increased from 22 to 1530 mm^3 (around 70-fold increase) over 18 days duration of the experiment. Mice treated with LDM at the tolerated dose of 0.05 mg/kg showed an inhibition rate of 53%. VH-LDP-AE at 0.25 mg/kg suppressed the tumor growth by 76%, which demonstrated statistically significant differences ($P < 0.05$) compared with that of LDM at 0.05 mg/kg. The therapeutic efficacy in groups of VH-LDP-AE was dose-dependent. Almost no therapeutic benefit could be achieved in the group treated with MMC at doses of 1 mg/kg (around 28% inhibition) or fusion protein VH-LDP at 0.25 mg/kg (around 25% inhibition) compared with the control group. No obvious toxic effects were observed in all groups during treatments. These results suggested that both VH-LDP-AE

Fig. 6



The antitumor activity of energized fusion protein VH-LDP-AE and LDM *in vivo*. (a) Growth inhibition of hepatoma 22 in mice. Drugs were administrated intravenously on day 1. Numbers in parentheses represent doses at mg/kg. (b) Effects on the growth of HT-1080 fibrosarcoma xenografts in nude mice. Drugs were administrated intravenously on day 4. Numbers in parentheses represent doses at mg/kg. MMC, mitomycin; LDM, lidamycin.

and LDM could retard the growth of HT-1080 xenografts significantly in nude mice; however, the therapeutic efficacy of energized fusion protein VH-LDP-AE was stronger than free LDM, as compared with the tolerated dosage level.

Discussion

The antibody Fv fragments can be further decreased in size by splitting them into VH and VL domains. Some experimental evidence indicated that the VH domain alone derived from the intact antibody or separated from the VL fragment can retain a significant portion of the original antigen affinity, but the isolated VH domains were often insoluble and prone to aggregation [33–35]. Worn *et al.* [36] reported that the isolated VH domains of A48 antibody, with a yield of 1.4 mg of refolded protein/l *E. coli*, were very prone to aggregation and could not be concentrated above 30–40 μg/ml, and showed no binding affinity to antigen. The VH domain of anti-influenza neuraminidase antibody NC41 dimerized in solution and then partly precipitated at concentrations of 5–10 mg/ml, showing binding affinity with a K_a lower than 10^4/mol/l [37]. With antibody McPC603, no binding of VH to the antigen could be shown and the VH domain also showed low solubility at temperatures above 4°C [38]. In the present study, the recombinant fusion construct VH-LDP consisting of the VH domain derived from mAb 3G11 and the apoprotein LDP accumulated in *E. coli* as inclusion bodies. After purification and refolding, the active fusion protein was soluble at a concentration of 15 mg/ml and with a yield of 60 mg/l culture medium. Compared with the other isolated VH domain mentioned above, it was noteworthy that the fusion protein VH-LDP was characterized by high-yield active production and good solubility. Taking into consideration the fact that the properties of the isolated domains VH and VL would vary depending upon the antibody under study [39], the good solubility of VH-LDP may probably be associated with the characteristic of mAb 3G11 itself. As reported, the fusion of a single antibody VL domain to barnase can increase solubility [40]. Therefore, we further speculated that the characteristic of VH-LDP different from the general isolated VH domain could be related to the occurrence of the apoprotein LDP. Natural LDM itself was highly soluble in aqueous environments, and the study in our laboratory showed that recombinant LDP alone was expressed in *E. coli* mainly as a soluble form and was above 70% of the total protein in supernatant of the culture. It is possible that the existence of LDP might affect the solubility behavior and prevent the aggregation of VH-LDP. A putative explanation is that the linking to LDP might partly cover the exposed hydrophobic surface of the VH domain and then prevent its sticky aggregation. Thus, VH-LDP could appear as a soluble form at higher concentrations.

Brinkmann *et al.* [41] had previously described immunotoxins in which the VH or VL domain of mAb B3 was fused to a truncated form of *Pseudomonas* exotoxin, and found that the cytotoxicity of these molecules is between 20- and 100-fold less than the corresponding Fv immunotoxin. In another study, the isolated VL domain derived from anti-ferritin antibody F11 preserved the antigen-binding activity; however, it was 4- to 30-fold lower than that of the parental antibody F11 in the absence of the partner VH domain [42]. Our results were consistent with the above studies. ELISA data showed that the fusion protein VH-LDP retained part of the antigen a binding activity of the parent mAb 3G11 and interacted with type IV collagenase with a K_a of 0.5×10^7/

mol/l, about 12-fold lower than that of the corresponding scFv (3G11). VH-LDP also showed immunoreactivity with various antigen-related cancer cells, and markedly inhibited the gelatinase activity in a dose-dependent and staturable manner. These data strongly suggested that in the case of mAb 3G11, the VH domain alone contained sufficient structural information for specific antigen recognition.

As one of the most potent antitumor antibiotics ever reported, LDM is a 1:1 complex of an enediyne chromophore AE having DNA-cleaving ability and a carrier apoprotein LDP. The chromophore is bound to the hydrophobic pocket formed by the apoprotein, and the major binding interactions between AE and LDP are hydrophobic contacts between the core of the chromophore and the hydrophobic side-chains of the pocket-forming residues [27]. This lays the basis on which the LDM molecule can be separated and reconstituted. In the present study, we obtained an energized fusion protein VH-LDP-AE by molecular reconstitution, integrating the VH-LDP fusion protein with an active enediyne chromophore. It showed potent cytotoxicity to various cancer cell lines *in vitro*, significant inhibitory activity on angiogenesis and effective tumor-growth suppression *in vivo*. All these demonstrated that VH-LDP-AE preserved the biological activity of natural LDM and indicated that AE was added accurately into the hydrophobic pocket of apoprotein LDP. The molecule of the energized fusion protein VH-LDP-AE contains three parts including VH, LDP and AE, and each of them plays different roles in exerting the biological function of the whole molecule. The single-domain antibody VH portion, derived from an antibody directed against type IV collagenase, which is related to cancer invasion, metastasis and angiogenesis, may not only serve as a tumor-targeting vehicle, but also exert its own antitumor activity by inhibiting the target enzyme. The active enediyne AE, derived from LDM, may serve as a 'warhead' with highly potent cytotoxicity. In addition, LDP may work as a carrier for AE by offering a hydrophobic pocket. As expected, VH-LDP-AE had realized the integration of the above-mentioned activities in the energized fusion protein molecule.

With a theoretical molecular mass of 26.2 kDa, the engineered and energized fusion protein VH-LDP-AE can be considered as one of the antibody-based fusion proteins that have the smallest molecular size and show antitumor efficacy *in vivo*. First, the recognition and targeting module was composed of a single variable domain of the antibody. Second, the 'warhead' molecule LDM (around 11 kDa) was much smaller than the conventional toxin or cytokines employed in the construction of fusion proteins, such as gelonin (28 kDa), a truncated form of *Pseudomonas* exotoxin (PE38KDEL, 38 kDa), cytokine interleukin-2 (15.5 kDa) and interleukin-12 (75 kDa) [43–46]. With much smaller molecular size than other antibody-based fusion proteins, this molecule might show better tumor penetration and reduced immunogenicity. To our knowledge, VH-LDP-AE is the first ever reported single-domain VH-based fusion protein energized with enediyne.

In conclusion, the engineered and energized fusion protein VH-LDP-AE showed not only potent cytotoxicity to cancer cells and inhibitory activity on angiogenesis, but was also highly effective *in vivo*. These properties, together with its much smaller size than the conventional antibody-based drugs, suggested that VH-LDP-AE would be a promising candidate for targeting cancer therapy. In addition, this approach to manufacturing energized fusion proteins might serve as a technology platform for the development of new antibody-based therapeutics.

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