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A Gelatinases-targeting scFv-based Fusion Protein Shows Enhanced Antitumour Activity with Endostar against Hepatoma

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Abstract: Gelatinases play important roles in tumour invasion and metastasis and are thus considered promising targets for cancer therapy. In this study, a new single-chain variable fragment (scFv)-based fusion protein Fv-LDP, composed of the anti-gelatinases scFv and lidamycin apoprotein (LDP), was prepared, and its combination with angiogenesis inhibitor Endostar was then investigated. The fusion protein Fv-LDP specifically bound to various tumour cells, and its binding capability to human pulmonary giant cell carcinoma (PG) cells was higher than that of LDP. Fv-LDP inhibited the expression and secretion of gelatinases and could be internalized into tumour cells via endocytosis. Fv-LDP also suppressed the growth of human hepatoma cells and murine hepatoma 22 transplanted in Kunming mice in various degrees. In addition, Endostar could enhance the synergistic or additive inhibition of Fv-LDP on the growth, migration or invasion of human hepatoma cells shown by a colony formation assay and a transwell-based migration or invasion assay, respectively. *In vivo*, Fv-LDP/Endostar combination showed a significantly synergistic effect on the growth of a human hepatoma xenograft, with an inhibition rate of 80.8% compared with the Fv-LDP (44.1%) or Endostar (8.9%)-treated group. The above-mentioned results indicate that the fusion protein Fv-LDP is effective against transplantable hepatoma in mice and human hepatoma xenografts in athymic mice. Moreover, Endostar can potentiate the inhibition effect of Fv-LDP on the growth of human hepatoma cells and xenografts. These data will provide a new combined strategy for improving the therapeutic efficacy of treatments for hepatoma or other gelatinase-overexpressing tumours.

Gelatinases are the most important cancer-related matrix metalloproteinases (MMPs). They can degrade various components of the extracellular matrix and play pivotal roles in tumour angiogenesis, invasion and metastasis [1–3]. Gelatinases consist of MMP-2 and MMP-9, which are abundantly expressed in a variety of malignant tumours and the stromal cells in the tumour microenvironment [4–6]. Gelatinases can promote tumour cell growth, angiogenesis, invasion and metastasis; conversely, the inhibition of gelatinases can result in the suppression of these tumour-developing processes [1–3]. Furthermore, the expression of gelatinases highly correlates with the poor outcome of tumours [7,8]. Gelatinases have been considered important biomarkers for cancer therapy [9,10]. However, the disappointing performance of synthetic MMP inhibitors in clinical trials for cancer therapy may be attributed to a lack of specificity and efficacy or undesirable side effects [3]. Developing antibody-based drugs may be a promising avenue to overcome these drawbacks of MMP inhibitors. In recent years, a series of low molecular weight antibody constructs containing antigen-binding domain have been explored to develop antibody-based drugs with better tumour penetration, such as antigen-binding fragment (Fab) [11], single-chain variable fragment (scFv) [12], single-domain antibody [13] and oligopeptides [14]. scFv is considered the most frequently used and favoured fragment to deliver protein-based drugs to cancer cells because of its moderate molecular size and faster tissue penetration [15]. Therefore, scFv-integrated fusion proteins may be of importance for the development of antibody-based drugs directed against gelatinases due to their obvious attributes.

Lidamycin (LDM), originally designated as C-1027, belongs to the family of enediyne antitumour antibiotics and has been known for its highly potent antitumour activity because of its unique ability to break single strand and/or double strand of DNA via radical-mediated hydrogen abstraction [16]. In terms of IC_{50} (50% inhibitory concentration) values, LDM is 10,000-fold more cytotoxic than mitomycin or doxorubicin [17]. *In vivo*, LDM can significantly inhibit the growth of transplantable tumours in mice [18]. LDM is also famous for its novel structure, which consists of a non-covalently bound LDM apoprotein LDP and a highly active enediyne chromophore AE [19]. LDP (10,500 Da) and AE (843 Da) can be dissociated and reconstituted, but the extremely potent antitumour activity of the rebuilt molecule is almost identical to that of natural LDM [20]. Therefore, LDM is a unique 'warhead' to generate novel scFv-based fusion proteins for cancer therapy via two-step procedure, including DNA recombination and molecular reconstitution [11–14,21]. Phase II clinical trials of LDM are underway in China.

LDP is composed of 110 amino acid residues with two pairs of disulphide bonds that are positioned between Cys36 and Cys45 as well as between Cys86 and Cys91 [22]. LDP can form a hydrophobic pocket for protecting and stabilizing AE [23]. However, LDP alone does not show aminopeptidase activity, which is different from the holo-antibiotic [24]. In addition, LDP can specifically bind to various human tumour tissues, and tumour tissues significantly differ from the corresponding normal tissues. The binding reactivity of LDP posi-

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tively correlates with the expression of VEGF and EGFR in lung carcinoma tissues and the expression of VEGF and HER2 in breast carcinoma tissues [21,25]. Furthermore, integrating LDP into a ligand oligopeptide or scFv that targets EGFR has resulted in augmented binding capability to cancer cells [12,14]. These results lay the basis for the preparation of scFv-based fusion protein Fv-LDP directed against gelatinases.

Endostar, a modified and recombinant human endostatin, has received great attention due to its anti-angiogenesis and tumour suppression activity [26]. Endostar alone can inhibit the wound healing migration and invasion of human breast cancer cells by down-regulating gelatinases [27]. In 2005, the Chinese State Food and Drug Administration approved Endostar for the treatment of non-small-cell lung cancer. Because the efficacy of single endostatin administration is limited, Endostar is often explored in combination with chemotherapeutic drugs in the treatment of various cancers. Notably, the results have shown that the combination of endostatin with chemotherapy or chemo-radiotherapy can significantly or synergistically inhibit the growth, invasion and metastasis of several tumours [28–30].

3G11 is a murine IgG_1-type monoclonal antibody (mAb) prepared by our laboratory that can specifically react with gelatinases. mAb3G11 shows positive immunoreactivity in most cases of colorectal carcinoma and can inhibit the secretion of gelatinases. 3G11-LDM conjugated via chemical cross-linking is highly effective against colon carcinoma in mice [31]. Based on the above data, a new scFv and LDP-based fusion protein directed against gelatinases, Fv-LDP, was constructed via recombinant DNA techniques. In addition, considering the inhibition of tumour migration and invasion by Endostar and MMP inhibitors as well as the enhanced or synergistic efficacy of Endostar and other chemotherapy drugs, the combination of Fv-LDP with Endostar may be a reasonable choice for cancer treatment. This study mainly documented the construction, expression, identification, bioactivity and significant therapeutic efficacy of Fv-LDP in a mouse transplantable hepatoma 22 model and the Fv-LDP/Endostar combination in a human hepatoma BEL-7402 xenograft model in athymic mice. The data confirmed that the scFv-based fusion protein consisting of LDP and an antibody fragment to gelatinases showed specific and augmented binding capability to cancer cells compared with LDP, and the Fv-LDP/Endostar combination displayed a potent synergistic effect on human tumour growth.

Materials and Methods

Construction of expression vector pET-30a(+)/fv-ldp. To obtain the recombinant plasmid pET-30a(+)/*fv-ldp*, the gene encoding scFv was first amplified via polymerase chain reaction (PCR) from the pKFv1027 vector carrying the gene encoding anti-gelatinases scFv from mAb3G11 in the format of VH-$(G_4S)_3$-VL using the forward primer 5′-CGCATATGCAGGTGAAGCTGCAGCAGTCT-3′ (the *Nde* I site is underlined) and the reverse primer 5′-CGGAATTCTGAACCGCCTCCACCACGTTTGATTTCCAG-3′ (the *EcoR* I site is underlined, and a G_4S spacer was added between the *scfv* sequence and *EcoR* I site). The LDP-encoding fragment was then amplified by PCR from the pIJ1027-GRGDS vector carrying the LDP-encoding gene using the sense primer 5′-CGGAATTCGCGCCCGCCTTCTCCGTCAGTCCC-3′ (the *EcoR* I site is underlined) and the antisense primer 5′-CCGCTCGAGTCAGCCGAAGGTCAGAGCCACGTG-3′ (the *Xho* I site is underlined). Both the *scfv* and *ldp* gene fragments amplified above were digested by the endonucleases *Nde* I/*EcoR* I and *EcoR* I/*Xho* I, respectively, and then mixed with the *Nde* I/*Xho* I-cleaved pET-30a(+) expression vector followed by overnight ligation at 16°C to generate the recombinant plasmid pET-30a(+)/*fv-ldp*. Finally, DNA sequencing analysis was carried out to verify the correct insertion of the *fv-ldp* gene.

Expression, purification, refolding and identification of fusion protein Fv-LDP. The pET-30a(+)/*fv-ldp* expression plasmid was transformed into the host strain *E.coli* BL21(DE3)star™ (Novagen, Darmstadt, Germany). The expression of Fv-LDP from different generations of recombinant strains was confirmed by SDS-PAGE and Western blotting using anti-his•tag mAb (Novagen, Darmstadt, Germany) as the primary antibody, as described previously [32]. Fv-LDP was then purified by ion metal affinity chromatography (IMAC) using AKTA purifier 10 according to the manufacturer's instructions followed by a stepwise dialysis, as reported previously [33]. To validate the correctness of gene translation, the molecular weight and 5′-terminal amino acid sequence were measured after the refolded Fv-LDP was refined using the pre-packed Superdex™ 75 column (GE Healthcare, Piscataway, NJ, USA).

Cell culture and reagents. Human hepatoma BEL-7402 and HepG2 cells, PG cells and the immortalized human white cells BJH-1 (presented by Professor Jianping, Cai, Beijing Hospital, China) were routinely cultured in modified RPMI-1640 or MEM medium (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Murine hepatoma 22 cells expressing gelatinases were ascites-passaged in female Kunming mice [34]. Endostar was purchased from Shandong Simcere-Medgenn Bio-pharmaceutical Co., Ltd (Yantai, Shandong, China).

Enzyme-linked immunosorbent assay (ELISA). The expression levels of gelatinases in various cell lines were first analysed by Western blotting as described by Gao *et al.* [32] using anti-MMP-2 mAb (CST, Danvers, MA, USA) and anti-MMP-9 mAb (Abcam, Cambridge, UK) as the primary antibodies. To assess the antigen-binding activity of Fv-LDP to gelatinases and various cell lines, ELISA was performed as described previously with some modification [13]. Briefly, 50 μl/well of 1500-fold diluted mouse anti-his·tag mAb (Novagen, Darmstadt, Germany) was first used as the primary antibody to recognize bound Fv-LDP protein after a 2-hr incubation of Fv-LDP with gelatinases or tested cells. Subsequently, 50 μl/well HRP-conjugated goat anti-mouse IgG (CST, Danvers, MA, USA) at a dilution of 1:2000 was added to the wells as the second antibody after a 1-hr incubation of the primary antibody. Unlike Fv-LDP, mAb3G11 could directly bind to HRP-conjugated goat anti-mouse IgG. Finally, the colour was developed using O-phenylenediamine (Sigma, St.Louis, MO, USA) substrate solution and stopped with H_2SO_4 solution, followed by the measurement of the absorbance at 492 nm (A_{492}). All assays were performed in triplicate.

Immunofluorescent cytochemical staining. The tested cells were fixed, permeabilized and blocked. These cells were then incubated with FITC-labelled Fv-LDP prepared according to a previously published method [12]. To compare the binding capability of Fv-LDP and LDP, PG cells were synchronously treated with FITC-labelled LDP and counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, St.Louis, MO, USA). All images were photographed using a fluorescent microscope or a laser scanning confocal microscope.

Co-immunoprecipitation (coIP) assay. The cells were routinely cultured and lysed. One milligram of total protein from cell lysates was incubated with 100 μg of Fv-LDP for 8 hr at 4°C after removing non-specific binding proteins via co-incubation with normal mouse IgG. The mixtures were then incubated overnight with 1 μg anti-MMP-2 or anti-MMP-9 mAb (Abcam, Cambridge, UK) at 4°C. The formed complexes were collected with protein A+G agarose beads (Beyotime, Jiangsu, China), and the precipitates were thoroughly washed with ice-cold PBS. The captured Fv-LDP protein was detected by Western blotting using anti-his·tag mAb (Novagen, Darmstadt, Germany) as the primary antibody.

Gelatin zymography assay. The expression level of gelatinases was detected by Western blotting as mentioned above in PG cells treated with various concentrations of Fv-LDP for 24 hr. Next, gelatin zymography was performed according to a previously published method with some modification [13]. The cells were incubated with serum-free RPMI 1640 medium and Fv-LDP for 24 hr to generate the conditioned medium as the tested samples. The samples corresponding to the same number of cells were then separated by SDS-PAGE, and the gel was washed twice with 2.5% Triton X-100 (Sigma, St.Louis, MO, USA) followed by 16-hr incubation at 37°C in the gelatin buffer (50 mmol/l Tris-HCl, 200 mmol/l NaCl, 10 mmol/l $CaCl_2$, 1 μmol/l $ZnCl_2$, pH 7.5). White zones of lysis indicate gelatin-degrading activity after the staining and the de-coloration of the gel.

Fluorescence-activated cell sorter (FACS)-based internalization assay. BEL-7402 cells (5×10^5) were incubated with 10 μmol/l FITC-labelled Fv-LDP for 1 hr in 100 μl of FACS buffer (2% FBS/PBS) at 4°C to prevent internalization. After washing three times with FACS buffer to remove unbound protein, these cells were resuspended and placed at 37°C. At the indicated time-points, cells were centrifuged and resuspended in 1% trypan blue solution to quench the fluorescence of cell surface-bound FITC. These cells were then re-centrifuged, resuspended and detected by FACS. In this experiment, one sample without FITC-labelled Fv-LDP incubated at 4°C was defined as a blank for background correction; one sample incubated at 4°C without trypan blue dye was used to measure the initial bound fluorescence intensity, and another incubated at 4°C including trypan blue dye was utilized to determine the fluorescence intensity incompletely quenched by trypan blue. The data are shown as the internalization efficiency (% internalized), which was calculated according to a previously reported method [35].

Colony formation assay. The cells were seeded in 96-well plates at a density of 50 cells/well. After 24 hr, the cells were treated with various concentrations of Fv-LDP and Endostar alone or three doses of Fv-LDP (0, 20 and 40 μmol/l), five doses of Endostar (0, 12.5, 62.5, 125 and 250 μg/ml) and their combinations. After drug exposure for 5–6 days, the colony was enumerated if it contained more than 30 cells. Survival colony formation was calculated with the following equation:

$$\text{surviving fraction} = \frac{\text{colonies counted of drug treated group}}{\text{colonies counted of control group}} \times 100\%.$$

All experiments were carried out in triplicate and repeated at least three times. The combination effect of Fv-LDP and Endostar was evaluated according to the coefficient of drug interaction (CDI) [30,36,37]. The CDI was calculated using the formula: $\text{CDI} = \frac{\text{AB}}{\text{A} \times \text{B}}$, where A and B are the fractions of cells that survived the respective single drug and AB is the fraction of cells that survived the two-drug combination. CDI < 1 indicates a synergism, CDI = 1 represents an additive effect, and CDI > 1 means an antagonistic effect.

Cell migration and invasion assay. To determine the effect of drugs on the ability of cells to migrate through a transwell filter (Costar, USA), cells suspended in 1% FBS/medium containing Fv-LDP (fixed dose 20 and 40 μmol/l), Endostar (fixed dose 250 μg/ml) or their combinations were added to the upper chamber. A total of 600 μl of 20% FBS/medium containing either a single agent or their combinations was applied to the lower chamber. The untreated cells were defined as a control group. After 24 hr of incubation, the membranes were fixed and stained with 0.1% crystal violet. The migrated cells were photographed (200×) and counted.

To elucidate the effect of Fv-LDP and Endostar on tumour invasion, a Matrigel invasion assay was carried out using transwell filters pre-coated with 2.5 mg/ml Matrigel (Corning, USA). BEL-7402 cells (10^5 per well) suspended in 1% FBS/medium containing Fv-LDP (fixed dose 20 and 40 μmol/l), Endostar (fixed dose 250 μg/ml) or their combinations were seeded onto the upper chambers. A total of 600 μl of 20% FBS/medium containing either a single agent or combinations was added to the lower wells of the chambers. The untreated cells served as a control group. After 24 hr of incubation, the membranes were fixed, stained, photographed and counted as mentioned above.

In vivo *therapeutic studies.* Female Kunming mice and BALB/c athymic mice (6 week old) were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). All animal experiments were conducted in accordance with internationally accepted principles for the care and use of laboratory animals.

To evaluate the efficacy of Fv-LDP, murine hepatoma 22 cells (1.5×10^6) ascites-passaged in female Kunming mice were directly transplanted subcutaneously into the right axilla of mice (day 0), and the mice were then randomized into groups (n = 10 per group). At day 3, 6 and 10, Fv-LDP was intravenously administered at dosages of 30, 60 and 100 mg/kg of body-weight, respectively. All tested mice were sacrificed at day 14. The final tumour weight of every group was measured, and the tumour growth inhibition (TGI) was calculated using the following formula:

$$\%\text{TGI} = (1 - \frac{\text{mean tumour weight of indicated group}}{\text{mean tumour weight of control group}}) \times 100\%.$$

The *in vivo* therapeutic efficacy of Fv-LDP and Endostar was explored in BALB/c athymic mice bearing human hepatoma BEL-7402 xenografts. The athymic mice were first inoculated subcutaneously in the right flank with 1×10^7 BEL-7402 cells suspended in 200 ml PBS. After 3–4 weeks, tumours were dissected aseptically from donor animals and cut into pieces of 2 mm^3, and the pieces were then transplanted subcutaneously in the right flank of mice. When the BEL-7402 xenograft reached approximately 100 mm^3 in size, the tumour-bearing animals were randomly divided into four groups of six animals per group. Fv-LDP was intravenously administered five times twice per week, and Endostar was synchronously given to the tumour-bearing mice by intraperitoneal injection. The tumour size and body-weight were measured twice per week, and the tumour volume was calculated using the following formula: tumour volume = 0.5 × length × $(\text{width})^2$. The TGI (%) was then calculated according to the above formula. The CDI value of Fv-LDP plus Endostar was calculated as previously described [30,36,37].

Statistical analysis. Significant differences between two groups were determined with Student's *t*-test. $p < 0.05$ was considered statistically significant, and $p < 0.01$ was thought highly significant.

Results

Construction, expression, preparation and identification of fusion protein Fv-LDP.

To obtain the fusion gene *fv-ldp*, gene fragments encoding scFv(3G11) and LDP were amplified by PCR using the

corresponding primers mentioned above. Subsequently, *scfv* and *ldp* were directly cloned into the *Nde* I/*Xho* I restriction sites of the expression vector pET-30a(+) in the format *scfv-ldp* to generate the plasmid pET-30a(+)/*fv-ldp* (fig. 1A). After verifying by DNA sequencing that the correct gene was inserted, the plasmid pET-30a(+)/*fv-ldp* was transfected into *E. coli* BL21 (DE3) star™ to produce a recombinant strain expressing Fv-LDP (fig. 1B). Furthermore, the anti-his•tag mAb and Fv-LDP from different generations of recombinant strains specifically interacted, which was consistent with the fact that pET-30a(+) carried an optional C-terminus his•tag sequence (fig. 1B).

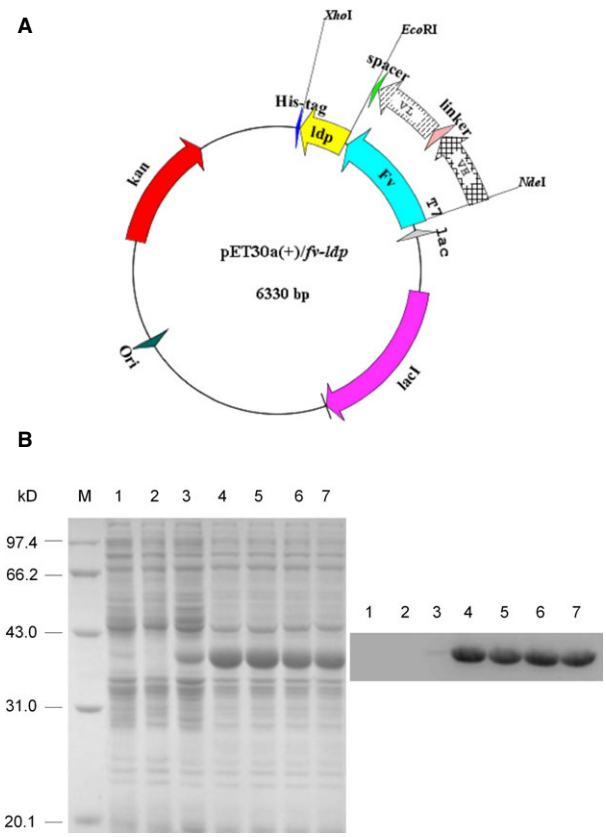

Fig. 1. Construction, expression and Western blotting of fusion protein Fv-LDP. (A) The construction of the recombinant plasmid pET-30a(+)/*fv-ldp* carrying fusion gene *fv-ldp*. kan, the kanamycin resistance gene; Ori, origin of DNA replication; lacI, lacI coding sequence; T7 lac, T7 promoter and lac operator; *fv*, sequence encoding for scFv fragment from the parental mAb3G11, which consists of *vh* (a heavy chain variable domain), a linker (3 × G_4S) and *vl* (a light chain variable domain); *spacer*, sequence encoding for the G_4S spacer peptide located between *fv* and *ldp*; *ldp*, sequence encoding for lidamycin (LDM) apoprotein. *his·tag*, C-terminal tag of six histidine residues. (B) SDS-PAGE under reducing conditions (left) and Western blotting (right) of the recombinant strains expressing Fv-LDP. Lanes: M, low molecular weight markers; 1, total proteins of *E. coli* carrying plasmid pET-30a (+) without IPTG induction; 2, total proteins of *E. coli* carrying plasmid pET-30a (+) with IPTG induction; 3, total proteins of *E. coli* carrying plasmid pET-30a(+)/*fv-ldp* without IPTG induction; 4–7, total proteins of *E. coli* carrying plasmid pET-30a(+)/*fv-ldp* with IPTG induction which was the original, 35th, 65th and 100th strain from the same seed, respectively.

Because of the his•tag sequence located at the C-terminus, most of the Fv-LDP could be eluted with 100 mmol/l imidazole-containing buffer and only a small amount of Fv-LDP was found when the concentration of imidazole was further increased (fig. 2A). After chromatography using a pre-packed Superdex™ 75 column, the refolded Fv-LDP was visualized at a purity of over 95% as analysed by SDS-PAGE (fig. 2B), and the molecular weight of 38.6 kD was determined by MALDI-TOF-MASS (fig. 2C), which was approximately the theoretical value predicted by the Lasergene software. Furthermore, the first five amino acids at the 5′-terminal end of Fv-LDP were M (methionine), Q (glutamine), V (valine), K (lysine) and L (leucine), which were identical to those predicted by the DNA translation software (fig. 2D).

Binding of fusion protein Fv-LDP.

The binding assays of Fv-LDP were carried out using the immortalized human white cell line BJH-1 that expresses relatively low levels of gelatinases and three high gelatinase-expressing cancer cells, BEL-7402, PG and HepG2 (fig. 3A). ELISA was first used to compare the binding activity of Fv-LDP and the parental mAb3G11 to gelatinases and various cell lines. The results indicated that Fv-LDP could bind to gelatinases and the tested cell lines in a dose-dependent manner, but the binding capability of Fv-LDP was lower than that of mAb3G11 (fig. 3B). In addition, the binding activity of Fv-LDP to the gelatinase-low BJH-1 cells was much lower than that of human cancer cells expressing relatively high levels of gelatinases, BEL-7402 or PG, which suggested that the binding of Fv-LDP specifically depended on the expression of gelatinases. The data from the immunofluorescent cytochemical staining also showed that Fv-LDP could bind to the above-mentioned tumour cells and make them display intensively green fluorescence due to FITC, while only weakly green fluorescence was observed in BJH-1 cells expressing relatively low gelatinases, which further confirmed that the binding activity of Fv-LDP depended on the level of gelatinases (fig. 3C). To further identify whether Fv-LDP could interact with gelatinases located in BEL-7402 and PG cells, coIP assays were carried out. The results revealed that Fv-LDP was precipitated as part of the complex with MMP-2 or MMP-9, while pre-immune serum precipitated almost no Fv-LDP protein (fig. 3D). Next, we compared the binding capabilities of Fv-LDP and LDP to PG cells. As indicated in fig. 3E, the nuclei of PG cells were stained blue by DAPI while the cell surface and cytoplasm of drug-treated PG cells displayed green fluorescence derived from FITC. Furthermore, the fluorescence intensity of PG cells treated with FITC-labelled Fv-LDP was evidently stronger than that of cells incubated with FITC-labelled LDP, suggesting that the introduction of scFv against gelatinases enhanced the selectivity of LDP.

The inhibited expression and secretion of gelatinases by fusion protein Fv-LDP and its internalization via endocytosis.

The influence of Fv-LDP on the expression of gelatinases was first detected by Western blotting, and the inhibition of gelatinases was observed in PG cells treated with different

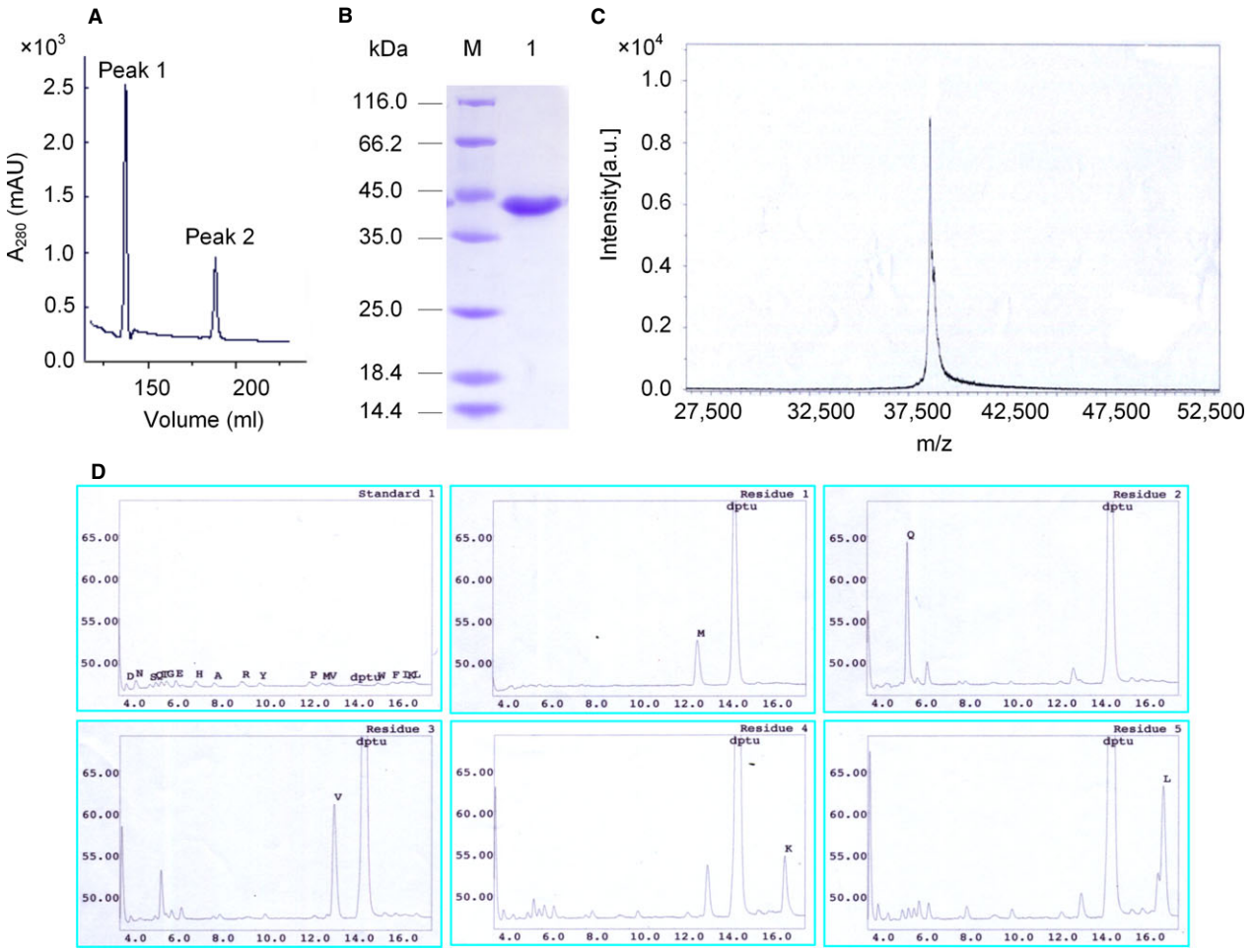

Fig. 2. Purification, refolding and identification of fusion protein Fv-LDP. (A) Purification of Fv-LDP by IMAC using AKTA purifier 10. Peak 1 and 2, denatured Fv-LDP eluted with 100 and 250 mmol/l imidazole-containing buffer, respectively. (B) SDS-PAGE analysis of refolded Fv-LDP under the reducing conditions. M, Low molecular weight protein marker; 1, Fv-LDP. (C) Molecular weight of Fv-LDP measured by MALDI-TOF-MASS. (D) The first five amino acids of Fv-LDP at the N-terminus. M, Q, V, K and L represent the abbreviation of methionine, glutamine, valine, lysine and leucine, respectively.

concentrations of Fv-LDP for 24 hr (fig. 4A). We then examined the effect of Fv-LDP on the secretion of gelatinases by gelatin zymography. The lysis of gelatin by gelatinases was detected as the negative-stained bands, and the activity of gelatinases in the culture supernatant of PG cells was inhibited by Fv-LDP in a dose-dependent manner (fig. 4B). In addition, to determine whether Fv-LDP could enter tumour cells, the internalization efficiency was measured by determining the fluorescence intensity of FITC in the cytoplasm of BEL-7402 cells. As shown in fig. 4C, Fv-LDP could be internalized into BEL-7402 cells in a time-dependent manner, and the internalization efficiency at 4 hr reached 9.8% when the cells were incubated at 37°C.

Cytotoxicity of fusion protein Fv-LDP to human hepatoma cells.

To explore the *in vitro* antitumour effect of Fv-LDP, human hepatoma cells were treated with different concentrations of Fv-LDP. The results of colony formation assay indicated that Fv-LDP could inhibit the proliferation of hepatoma BEL-7402 and HepG2 cells in a dose-dependent manner (fig. 5). The calculated IC_{50} value of Fv-LDP was 190 and 207 μmol/l for BEL-7402 and HepG2 cells, respectively, which suggested that Fv-LDP was slightly cytotoxic to these two types of hepatoma cells.

Antitumour effect of fusion protein Fv-LDP in vivo.

As shown in table 1, the growth of transplanted hepatoma 22 cells in Kunming mice treated with various doses of Fv-LDP was inhibited by 26.0% to 57.6% at day 14. Although the final average body-weight of each Fv-LDP group was less than that of the control group ($p < 0.05$ *versus* 60 or 100 mg/kg Fv-LDP), it gradually increased in the days after tumour inoculation. Throughout this experiment, all treated mice survived, and no remarkable side effects were observed.

Enhanced inhibitory effect of fusion protein Fv-LDP plus Endostar on human hepatoma cell growth, migration and invasion in vitro.

To test the impact of Endostar on the growth of human hepatoma cells, BEL-7402 cells were treated with increasing

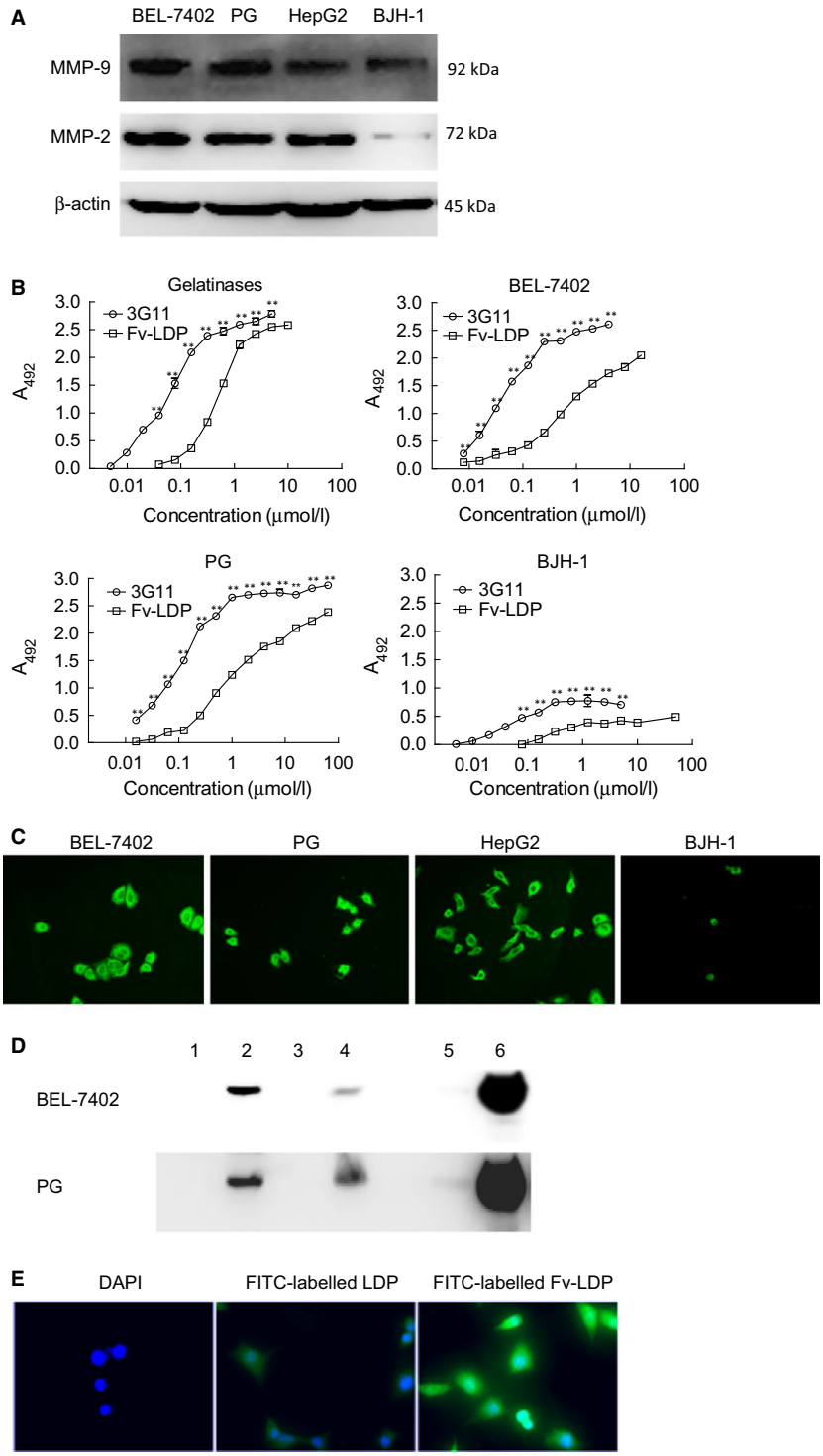

Fig. 3. The binding of fusion protein Fv-LDP. (A) The expression levels of gelatinases in different human cell lines analysed by Western blotting. (B) The immunoreactivity of Fv-LDP to gelatinases, BEL-7402, PG and BJH-1 cells by ELISA. (C) Immunofluorescence detection of FITC-labelled Fv-LDP bound to the immortalized human cells with different levels of gelatinases. The images were observed under a laser scanning confocal microscope at 20×. (D) The binding specificity of Fv-LDP to gelatinases verified by coIP assays. Total proteins extracted from BEL-7402 cells and PG cells were incubated with PBS (lane 1 and 3) or Fv-LDP (lane 2, 4–5). The mixture was then incubated with either anti-MMP-2 mAb (lane 1–2) or anti-MMP-9 mAb (lane 3–4), or pre-immune serum (lane 5). Subsequently, the formed complexes were collected with protein A + G agarose and analysed by Western blotting using anti-his·tag mAb as the primary antibody. Fv-LDP (lane 6) served as the positive control. (E) Immunofluorescence comparison between FITC-labelled LDP and FITC-labelled Fv-LDP bound to PG cells that were counterstained with DAPI. The images were obtained using a fluorescence microscope at 400×.

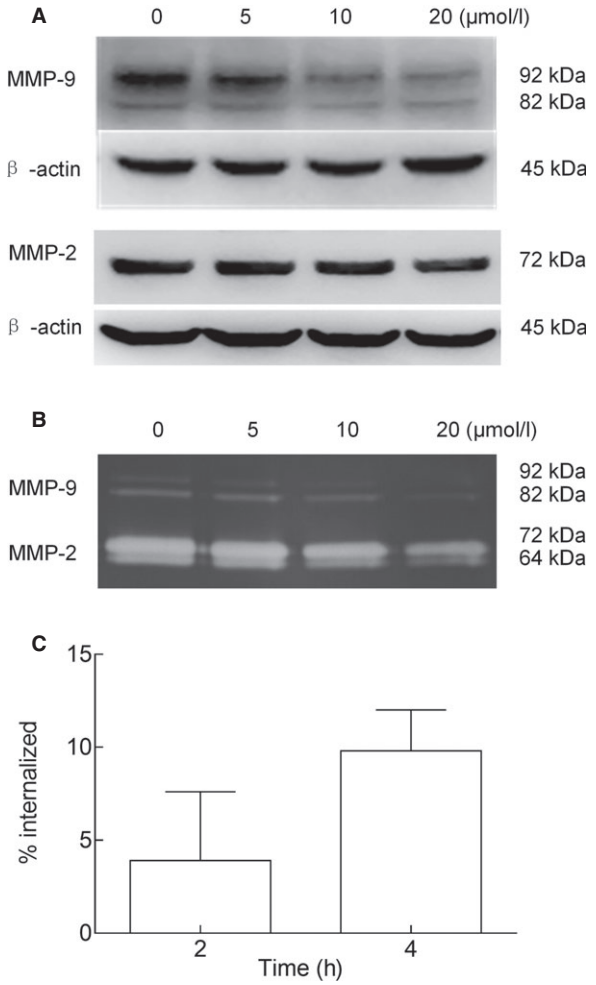

Fig. 4. Fusion protein Fv-LDP can inhibit the expression and secretion of gelatinases and be internalized into tumour cells. (A) The inhibition of gelatinases expression in Fv-LDP-treated PG cells. (B) Gelatin zymography analysis of PG cells treated with different concentrations of Fv-LDP. (C) The internalization efficiency of bound Fv-LDP determined by fluorescence-activated cell sorter (FACS)-based internalization assay. To differentiate internalized protein from the extracellular one, trypan blue was applied to quench the fluorescence of FITC on cell surfaces.

concentrations of Endostar (0, 12.5, 31.25, 62.5, 125, 250, 500, 750 and 1000 μg/ml) for 6 days. As shown in fig. 6A, Endostar inhibited cell growth in a dose-dependent manner. We subsequently explored the effect of Fv-LDP (fixed dose 0, 20 and 40 μmol/l) and Endostar (fixed dose 0, 12.5, 62.5, 125 and 250 μg/ml) on the growth of human hepatoma cells. The results indicated that Endostar enhanced the antiproliferation effect of Fv-LDP on BEL-7402 cells. Furthermore, the CDI value for each combination of Fv-LDP and Endostar was less than 1.00, which implied that the combination of Fv-LDP and Endostar exerted synergistic effects on inhibiting the proliferation of BEL-7402 cells (fig. 6B). We then examined whether Fv-LDP plus Endostar could affect the migration and invasion of human hepatoma cells. As indicated in fig. 6C,D, Fv-LDP and Endostar alone inhibited the migration of BEL-7402 and HepG2 cells; Fv-LDP combined with Endostar significantly

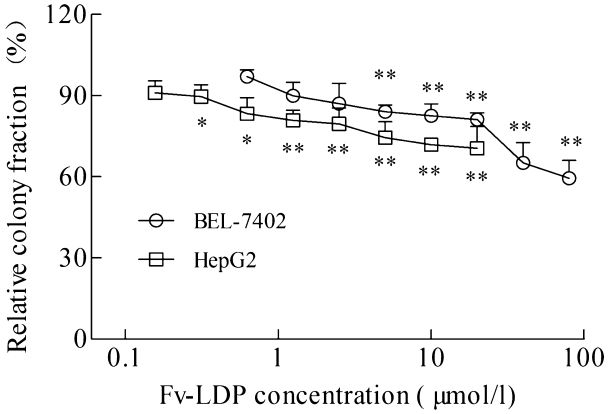

Fig. 5. Cytotoxicity of fusion protein Fv-LDP to human hepatoma cells determined by colony formation assay. $*p < 0.05$ and $**p < 0.01$ *versus* the control group.

further inhibited the migration of these hepatoma cells ($p < 0.01$ *versus* equimolar Fv-LDP or Endostar). The CDI value of Fv-LDP (20 and 40 μmol/l) plus Endostar (250 μg/ml) was 0.81 and 1.00 for BEL-7402 cells, and 0.67 and 0.75 for HepG2 cells, respectively, which suggested that the combination of Fv-LDP and Endostar had a synergistic or additive effect on inhibiting the migration of human hepatoma cells. Similarly, each combination treatment for 24 hr also resulted in a markedly inhibitory effect on the invasion of BEL-7402 cells ($p < 0.01$ *versus* equimolar Fv-LDP; $p < 0.05$ or 0.01 *versus* Endostar; fig. 6E).

Combination of fusion protein Fv-LDP and Endostar synergistically inhibited the growth of human hepatoma xenografts in athymic mice.

To further assess whether Fv-LDP plus Endostar had a synergistic effect on inhibiting the growth of tumours, we conducted another experiment in athymic mice bearing human hepatoma BEL-7402 xenografts. As shown, Fv-LDP treatment alone significantly inhibited the growth of tumours ($p < 0.05$ *versus* control group). Although Endostar given alone slightly inhibited tumour growth, the combination of Fv-LDP and Endostar exerted a significant synergistic antitumour effect with a CDI value of 0.38 ($p < 0.05$ *versus* Fv-LDP or $p < 0.01$ *versus* Endostar). The body-weight remained almost constant in the combined group (fig. 7, table 2).

Discussion

Carcinoma invasion and metastasis is the main reason for high mortality and the key factor to influence the prognosis of patients [38]. Gelatinases, the important members of the MMP family, can promote tumour cell invasion and metastasis, and the inhibition of gelatinases can suppress the invasion and metastasis of tumour cells [1,2]. Furthermore, gelatinases, which are overexpressed in most malignant tumours and in the stromal cells in the tumour environment, can anchor to the cell surface via integral membrane proteins, such as DNA

Table 1.
The growth inhibition of murine hepatoma 22 cells in Kunming mice by fusion protein Fv-LDP.

Groups	Dose (mg/kg)	No. of mice (Begin/End)	Body-weight (g)		Tumour weight (g)	TGI[1] (%)
			Day 0	Day 14		
Control	–	10/10	19.10 ± 0.90	32.90 ± 3.10	4.90 ± 1.93	
Fv-LDP	30	10/10	19.20 ± 0.70	30.70 ± 2.50	3.62 ± 1.79	26.0
	60	10/10	18.70 ± 0.70	30.70 ± 1.00*	3.44 ± 1.49	29.7
	100	10/10	19.30 ± 0.70	29.60 ± 1.40**	2.08 ± 1.30**	57.6

At day 0, 1.5×10^6 murine hepatoma 22 cells ascites-passaged were directly transplanted subcutaneously into the right axilla of mice. At day 3, 6 and 10, different doses of Fv-LDP were intravenously administered by tail vein. Then, the animals were sacrificed at day 14. The body-weight and the final tumour weight are shown as mean ± S.D.
[1]TGI, tumour growth inhibition rate, was calculated using the final mean tumour weight of every group.
*$p < 0.05$ and **$p < 0.01$ *versus* the control group.

repair protein Ku (via its integrin I-like domain), CD44 and integrins (αvβ3, αvβ1, αβ2, αvβ5, α4β1 and α5β1), and cell surface associations, such as collagens and low-density lipoprotein-related scavenger receptor (LRP) [3–6,39]; gelatinases can also regulate key signalling pathways in cell growth, invasion and metastasis by interacting with cell surfaces [40,41]. In addition, the increased expression of gelatinases is positively correlated with poor prognosis. Therefore, gelatinases possess the characteristics of a tumour cell membrane antigen and are promising targets for cancer therapy. To date, a considerable body of research has focused on neutralizing gelatinases via antigen–antibody reaction, inhibiting gelatinase activity with MMP inhibitors or using a gelatinase-stimuli strategy via pro-drug, liposomes and nanoparticles to develop gelatinase-related drugs for tumour therapy [4,13,42]. mAb 3G11 is an anti-gelatinases mAb developed by our laboratory, and its specific binding activity to gelatinases and tumour cells as well as its certain curative efficacy in tumour-bearing mice has been confirmed [31]. In addition, LDP is a carrier protein able to bind to tumour cells or tissues [21,25]. These data form the basis for generating the new, targeted fusion protein Fv-LDP via the integration of the scFv fragment of mAb3G11 with the LDM apoprotein LDP.

In this article, a gelatinases-targeting scFv-based fusion protein was successfully prepared and identified (figs 1 and 2). Fusion protein Fv-LDP retained the partial activity of mAb 3G11, suggesting that Fv-LDP could function as the antibody and delivery itself to the location where gelatinases were overexpressed. Furthermore, the binding activity of Fv-LDP strongly depended on the level of gelatinases (fig. 3A–C). The specific binding of Fv-LDP to gelatinases was also verified in different tumour cells by coIP assays (fig. 3D). Previous reports elucidated that the binding capability of LDP to various tumour cells or tissues was related to the expression of VEGF and EGFR or Her2; evidently, LDP has the potential to act as a targeting drug carrier in the manufacture of new anticancer therapeutics [21,25]. Here, we continued to observe the binding capability of LDP to tumour cells, but the binding intensity was much weaker than that of Fv-LDP (fig. 3E). In addition, considering the anchoring of gelatinases to the cell surface, Fv-LDP is speculated to target the tumour cell surface mainly via the interaction of the scFv moiety with gelatinases, while the LDP moiety may play a supporting role by targeting other overexpressed tumour antigens, such as EGFR and VEGF.

Matrix metalloproteinases are reportedly regulated at multiple levels including transcription, secretion, activation of zymogen forms, extracellular inhibition and internalization via endocytosis [3]. In the present study, we observed that Fv-LDP could inhibit the expression and secretion of gelatinases in a dose-dependent manner (fig. 4A,B). We then sought to investigate whether Fv-LDP directed against gelatinases on the cell surface could enter the tumour cells via endocytosis. To this end, we measured the internalization efficiency of Fv-LDP. The results showed that approximately 4% of Fv-LDP was internalized into BEL-7402 cells at 2 hr (fig. 4C). The internalization rate of Fv-LDP was lower than that of other antibody-based fusion proteins bound to the cell membrane, which suggested that most of the Fv-LDP might act on the surface of tumour cells but did not directly enter the tumour cells [12,14].

Taken together, these results indicate that Fv-LDP can function not only as an antibody to neutralize gelatinases via antigen–antibody interaction, but can also act as a MMP inhibitor to suppress the expression and secretion of gelatinases.

The AE moiety of LDM has long received much attention because of its extremely potent cytotoxicity and unique molecular structure [43]. However, the bioactivity of LDP and LDP-based fusion proteins also has gradually garnered our interest in recent years. LDP-based fusion protein can reportedly inhibit the growth of tumours [12]. In the current study, similar results for Fv-LDP were also obtained based on the colony formation assay and the *in vivo* experiments using mice bearing human hepatoma BEL-7402 and murine transplantable H22 cells (fig. 5, fig. 7, tables 1–2). The specific binding of Fv-LDP to gelatinases as well as the inhibition of gelatinases expression and secretion should also contribute to the antitumour effect of Fv-LDP.

To further increase the antitumour activity of Fv-LDP, we explored a new strategy for cancer therapy by combining Fv-LDP and Endostar, which was not related to the cytotoxicity of AE (the active moiety of LDM). Endostar is a known modified recombinant human endostatin and has been approved for clinical use in China [26]. Endostar can also

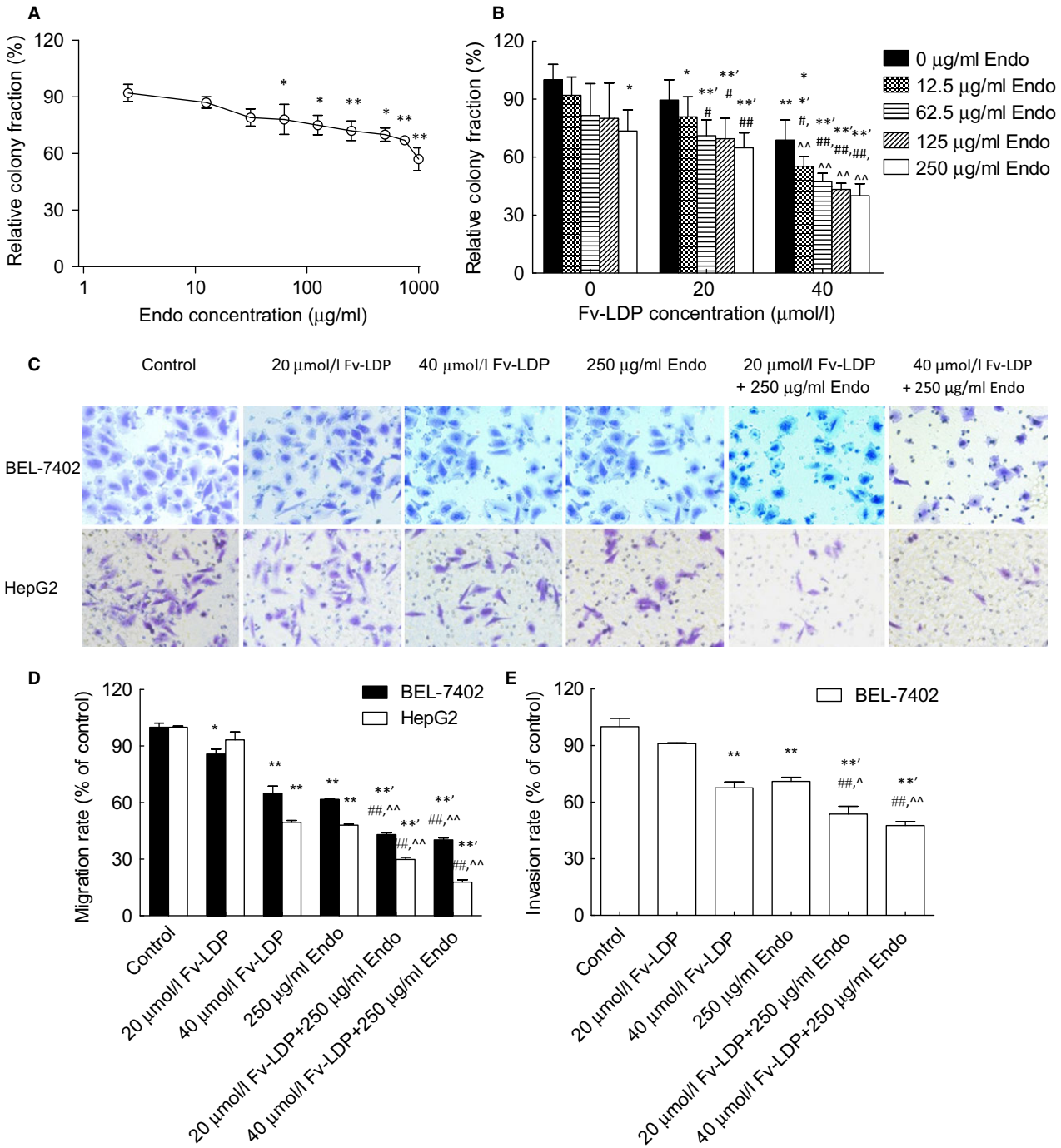

Fig. 6. Fusion protein Fv-LDP and Endostar inhibit human hepatoma cell growth, migration and invasion *in vitro*. (A) The effect of Endostar on the growth of BEL-7402 cells by colony formation assay. *$p < 0.05$ and **$p < 0.01$ *versus* the drug-free group. (B) The synergistic cytotoxicity of Fv-LDP plus Endostar to BEL-7402 cells by colony formation assay. (C and D) The suppression of cell migration by Fv-LDP, Endostar and their combinations in a transwell-based migration assay. (E) The enhanced inhibition of cellular invasion in BEL-7402 cells treated with Fv-LDP and Endostar. The cells were treated for 24 hr, and the membranes were then fixed and stained with 0.1% crystal violet. The migrated and invasive cells were photographed using a fluorescence microscope at 200× and counted. *$p < 0.05$ and **$p < 0.01$ *versus* control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* equimolar Fv-LDP group; ^$p < 0.05$ and ^^$p < 0.01$ *versus* Endostar group. Endo, Endostar.

inhibit the migration and invasion of human breast cancer cells by down-regulating gelatinases [27]. Furthermore, Endostar combined with chemotherapy or chemo-radiotherapy can significantly inhibit tumour growth *in vivo* [28–30]. Considering the interaction of both proteins with gelatinases and the effective combination of other drugs with Endostar, a combination protocol of Fv-LDP/Endostar was designed and investigated. As expected, the efficacy of Endostar on

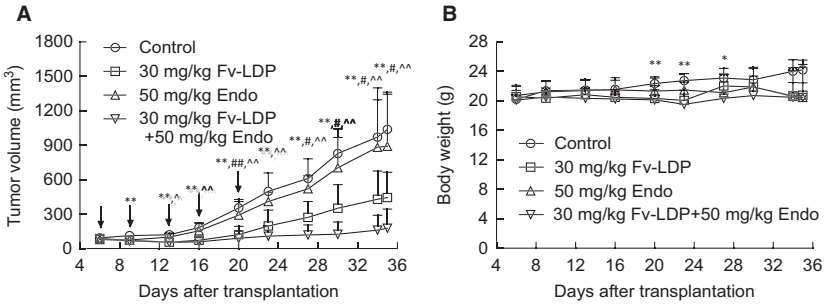

Fig. 7. Antitumour efficacy of fusion protein Fv-LDP and Endostar on human hepatoma BEL-7402 xenografts in athymic mice. Endostar was intraperitoneally administered, while Fv-LDP was injected via the tail vein. Black arrows indicate the administration time. The significance levels of the combination group are shown. (A) Tumour growth in each treatment group. Tumour volume was measured twice per week after treatment. (B) The body-weight of experimental mice from each group. All data are shown as the mean tumour volume ± S.D. or animal weight ± S.D. *versus* experimental days. Endo, Endostar. $*p < 0.05$ and $**p < 0.01$ *versus* control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *versus* Fv-LDP group; $\wedge p < 0.05$ and $\wedge\wedge p < 0.01$ *versus* Endo group.

Table 2.
Synergistic inhibitory effect of the fusion protein Fv-LDP and Endostar on human hepatoma BEL-7402 xenografts in BALB/c mice.

Groups	Dose (mg/kg)	No. of mice (Begin/End)	Body-weight (g)		Tumour weight (g)	TGI[1] (%)	CDI[2]
			Day 6	Day 35			
Control	–	6/6	20.2 ± 1.75	24.19 ± 1.33	0.77 ± 0.20		
Fv-LDP	30	6/6	20.55 ± 0.86	20.76 ± 4.17	0.43 ± 0.28*	44.1	
Endo	50	6/6	20.72 ± 1.38	20.46 ± 1.97**	0.70 ± 0.31	8.9	
Fv-LDP + Endo	30 + 50	6/6	20.09 ± 0.71	20.45 ± 4.06	0.15 ± 0.13*,#,^^	80.8	0.38

The nude mice were treated with Fv-LDP by tail vein injection at day 6, 9, 13, 16 and 20, respectively. Endostar was synchronously given to the tumour-bearing mice by intraperitoneal injection. The body-weight and the final tumour weight are presented as mean ± S.D.
[1]TGI, tumour growth inhibition rate, was calculated using the final mean tumour weight of every group.
[2]CDI, coefficient of drug interaction, was calculated as the formula elucidated in the references, a CDI of <1 represents a synergy effect (more than additive), a CDI of = 1 denotes an additive effect, and a CDI of >1 demonstrates an antagonistic effect. Endo, Endostar.
$*p < 0.05$ and $**p < 0.01$ *versus* the control group, $^{\#}p < 0.05$ *versus* Fv-LDP group and $\wedge\wedge p < 0.01$ *versus* Endo group.

the growth of human hepatoma cells was very limited; however, the combination of Fv-LDP and Endostar resulted in a significant synergistic antitumour effect on the growth of BEL-7402 cells *in vitro* (fig. 6A,B). Fv-LDP plus Endostar also exerted an additive or synergistic effect on the migration of BEL-7402 and HepG2 cells (fig. 6C,D). Similar results were obtained from human hepatoma BEL-7402 cells in a Matrigel invasion assay (fig. 6E). Furthermore, the *in vitro* synergistic effect was also confirmed in a human hepatoma BEL-7402 xenograft model, in which Fv-LDP combined with Endostar showed a significant synergistic effect in suppressing tumour growth at a tolerated dose (fig. 7, table 2). These results suggest that the combination of Fv-LDP and Endostar is feasible. Nevertheless, other mechanisms can also contribute to the combination effect. For example, Endostar may very well contribute to the synergism via anti-angiogenesis [26,30].

For the first time, the current study evaluated the antitumour activity of a scFv-based engineered fusion protein, Fv-LDP, that increased the selectivity of LDP via the integration of scFv directed against gelatinases. In addition, Endostar exerted a potentiation effect of the fusion protein Fv-LDP in the treatment of human hepatoma BEL-7402 cells or tumours. The combination of Fv-LDP and Endostar will provide a new strategy for improving the therapeutic efficacy of treatments for hepatoma or other gelatinase-overexpressing malignant tumours.

Acknowledgements

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Conflict of interest

The authors report no conflict of interests.

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