Antitumor efficacy of the scFv-based fusion protein and its enediyne-energized analogue directed against epidermal growth factor receptor

Weijin Sheng, Yue Shang, Qingfang Miao, Yi Li and Yongsu Zhen

Epidermal growth factor receptor (EGFR), overexpressed in many epithelial tumors, plays important roles in the formation and the development of tumors, and thus it is regarded as a promising target for cancer therapy. Single-chain variable fragment (scFv), an engineered antibody fragment, is generally used for constructing antibody-targeted drugs, owing to its low immunogenicity and high penetration capability into solid tumors. A fusion protein ER(Fv–LDP), consisting of an anti-EGFR scFv and the apoprotein (LDP) of lidamycin (LDM), was prepared and then assembled with the active chomophore [active enediyne (AE)] of LDM to generate enediyne-energized analogue ER(Fv–LDP–AE). The fusion protein ER(Fv–LDP) bound specifically to EGFR-overexpressing cancer cells and internalized into the cytoplasm through receptor-mediated endocytosis. ER(Fv–LDP) possessed cytotoxicity against carcinoma cell lines, which was hundreds of times more potent than the separate moiety of ER(Fv) and LDP. The enediyne-energized fusion protein ER(Fv–LDP–AE) also showed stronger cytotoxicity to target-relevant cancer cells than LDM *in vitro*. In human epidermoid carcinoma A431 xenografts, ER(Fv–LDP) presented higher antitumor efficacy than that of ER(Fv), LDP, and their mixture, with tumor growth inhibition rates of 63.6, 46.7, 48.5, and 49.9%, respectively. The enediyne-energized fusion protein ER(Fv–LDP–AE) at a dose of 0.4 mg/kg inhibited tumor growth by 89.2%, while no significant body weight loss was seen in treated animals. The results show that an anti-EGFR scFv-based fusion protein and its enediyne-energized analogue can be prepared by DNA recombination and molecular reconstitution. Both ER(Fv–LDP) and ER(Fv–LDP–AE) are effective against EGFR-overexpressing cancer xenograft in athymic mice. An integrated technical platform for scFv-based enediyne-energized fusion proteins has been established. *Anti-Cancer Drugs* 23:406–416 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

In view of high specificity and efficacy, antibody targeting therapy has been a focal point of cancer therapy in recent years [1]. Previous research has shown that antibody-targeted drugs were proved to be effective for cancer treatment. Differing from traditional antibody therapeutics, antibody–drug conjugates or antibody-based fusion proteins are generally composed of a target-specific molecule (e.g. intact IgG monoclonal antibody or antibody fragment) and an effector agent (e.g. drug, toxin, or cytokine) by chemical coupling or by genetic engineering, respectively, which are effective in reducing toxicity to normal tissues and augmenting antitumor efficacy [2–4].

Epidermal growth factor receptor (EGFR) is a transmembrane receptor belonging to a family of four homologous proteins, including EGFR/ErbB1/HER1, ErbB2/HER2/*neu*, ErbB3/HER3, and ErbB4/HER4. Overexpression of EGFR has been observed in a variety of human carcinomas, such as head and neck squamous cell carcinoma, non-small-cell lung carcinoma, and breast cancer. Clinical studies indicate that EGFR, which is associated with poor prognosis, recurrence, and drug resistance, should be an important target for anticancer therapy [5]. At present, several monoclonal antibodies (e.g. cetuximab, nimotuzumab, and panitumumab) and small-molecule kinase inhibitors (e.g. gefitinib, erlotinib, and lapatinib) targeting EGFR have been approved by the US Food and Drug Administration for clinical therapy of tumors, and preclinical and clinical research is being carried out on many more [6–9].

The single-chain variable fragment (scFv), which is composed of a heavy chain variable domain and a light chain variable domain of the antibody molecule, is a target-specific molecule and can be used for constructing molecule-targeted drugs. As we previously reported, an scFv, which displays specific binding to purified EGFR and the EGFR located on carcinoma cell membrane, has been prepared through the construction and screening of phage antibody libraries against EGFR [10]. It is of interest that linking a 'warhead' molecule to the scFv moiety for preparation of a target-specific and highly potent fusion protein.

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Lidamycin (LDM, also called C-1027), a peptide antibiotic produced by *Streptomyces globisporus* strain isolated in the Institute of Medicinal Biotechnology, CAMS, displays extremely potent cytotoxicity against tumor cells [11]. LDM is composed of an active enediyne (AE, 843 Da) chomophore with extremely potent cytotoxicity and a noncovalently bound apoprotein (LDP, 10.5 kDa) that forms a hydrophobic pocket for protecting and stabilizing the chromophore. These two parts can be dissociated and reconstituted, and the biological activity of the rebuilt molecule is comparable to that of natural LDM [12].

In this study, for the purpose of manufacturing highly effective antibody-based drugs directed against EGFR, a fusion protein ER(Fv–LDP) and its enediyne-energized analogue ER(Fv–LDP–AE) were prepared. The fusion protein was produced by linking an scFv against EGFR to the apoprotein of LDM by DNA recombination, and then the fusion protein ER(Fv–LDP) was assembled with the enediyne chomophore AE. The binding affinity of ER(Fv–LDP) and its separated moieties, ER(Fv) and LDP, to cancer cells was compared *in vitro* and their internalization processes were determined by fluorescence-activated cell sorting (FACS) analysis. The antitumor activity of fusion protein ER(Fv–LDP) and its energized analogue ER(Fv–LDP–AE) was determined *in vitro* and *in vivo*, respectively.

Methods

Cell culture

Human lung carcinoma A549, breast cancer MCF-7, and embryonic kidney HEK293 cells were cultured in RPMI 1640-Glutamax-I medium (Invitrogen Life Technologies, Carlsbad, California, USA), containing 10% fetal bovine serum (FBS) (Gibco/BRL, New York, New York, USA) and penicillin and streptomycin at 100 units/ml and 100 μg/ml, respectively. Human epidermoid carcinoma A431 cells were grown in DMEM-Glutamax-I medium (Invitrogen Life Technologies) containing 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

Construction of the expression vector pET30(a)-*scfv-ldp*

The vector *p*CANTAB 5E-*scfv* carrying the gene of anti-EGFR scFv was constructed in our laboratory. The scFv DNA fragments were amplified from the *p*CANTAB 5E-*scfv* using primers VH (5′-GGAATTCCATATGGCC CAGGTCCAGCTGCAG-3′) and primers VL (5′-CGGAA TTCGGATCCGCCACCGCCCCGTTTTATTTCCAAC TTTGT-3′); space sequences encoding Gly_4Ser were added to the C-terminus. The PCR profile was as follows: 30 cycles of 1 min degeneration at 94°C, 2 min annealing at 58°C, 2 min extension at 72°C, followed by a final extension for 10 min at 72°C. After purification, *scfv* PCR product and the vector *p*ET30(a)-*ldp* containing the LDP-encoded fragment [13] were digested with *Nde I* and *EcoR I* restriction enzymes (Takara, Dalian, China), and were then ligated and used to transform competent *Escherichia coli* (*E. coli*) strain DH5α (Novagen, Madison, Wisconsin, USA). Transformed cells were plated onto Luria–Bertani agar plates containing 50 μg/ml of kanamycin, and then the plates were inverted and incubated at 37°C overnight. The positive colony on the plates was selected and sequenced to confirm the DNA fragment.

Expression of fusion protein ER(Fv–LDP)

A single clone *p*ET30(a)-*scfv-ldp* was used to transform fresh competent *E. coli* strain BL21(DE3) star (Novagen). Colonies were incubated in Luria–Bertani medium containing 50 μg/ml of kanamycin at 37°C with shaking at 250 rpm until an A_{600} of 0.5–0.7 was achieved. The target protein was then expressed by inducing it with 0.3 mmol/l isopropyl β-D-thiogalactopyranoside at 30°C for 12 h. Five samples of recombinant bacteria, including supernatant of media, total protein of cells, periplasmic fraction, cytoplasmic soluble fraction, and inclusion bodies, were prepared and analyzed using 10% SDS-PAGE gel.

Purification and refolding of fusion protein ER(Fv–LDP)

The recombinant bacteria were harvested by centrifugation at 10 000*g* for 15 min at 4°C. The cell paste was resuspended in the buffer (20 mmol/l Tris-HCl, pH 8.0) and sonicated on ice. After centrifugation, the pellet was resuspended in cold isolation buffer (20 mmol/l Tris-HCl, 0.5 mol/l NaCl, and *2%* Triton-X 100, pH 8.0) and washed. The washing step was repeated, and then the inclusion body pellet was dissolved in binding buffer (20 mmol/l Tris-HCl, 0.5 mol/l NaCl, 5 mmol/l imidazole, and 8 mol/l urea, pH 8.0). After 1 h incubation at room temperature, the insoluble particle was removed by centrifugation at high speed for 30 min at 4°C. The supernatant was filtered through a 0.45-μm filter to remove any remaining particles. Fusion protein ER(Fv–LDP) was purified with affinity columns prepacked with precharged Ni Sepharose (GE Healthcare Life Science, Uppsala, Sweden) according to the operation manual. The purified protein was refolded by stepwise dialysis from a high concentration to a low concentration of denaturant and was then concentrated by ultrafiltration (Millipore, Belford, Massachusetts, USA).

Preparation of enediyne-energized fusion protein ER(Fv–LDP–AE)

The fusion protein ER(Fv–LDP) was assembled with the AE derived from LDM. Briefly, 10 mg LDM were suspended in 5 ml of cold methanol and whisked at 4°C for 5 min, and the mixture was placed at −20°C for 1 h. The AE fraction was collected in a brown bottle after gel-filtration with Sephadex G-75 column (GE Healthcare Life Science) and stored at −70°C. To obtain enediyne-energized ER(Fv–LDP–AE), ER(Fv–LDP) and AE were mixed at a 1 : 3 molar ratio in 10 μmol/l PBS (pH 7.0), and

reacted at room temperature for 12 h. Finally, ER (Fv–LDP–AE) was separated from free AE with Sephadex G-25 gel-filtration column (GE Healthcare Life Science).

Binding affinity assay

Enzyme-linked immunosorbent assay was used for measuring the binding efficiency of ER(Fv–LDP) to EGFR (Sigma-Aldrich, St Louis, Missouri, USA). Ninety-six-well plates were coated with EGFR (100 μl/well) by addition of 5 μg/ml of EGFR in PBS (pH 7.4) at 4°C overnight. The plates were incubated with PBS containing 2% BSA to block nonspecific binding, and then 100 μl of five-fold dilutions of ER(Fv–LDP) ranging from 15 000 to 0.2 nmol/l were added and incubated at 37°C for 2 h. A 2000-fold diluted mouse anti-His tag monoclonal antibody (Tiangen Biotechnology, Beijing, China) and a 5000-fold diluted horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Zhongshan Golden Bridge Biotechnology, Beijing, China) were used as the primary antibody and the secondary antibody, respectively. A total of 100 μl of 0.01% 3,3′,5,5′-tetramethylbenzidine in 0.1 mol/l phosphate–citrate buffer (pH 5.0) was added as substrate solution, followed by terminating reaction with 2 mol/l H_2SO_4. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Bremen, Germany), and the affinity constant (K_a) was calculated. All assays were carried out in triplicate.

Expression levels of EGFR in various cell lines were analyzed by western blot as described by Guo *et al.* [14]. Immunofluorescence assay was applied to compare the binding activity of ER(Fv–LDP) to four cell lines that express EGFR at different levels. Cells were seeded on coverslips and incubated at 37°C overnight, and then fixed with methanol and blocked with goat serum. After washing with PBS, the cells were incubated sequentially with 50 μg/ml of ER(Fv–LDP), mouse anti-His tag monoclonal antibody, and fluorescein isothiocyanate (FITC)-conjugated goat antimouse antibody (Zhongshan Golden Bridge Biotechnology). Fluorescence was observed under a fluorescence microscope and recorded by a camera.

To compare the binding activity of ER(Fv–LDP), ER(Fv), and LDP to EGFR-overexpressing tumor cells, FACS-based saturation binding assay was applied [15]. FITC dissolved in dimethyl sulfoxide was conjugated to proteins at a 1 : 24 molar ratio in carbonate buffer (0.16 mol/l Na_2CO_3 and 0.33 mol/l $NaHCO_3$, pH 9.5) at 4°C for 12 h. The unbound FITC was removed by ultrafiltration and washed with PBS. A431 cells of 5×10^5 cells/tube in the logarithmic phase were harvested and resuspended in 100 μl of reaction buffer (PBS + 2% FBS). FITC-labeled proteins were added and incubated with cells at room temperature for 2 h. Cells were resuspended in 100 μl of reaction buffer and washed three times, and were then analyzed using FACS Calibur (BD Biosciences, Franklin Lakes, New Jersey, USA). The dissociation constant (K_d) and the maximum number of binding sites (B_{max}) were determined by analysis of the mean fluorescence intensity versus concentration of proteins with GraphPad Prism 5 (GraphPad Software, San Diego, California, USA).

Internalization assay

A431 cells were seeded on coverslips and incubated for 24 h. ER(Fv–LDP) was added at a final concentration of 1 μmol/l, and then incubated at 37°C for another 8 h. After the cells had been fixed with paraformaldehyde and blocked, they were incubated sequentially with mouse anti-His tag monoclonal antibody and FITC-conjugated goat antimouse antibody. Then, 1 mg/ml of Hoechst 33342 (Beyotime Institute of Biotechnology, Shanghai, China) was added for nucleus staining. Fluorescence was observed with a fluorescence microscope.

FACS analysis was also carried out to detect the internalization efficiency of fusion protein ER(Fv–LDP) as described previously [16]. To prevent internalization, 5×10^5 cells/tube of A431 cells were suspended in 100 μl of precooled FITC-labeled ER(Fv–LDP) at 10 μmol/l and incubated at 4°C for 1 h. After washing twice with cold reaction buffer to remove unbound protein, cells were resuspended and incubated for 0.25, 0.5, 1, 1,5, or 2 h at 37°C for internalization. After centrifugation, the pellet was resuspended in 0.4% trypan blue, which is capable of quenching the fluorescence of FITC on the cell surface. One sample as blank was set without 37°C incubation; another sample without 37°C incubation and trypan blue dye was used to measure initial bound fluorescence intensity. The intracellular fluorescence intensity was detected using FACS Calibur, and data were reported as the percentage of initially bound fluorescence.

In-vitro bioactivity of ER(Fv–LDP) and ER(Fv–LDP–AE)

To assess the cytotoxicity of ER(Fv–LDP) and ER (Fv–LDP–AE) to EGFR-overexpressing cancer cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to determine cell proliferation. Cells were plated on 96-well plates at a density of 5000 cells/well, and placed at 37°C in 5% CO_2 overnight for adhesion. Different concentrations of proteins were added in triplicate and incubated for an additional 48 h. After adding 20 μl of MTT (5 mg/ml) and 4 h incubation, the culture supernatant was removed, and then 150 μl of dimethyl sulfoxide was added. Cell plates were read at 570 nm with a microplate reader (Thermo Fisher Scientific), and the 50% inhibitory concentration (IC_{50}) of the tested proteins was calculated.

Flow cytometry was employed to detect the influence of fusion protein on cancer cell cycle. A431 cells cultured on six-well plates were treated with ER(Fv–LDP) or

ER(Fv–LDP–AE) for 48 h. The cells were collected and fixed with 70% ethanol, and then resuspended in propidium iodide (PI) dye containing 100 μg/ml of RNaseA and 25 μg/ml of PI. Cell cycle test was performed after a light proof dyeing at room temperature.

As the fusion protein ER(Fv–LDP) and its energized ER(Fv–LDP–AE) were both targeted on EGFR, the effect of these recombinant proteins on EGFR expression level and phosphorylation was assayed. After incubation with ER(Fv–LDP) or ER(Fv–LDP–AE) for 48 h, the whole cell lysates were prepared and analyzed by western blot. The polyvinylidene fluoride membrane samples containing cell proteins were probed with anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) or phospho-EGFR antibody (Thr669) (Cell Signaling Technology, Beverly, Massachusetts, USA), then with antirabbit IgG HRP-linked antibody (Cell Signaling Technology, and finally visualized with Immobiolion Western Chemiluminescent HRP Substrate (Millipore).

In-vivo efficacy study

The therapeutic efficacy of ER(Fv–LDP) and ER (Fv–LDP–AE) was determined in BALB/c (*nu/nu*) athymic mice (4–6 weeks old) bearing human epidermoid carcinoma A431 xenograft. The female BALB/c (nu/nu) mice were purchased from the Institute for Experimental Animals, Chinese Academy of Medical Sciences and Peking Union Medical College. The experiments were performed according to the regulation of Good Laboratory Practice for nonclinical laboratory studies of drugs issued by the National Scientific and Technologic Committee of People's Republic of China.

Mice were inoculated subcutaneously in the right flank with 1×10^7 A431 cells suspended in 200 μl PBS. After 3–4 weeks, tumors were dissected aseptically from donor animals and cut into pieces of 2 mm^3, and then the pieces were transplanted subcutaneously in the right flank of mice. When the tumors were established, mice were randomly divided into groups of six mice each. The experimental mice were injected twice through the tail vein at an interval of 7 days. At the same time, one group of mice was given physiological saline as control. Every 3 days, tumor size was measured with a caliper, and tumor volume was calculated using the following formula: tumor volume $= 0.5 \times \text{length} \times (\text{width})^2$. When the experiment was finished, the mice were weighed and killed in order to dissect the tumors. Tumors were weighed and the tumor growth inhibition was calculated as follows: tumor growth inhibition $= (1 - T/C) \times 100\%$. T is the mean tumor weight of the treated group, whereas C represents the mean tumor weight of the control group [17].

Statistical analysis

Data were presented as the mean ± SD. Statistical comparisons between groups were executed by Student's t-test, and a significant difference was considered if $P < 0.05$.

Results

Preparation of fusion protein ER(Fv–LDP) and its enediyne-energized analogue ER(Fv–LDP–AE)

The expression vector *p*ET30(a)-*scfv-ldp* was presented as shown in Fig. 1b, including *scfv* gene encoding anti-EGFR ScFv, *ldp* gene, and the gene of a linker, which is located between the C-terminus of *scfv* and the N-terminus of *ldp*. As shown in Fig. 1c, fusion protein ER(Fv–LDP) with histidine tag was expressed in the form of an inclusion body by *E. coli* BL21(DE3) star after inducing it with isopropyl β-D-thiogalactopyranoside. After purification and refolding, approximately 10 mg ER(Fv–LDP) over 90% purity were yielded from 1 l fermentation broth, and migrated as a single band of about 38 kDa in SDS-polyacrylamide gel electrophoresis gel under reducing condition (Fig. 1d). Fusion protein ER(Fv–LDP) was mixed with AE at a molar ratio of 1 : 3 overnight, and the enediyne-energized product ER(Fv–LDP–AE) was purified by using a Sephadex G-25 column and monitoring the absorbance at 280 nm in real time. The chromatogram of energized fusion protein ER(Fv–LDP–AE) showed a fusion protein peak at 3.42 min and an AE peak at 7.60 min (Fig. 1e), which were the retention times for ER(Fv–LDP) and AE, respectively.

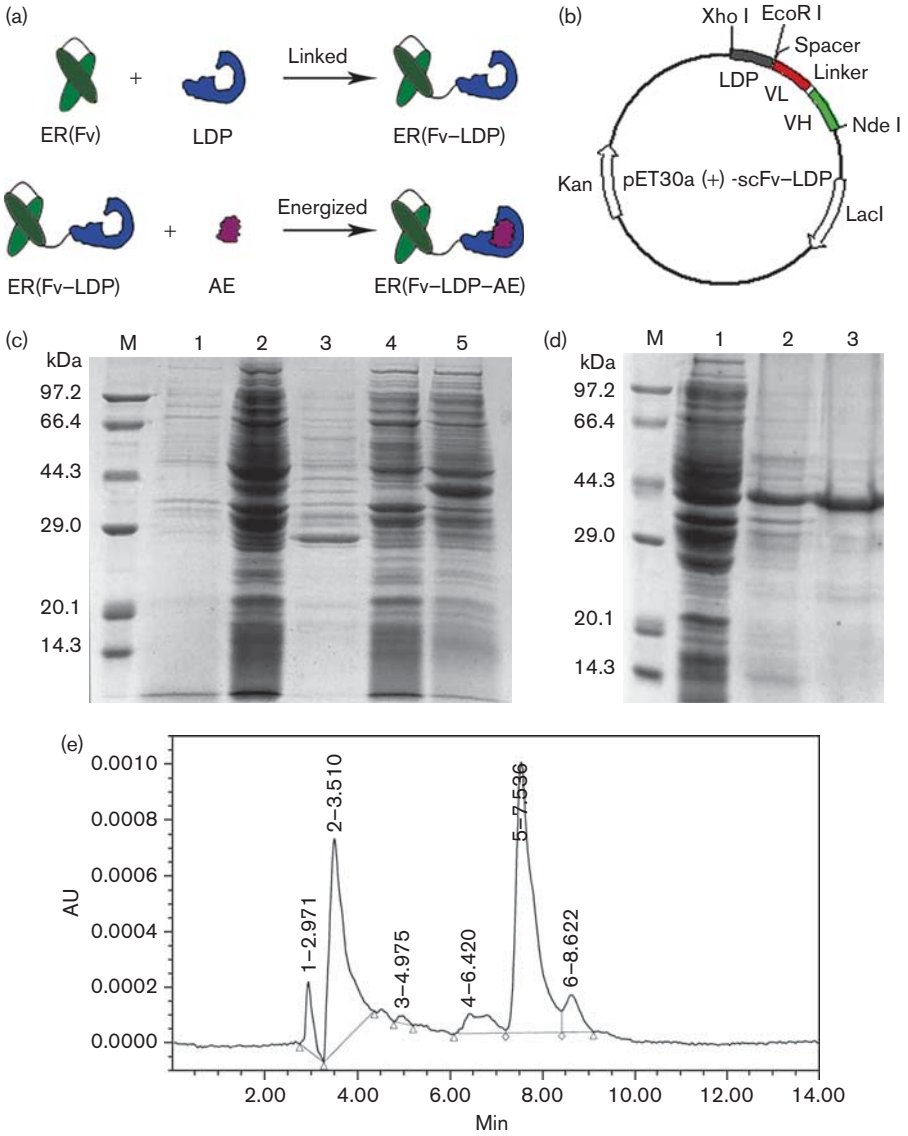
Binding and internalization of ER(Fv–LDP)

The binding efficiency of ER(Fv–LDP) to target antigen EGFR was examined by enzyme-linked immunosorbent assay. As shown in Fig. 2a, ER(Fv–LDP) possessed impressive binding affinity for EGFR with an association constant (K_a) of 1.4×10^8 l/mol determined with concentration-dependent dissociation curve.

Three EGFR-overexpressing cancer cell lines, A431, A549, and MCF-7, and one normal cell line HEK293 as EGFR negative control were chosen for immunofluorescence assay. As shown in Fig. 2c and d, the green fluorescence of FITC was observed on the membrane and in the cytoplasm of cancer cells under a fluorescence microscope, indicating that ER(Fv–LDP) protein bound specifically to the membrane of cancer cells, and then internalized into the cytoplasm through receptor-mediated endocytosis. No discernible fluorescence was observed around HEK293 cells, indicating little ER (Fv–LDP) bound to normal cells.

To compare the binding activity of ER(Fv–LDP), ER(Fv), and LDP, fluorescence intensity of FITC bound to the cells was measured by FACS Calibur. The results of binding-saturation analysis revealed that ER(Fv–LDP) and ER(Fv) presented greater binding affinity than LDP to EGFR-overexpressing cancer cells. The K_d of two fusion proteins were 212 and 336 nmol/l for A431 cells, respectively. However, the B_{max} of ER(Fv–LDP) was lower than that of ER(Fv), which suggested that the LDP

Fig. 1



Preparation of fusion protein ER(Fv–LDP) and its enediyne-energized analogue ER(Fv–LDP–AE). (a) Schematic presentation of ER(Fv–LDP) and ER(Fv–LDP–AE). Linking of ER(Fv) to LDP was carried out by recombination, and intercalation of AE into the hydrophobic pocket of ER(Fv–LDP) was mainly driven by hydrophobic interaction. (b) A diagram of expression vector *p*ET30(a)-*scfv-ldp*, with *NdeI*/*XhoI* gene fragment encoding fusion protein ER(Fv–LDP). ER(Fv–LDP) consisted of an scFv against EGFR, the apoprotein of LDM, and a (GGGGS) linker. (c) SDS-PAGE of fusion protein ER(Fv–LDP) location in recombinant *Escherichia coli* BL21. Lane 1, supernatant of media sample; lane 2, total protein; lane 3, periplasmic fraction; lane 4, cytoplasmic soluble fraction; lane 5, inclusion bodies. (d) SDS-PAGE analysis of purified ER(Fv–LDP) protein. Lane 1, total protein of recombinant *Escherichia coli* BL21; lane 2, inclusion bodies; lane 3, purified ER(Fv–LDP) protein. (e) Enediyne-energized fusion protein ER(Fv–LDP–AE) determined with reverse-phase high-performance liquid chromatography on C4 300A column at 350 nm.

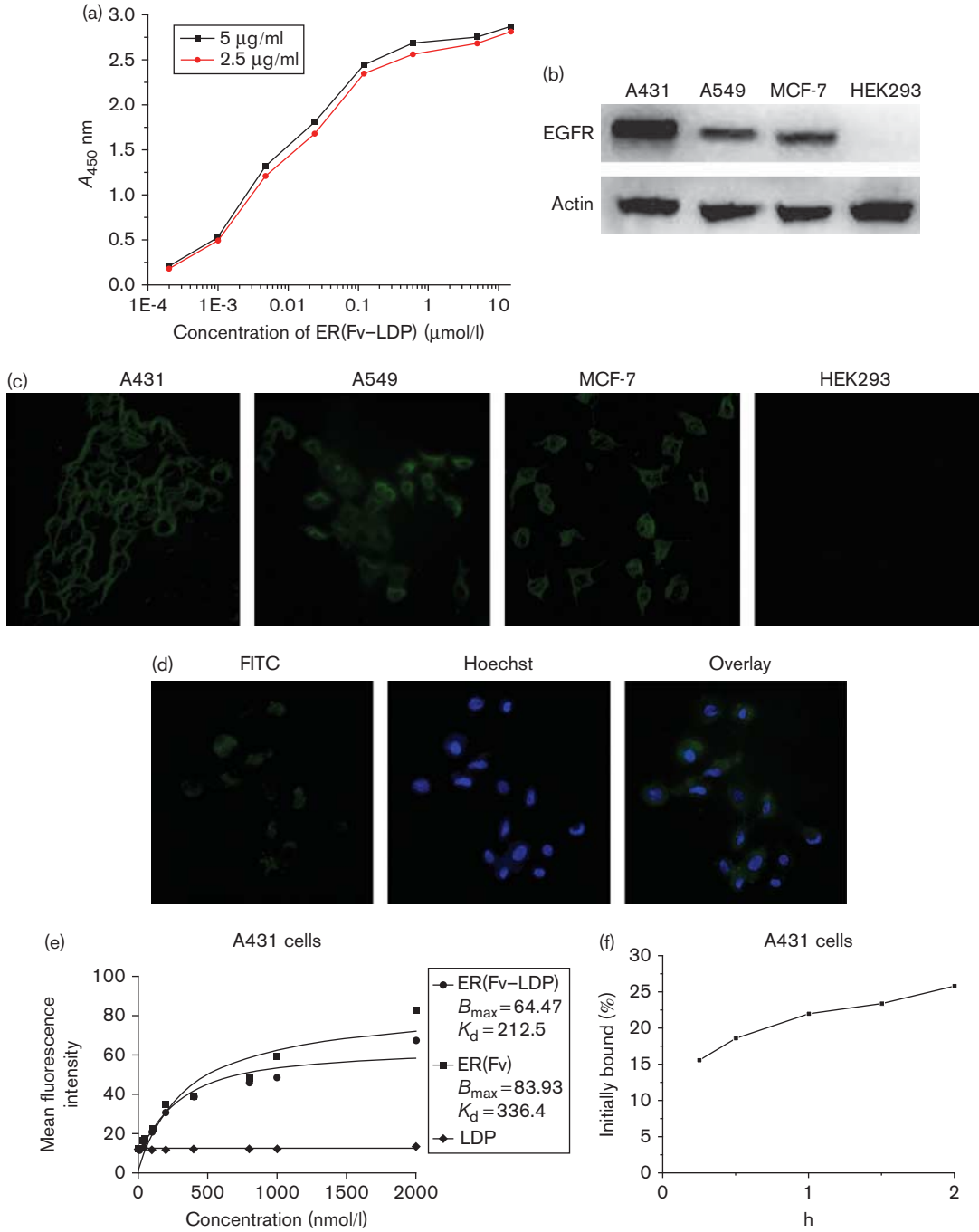
component probably blocked a number of binding sites on the cell surface. The K_d of LDP could not be calculated because of little LDP bound to cells at a concentration less than 2 μmol/l.

The internalization efficiency of ER(Fv–LDP) was analyzed by determining the fluorescence intensity of FITC in the cytoplasm of cancer cells. Figure 2f shows that in the first 2 h, 25% FITC-labeled ER(Fv–LDP) bound to cell membrane had moved into the cells, and the speed of endocytosis was reduced stepwise with time.

In-vitro efficacy of ER(Fv–LDP) and ER(Fv–LDP–AE)

The antiproliferative activity of ER(Fv–LDP) and its enediyne-energized analogue ER(Fv–LDP–AE) on three human carcinoma cell lines and the normal human cell line HEK293 was measured by using the MTT assay. ER(Fv), LDP, and LDM were also examined for

Fig. 2



Binding and internalization of fusion protein ER(Fv–LDP). (a) Enzyme-linked immunosorbent assay assay of ER(Fv–LDP) reacted with epidermal growth factor receptor (EGFR) antigen. (b) Expression of EGFR in different carcinoma cell lines analyzed by western blot. (c) Immunofluorescence assay of ER(Fv–LDP) bound to different carcinoma cells. Green fluorescence located around the cells indicated FITC-labeled ER(Fv–LDP) bound to EGFR on the cell membrane. The image was observed under fluorescence microscope at × 200. (d) Immunofluorescence assay of ER(Fv–LDP) internalized into the cytoplasm of A431 cells. (e) The binding activity of ER(Fv-LDP), ER(Fv), and LDP to A431 cells was compared by fluorescence-activated cell sorting (FACS)-based saturation binding assay. The mean fluorescence intensity was plotted versus the concentration of FITC-labeled protein, and K_d and B_{max} were determined using Prism 5 software (GraphPad Software, California, USA). (f) Internalization process of bound ER(Fv–LDP) protein was measured by FACS analysis. Trypan blue was applied to quench the fluorescence of FITC on cell surfaces, differentiating internalized proteins from the extracellular ones.

comparison. As shown in Table 1, the IC_{50} values of the fusion protein ER(Fv–LDP) against the three EGFR-overexpressing cancer cell lines ranged from 10^{-7} to 10^{-9} mol/l, being hundreds of times more cytotoxic than that of ER(Fv) and LDP. Compared in terms of IC_{50}, the energized fusion protein ER(Fv–LDP–AE) was seven-fold, 10-fold, and six-fold more potent than that of LDM in cytotoxicity to A431, A549, and MCF-7 cells, respectively. However, for normal human HEK293 cells, no obvious difference was observed either among ER(Fv–LDP), ER(Fv), and LDP, or between ER(Fv–LDP–AE) and free LDM. Three recombinant proteins, ER(Fv–LDP), ER(Fv), and ER(Fv–LDP–AE), presented minor cytotoxity to HEK293 cells, with the $IC_{50} > 10^{-4}$ mol/l. These results suggested that the selectivity and increased activity of fusion proteins were mainly due to the scFv binding that directs the recombinant proteins to target cancer cells.

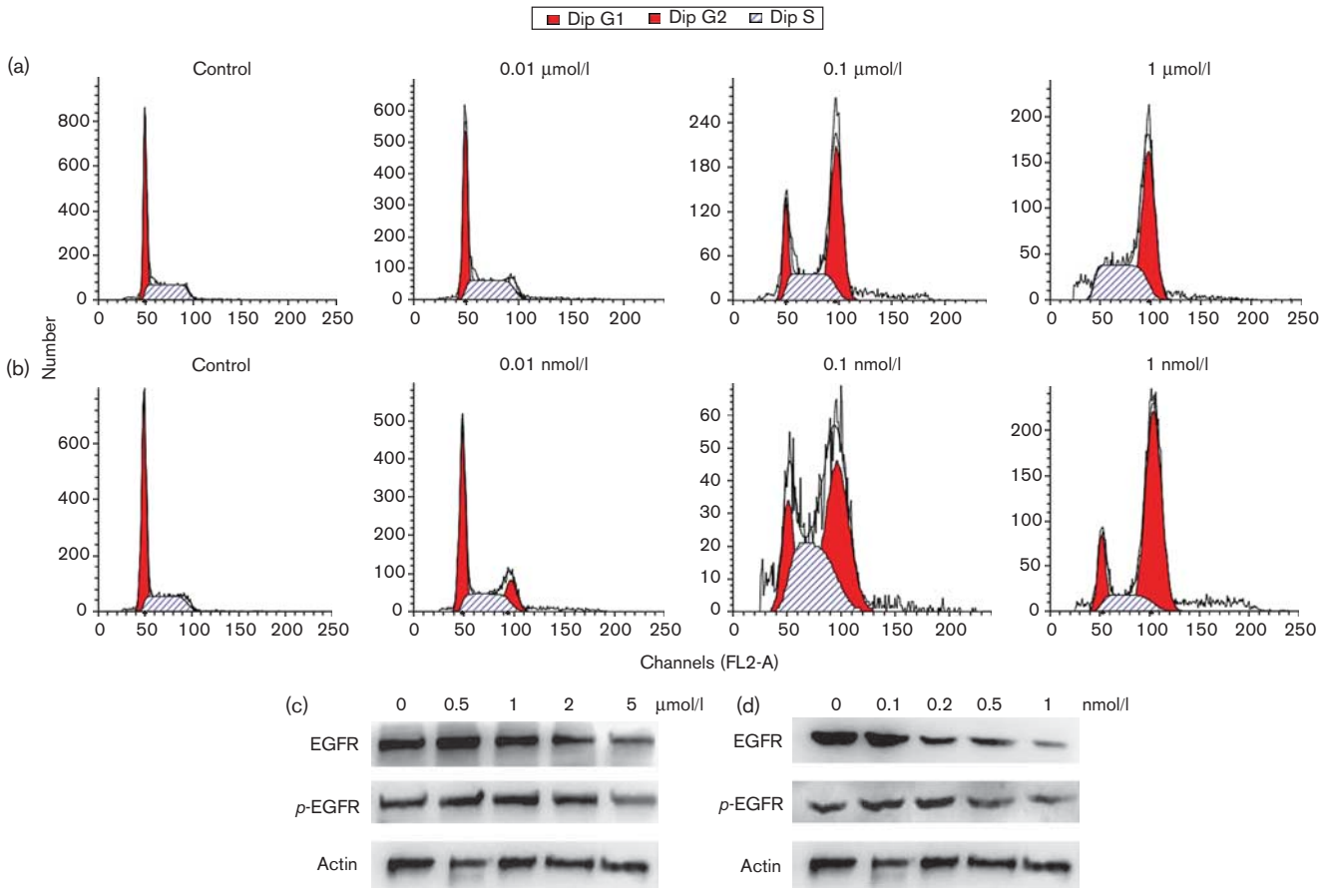
Table 1 Determination of IC_{50} values for different cancer cell lines

	IC_{50} (mol/l)			
Groups	A431	A549	MCF-7	HEK293
ER(Fv)	1.9×10^{-6}	8.0×10^{-8}	7.8×10^{-6}	9.8×10^{-5}
LDP	6.8×10^{-6}	1.6×10^{-6}	9.8×10^{-6}	1.1×10^{-4}
ER(Fv–LDP)	1.2×10^{-8}	4.1×10^{-9}	2.8×10^{-7}	7.3×10^{-5}
LDM	9.0×10^{-12}	9.3×10^{-13}	5.5×10^{-10}	3.0×10^{-9}
ER(Fv–LDP–AE)	1.4×10^{-12}	9.5×10^{-14}	9.2×10^{-11}	1.0×10^{-9}

IC_{50}, inhibitory concentration; LDM, lidamycin.

The cell cycle was checked by measuring PI fluorescence with flow cytometry. Figure 3a shows that ER(Fv–LDP) caused G2/M phase arrest of A431 cells, and the degree of arrest was concentration dependent. In addition, the cell cycle was also arrested by 0.1 nmol/l ER(Fv–LDP–AE) treatment; at 1 nmol/l, 71.6% of cells were in G2/M phase. Differing from ER(Fv–LDP), the energized fusion protein ER(Fv–LDP–AE) led to the reduction of not only the cells in G1 phase but also those in S phase.

Fig. 3



(a and b) Cell cycle distribution of human epidermoid carcinoma A431 cells after ER(Fv–LDP) and ER(Fv–LDP–AE) treatment, respectively. A431 cells were incubated with three doses of protein for 48 h, and then measured by flow cytometry after PI dying. (c and d) EGFR expression and phosphorylation level of A431 cells after ER(Fv–LDP) and ER(Fv–LDP–AE) treatment, respectively, were analyzed by western blot. Each protein was given at four different doses: 0.5, 1, 2, and 5 μmol/l for ER(Fv–LDP); 0.1, 0.2, 0.5, and 1 nmol/l for ER(Fv–LDP–AE). EGFR, epidermal growth factor receptor; PI, propidium iodide.

Because of these proteins targeting EGFR, western blot analysis reflected that, in addition to the cytotoxic effect, the fusion protein and energized fusion protein could affect EGFR signaling of cancer cells. As shown in Fig. 3c, ER(Fv–LDP) at the dose of 5 μmol/l down-regulated the EGFR expression level and its phosphorylation. ER(Fv–LDP–AE) at the concentration of 0.2 and 0.5 nmol/l caused EGFR and *p*-EGFR reduction, respectively.

In-vivo efficacy of ER(Fv–LDP) and ER(Fv–LDP–AE)

Three experiments were performed to evaluate the in-vivo efficacy of ER(Fv–LDP) and ER(Fv–LDP–AE). Experiment 1 was set to determine the efficacy of ER(Fv), LDP, ER(Fv–LDP), and the mixture of ER(Fv) plus LDP. In Fig. 4a, tumor growth curves show that both ER(Fv) and LDP inhibited the growth of carcinoma A431 xenograft in nude mice; in addition, the mixture of ER(Fv) and LDP displayed approximate efficacy. The fusion protein ER(Fv–LDP) showed much stronger antitumor effect than that of ER(Fv), LDP, or their mixture. As evaluated by tumor weight at the end of the experiment, the inhibitory rates were 46.7% by ER(Fv), 48.5% by LDP, 49.9% by the ER(Fv) plus LDP mixture, and 63.6% by ER(Fv–LDP), respectively. No significant difference in body weight was found among treated groups and the control, indicating that the administered doses of the recombinant proteins were well tolerated (Fig. 4b).

Experiment 2 was set to determine the efficacy of the energized fusion protein ER(Fv–LDP–AE) on carcinoma A431 xenograft in nude mice. Various dosage levels of ER(Fv–LDP–AE) including 0.2, 0.3, and 0.4 mg/kg, respectively, were tested. LDM at a tolerated dose of 0.05 mg/kg and ER(Fv–LDP) at 0.5 mg/kg were used for comparison. Figure 4c shows that ER(Fv–LDP–AE) significantly inhibited the growth of A431 xenografts. The inhibition rates by ER(Fv–LDP–AE) of 0.2, 0.3 , and 0.4 mg/kg were 75.4, 81.1 and 89.2%, respectively. For LDM and ER(Fv–LDP), the inhibition rates were correspondingly 64.1 and 58.3%. The groups treated with ER(Fv–LDP–AE) showed significant statistical difference from other treated groups and the control ($P < 0.01$). At the end of the experiment, the body weight loss of treated mice was less than 10% of the initial weight, considering that the treatment dosage was tolerated.

Experiment 3 was set to further confirm the therapeutic efficacy of the fusion protein ER(Fv–LDP) and the energized fusion protein ER(Fv–LDP–AE). As shown in Fig. 4e, both ER(Fv–LDP) and ER(Fv–LDP–AE) were highly effective when administered at appropriate dosage levels. No body weight loss was found in treated animals.

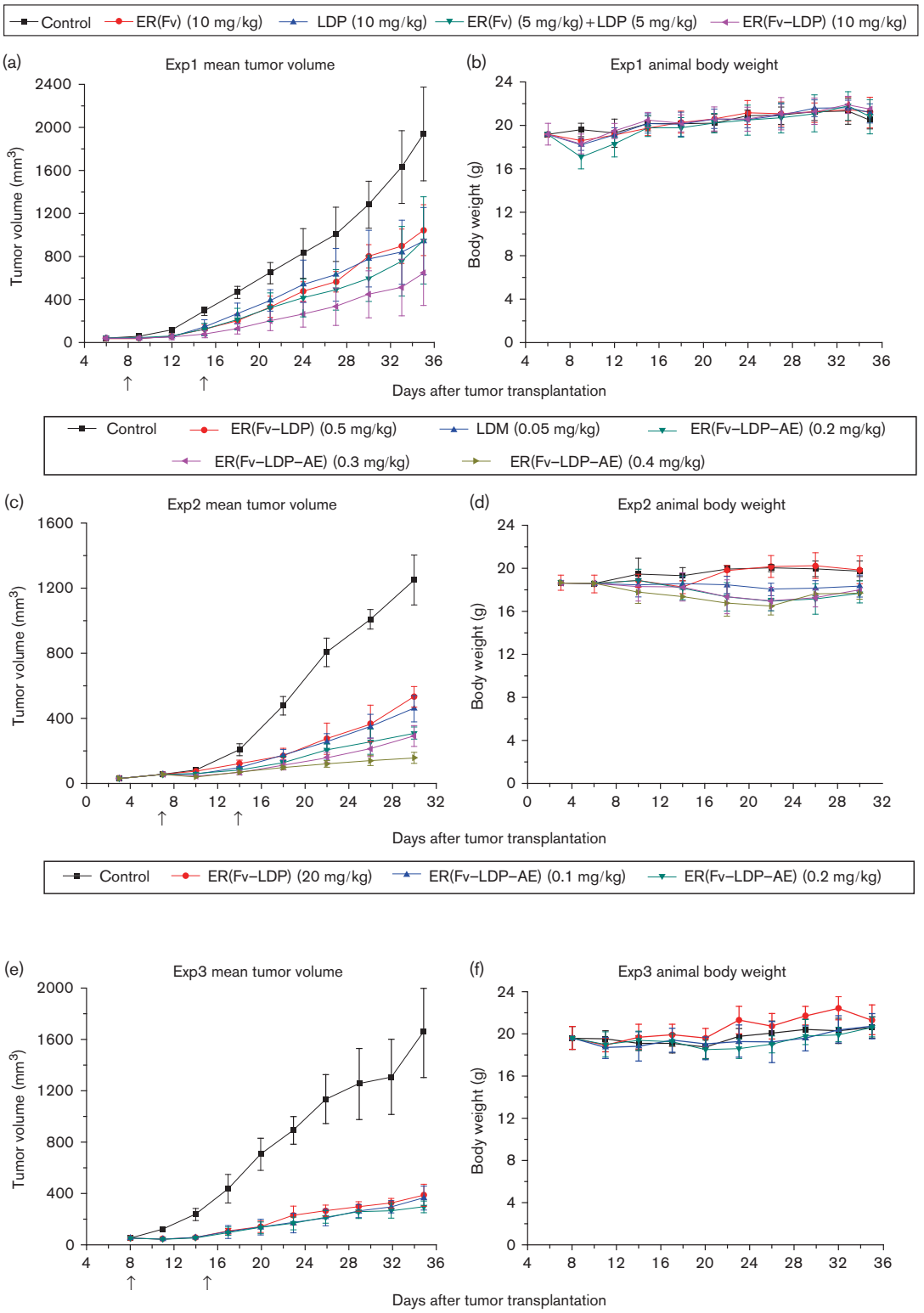
Discussion

EGFR, a tyrosine kinase receptor, is currently regarded as a promising and validated molecular target in cancer drug therapy. EGFR and its ligand are a part of cell signal transduction pathways, which are closely related to a number of processes important for cancer development and progression, including cell proliferation, differentiation, migration, and angiogenesis [18]. In clinical cancer therapy, two kinds of drugs against EGFR, monoclonal antibodies and small-molecule kinase inhibitors, have been applied for drug combination with conventional chemotherapy or radiotherapy, and showed better therapeutic effects than the separate treatment [19–21]. As the former affects ligand/receptor interaction through competitive binding of the ligand to the EGFR-binding site and triggers antibody-dependent cell-mediated cytotoxicity, the latter occupies the ATP binding site of EGFR and consequently abolishes protein–tyrosine kinase activity. These two targeting medicines could cause tumor cell cycle arrest, promote apoptosis, inhibit tumor cell invasion and metastasis, suppress angiogenesis, and increase sensitivity to radiotherapy and chemotherapy by blocking EGFR-related cell signal transduction pathways.

scFv, a miniaturized antibody, is a targeting molecule used for constructing antibody-targeted drugs. In comparison, single-chain antibody scFv of 27 kDa in molecular weight is much lower than the intact antibody of 150 kDa. Because of its small size, scFv shows stronger penetrating capability that could reach the solid tumor interior deeply [22], as well as lower immunogenicity that causes lower human antimouse antibody response [23,24]. Moreover, scFv could be expressed in genetically engineered bacteria for large-scale manufacture. Because the effector function of scFv is not comparable to natural antibody owing to Fc shortage, connecting to other effectors for construction of fusion proteins by genetic manipulation has become an important application of scFv [25]. As Wang *et al.* [26] reported, a recombinant e23sFv–TD–tBID, consisting of a single-chain anti-HER2 antibody fragment linked to a human active truncated Bid, selectively bound to HER2-positive tumor cells and induced apoptotic cell death *in vitro* and *in vivo*. A novel immunocytokine, in which murine interferon-α2 was fused with the tumor-targeting antibody fragment scFv(F8), specific to the alternatively spliced ethylenediamine domain of fibronectin, was shown to retain both antigen binding activity and interferon-α activity [27]. Preparation of scFv-based fusion proteins is effective in modifying the structure of antibodies and active proteins, and in acquiring a uniform product, avoiding heterogeneity caused by chemical coupling [28]. At present, a variety of fusion proteins based on anti-EGFR scFv, such as bispecific EGFR/CD3 diabody [29], anti-EGF receptor immunotoxin 425(scFv)-ETA′ [30], and anti-EGFR scFv-protein III fusion protein [31], presented significant cytotoxicity towards EGFR-positive carcinoma cell lines in preclinical study.

LDM, an enediyne antibiotic possessing extremely potent cytotoxicity to cancer cells and significant inhibitory

Fig. 4



Antitumor efficacy of fusion protein ER(Fv–LDP) and energized fusion protein ER(Fv–LDP–AE) on human epidermoid carcinoma A431 xenograft in nude mice. (a and b) experiment 1, nude mice bearing subcutaneous xenografts were treated with intravenous injection of ER(Fv), LDP, ER(Fv) plus LDP, and ER(Fv–LDP), respectively, at days 8 and 15 (shown as arrows). (c and d) experiment 2, nude mice were treated intravenously with ER(Fv–LDP), LDM, and different doses of ER(Fv–LDP–AE), respectively, at days 7 and 14 (shown as arrows). (e and f) experiment 3, nude mice were treated intravenously with a large dose of ER(Fv–LDP), or two small doses of ER(Fv–LDP–AE) at days 8 and 15 (shown as arrows). Data are shown as mean tumor volume ± SD or animal weight ± SD versus experimental days. LDM, lidamycin.

efficacy on the growth of cancer xenograft in nude mice, is considered an admirable effector molecule for the construction of molecule-targeted drugs [32]. Introduction of a target-oriented molecule into LDM is effective in promoting the antitumor efficacy of the drug and reducing the damage to normal tissues. At present, several scFv-based and LDM-containing energized fusion proteins have been manufactured with the above-mentioned two-step procedure in our laboratory. An energized fusion protein Fv–LDP–AE targeting IV collagenase was effective against hepatoma H22 in mice with an inhibitory rate of 95.5% [33]. HER2 (Fv–LDM), composed of anti-HER2 scFv and LDM, showed more than 10-fold stronger cytotoxicity to HER2-overexpressing cancer cells.

Recent studies indicated that LDP itself, the apoprotein of LDM, shows binding capability to a spectrum of human cancers, and notably that the binding capability correlates with the overexpression of EGFR and HER2 in a part of examined tumors with tissue microarray [34,35]. In addition, LDP displayed moderate cytotoxicity to human hepatoma Bel-7402 cells with a IC_{50} value of 7.05×10^{-5} mol/l that exerted tumor suppression of hepatoma H22 in Kunming mice. Therefore, in the case of the present study, scFv serves as the major target-oriented moiety in the fusion protein Fv–LDP. In addition, LDP moiety also plays an active role in the targeting process, suggesting that the fusion protein ER(Fv–LDP) might have concerted capability in targeting action. As to the effector function of the energized fusion protein ER(Fv–LDP–AE), certainly the enediyne AE moiety displays the major 'warhead' action in killing target cells; however, the LDP moiety can still play a role in cytotoxicity. In the animal experiment, the mixture of ER(Fv) and LDP could not attain the level of the antitumor effect of ER(Fv–LDP) at the equivalent dosage. ER(Fv–LDP) displays synergistic effects with ER(Fv) and LDP on target cancer cells. The functional mechanism of LDP has not been fully clarified, but we hypothesized that ER(Fv) was likely to work as a functional antibody, and influenced cell growth by acting on the cell signaling pathway relative to EGFR.

Through the combination of this study and previous researches, a technical platform for scFv-based enediyne-energized fusion protein has been developed in our laboratory. Three biological technologies are involved: screening of single-chain Fv against specific antigen by phage display, linking the scFv fragment with the apoprotein LDP for a fusion protein by DNA recombination, and inserting the AE chomophore for an energized fusion protein through molecular reconstitution, which displays potential application prospects for preparation of antibody-targeted drugs.

Conclusion

In this study, an scFv-based fusion protein and its enediyne-energized analogue targeting EGFR were prepared, which showed highly potent cytotoxicity to a variety of EGFR-overexpressing carcinoma cells and remarkable growth inhibition of human carcinoma A431 xenograft in nude mice. The scFv-based LDM-containing fusion proteins show target-oriented function, enhanced antitumor efficacy, and they interfere with the EGFR-related signaling pathway. This suggests that the fusion protein ER(Fv–LDP) and energized fusion protein ER(Fv–LDP–AE) would be promising candidates as antibody-based drugs for cancer therapy. In addition, an integrated technical platform including phage display, DNA recombination, and molecular reconstitution has been established to prepare scFv-based and enediyne-energized fusion protein.

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Conflicts of interest

There are no conflicts of interest.

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