

An albumin-binding domain and targeting peptide-based recombinant protein and its enediyne-integrated analogue exhibit directional delivery and potent inhibitory activity on pancreatic cancer with K-ras mutation

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Received August 4, 2019; Accepted November 1, 2019

DOI: 10.3892/or.2020.7468

Abstract. Efficient enrichment and transmembrane transport of cytotoxic reagents are considered to be effective strategies to increase the efficiency and selectivity of antitumor drugs targeting solid tumors. In the present study, a recombinant protein ABD-LDP-Ec consisting of the albumin-binding domain (ABD), the apoprotein (LDP) of lidamycin (LDM) and an EGFR-targeting oligopeptide (Ec) was prepared by DNA recombination and bacterial fermentation, and was integrated with the enediyne chromophore (AE) of lidamycin to generate its enediyne-integrated analogue ABD-LDP-Ec-AE. ABD-LDP-Ec exhibited high binding capacity to both albumin and EGFR-positive pancreatic cancer cells, and was internalized into the cytoplasm through receptor-mediated endocytosis and albumin-driven macropinocytosis of K-ras mutant cells. In animal experiments, ABD-LDP-Ec demonstrated notable selective distribution in pancreatic carcinoma xenografts by passive targeting of albumin captured in the

blood and was retained in the tumor for 48 h. ABD-LDP-Ec and ABD-LDP-Ec-AE exhibited inhibitory activity in cell proliferation and AsPC-1 xenograft growth, and ABD-LDP-Ec-AE increased the tumor growth inhibition rate by 20% compared with natural LDM. The results indicated that the introduction of ABD-based multi-functional drug delivery may be an effective approach to improve the efficacy of antitumor drugs, especially for K-ras mutant cancers.

Introduction

Although traditional chemotherapy drugs have obtained high clinical efficacy in the treatment of cancer, there are still a number of shortcomings, such as poor selectivity, strong side effects and rapid blood clearance. In order to achieve the characters of higher therapeutic effect, long half-life, and low side effects, the drug conjugate consisting of target molecules and chemotherapeutic drugs prepared by chemical coupling or genetic recombination, which could be delivered into solid tumors and cells, is a hot spot in anti-cancer drug research (1,2).

Human serum albumin (HSA), a single-chain aglycosylated protein consisting of 585 amino acids, is the most abundant protein in human plasma (3). Due to its non-immunogenicity, human compatibility and long half-life in the serum (~19 days) (4), HSA is a widely recognized carrier for the passive targeting to solid tumors and has been frequently applied to construct drug conjugates for longer plasma half-life (5-7). The albumin binding domain (ABD), a protein domain with three helix structures discovered in the surface proteins of gram-positive bacteria, exhibits the ability to bind albumin (8). A mutation of ABD (ABD035), obtained through screening and engineering and deimmunized by substituting residues in immunogenic regions, displays high affinity to both human serum albumin and mouse serum albumin (MSA) with a dissociation constant of 10^{-13} M (9). In addition, ABD035 exhibits good stability, which makes it an ideal skeleton structure for protein engineering (10). Considering the non-covalent binding between albumin and ABD, the construction of the recombinant protein containing ABD and a therapeutic protein is beneficial to prolong plasma half-life, increase treatment time and improve the efficacy of drugs (11-13).

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Abbreviations: ABD, albumin-binding domain; EGFR, epidermal growth factor receptor; LDM, lidamycin; LDP, lidamycin apoprotein; AE, enediyne chromophore; Ec, EGFR-targeting oligopeptide; HSA, human serum albumin; MSA, mouse serum albumin; IPTG, isopropyl β -D-thiogalactopyranoside; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; TEM, transmission electron microscopy; EIPA, ethyl-isopropyl amiloride; CCK-8, cell counting kit-8; TGI, tumor growth inhibition

Key words: albumin-binding domain, targeting peptide, albumin, epidermal growth factor receptor, lidamycin, K-ras mutation

Pancreatic carcinoma is an aggressive gastrointestinal malignancy with high morbidity and mortality rates worldwide; in 2018, the morbidity and mortality rates were 2.5 and 4.5%, respectively (14). Previous studies on the molecular pathogenesis have demonstrated that the occurrence, development and metastasis of pancreatic carcinoma are closely associated with a variety of gene mutations and abnormalities in cell signaling pathways, including K-ras and BRAF mutations (15,16) and the epidermal growth factor receptor (EGFR) and Hedgehog signaling pathways (17,18). K-ras mutations, which are frequently present in pancreatic and colon carcinomas, are regarded as an ideal therapeutic target owing to their close association with oncogenesis, poor prognosis and drug resistance (19), and they may provide an efficient drug delivery strategy due to the intensive macropinocytosis of extracellular nutrients observed in K-ras mutant cells (20,21). However, no clinical drug targeting K-ras mutations is currently available. EGFR upregulation in the majority of human carcinomas is a validated target for cancer therapy; therefore, a number of monoclonal antibodies and small-molecule kinase inhibitors against EGFR, such as cetuximab and erlotinib, have been applied for clinical treatment of pancreatic cancer (22,23).

Lidamycin (LDM), which is a peptide antibiotic in the process of phase II clinical trial, is considered to be an ideal 'warhead' molecule for targeted drugs against tumors; LDM is composed of an active enediynes chromophore (AE) with extremely potent cytotoxicity and a non-covalently bound apoprotein (LDP), which can be dissociated and reconstituted without the loss of natural activity (24). In recent years, a series of LDM modifications were performed to improve tumor targeting, increase cytotoxicity on tumor cells and reduce side effects. For example, the recombinant proteins integrating LDM with EGFR/HER2-targeted oligopeptide or HSA enriched in solid tumors and displayed stronger antitumor activity compared with LDM in athymic mouse xenograft models (25,26).

In this study, a recombinant protein ABD-LDP-Ec and its enediynes-integrated analogue ABD-LDP-Ec-AE were prepared and their antitumor activities were studied with an aim to achieve directional delivery of drugs against pancreatic cancer, especially K-ras mutant pancreatic cancer, with the help of EGFR-targeting binding, ABD-albumin combination and macropinocytosis.

Materials and methods

Preparation of the recombinant proteins and their enediynes-integrated analogues. The DNA sequence of ABD was synthesized and inserted into a pUC plasmid by GenScript Biotech Corporation, and the plasmid *pET30(a)-ldp* containing the LDP gene was constructed in our laboratory. The fragments of *abd*, *ldp* and *ldp-ec* were amplified by PCR using the following primers: *abd* forward (P1), 5'-GGAATTCATATGCTGGCGGAAGCCAAAGTC-3' and reverse (P2), 5'-GAAGATCTGGCGGTGGCGGATCCGCGGTGCGGATCCCTGGCGGAAGCCAAAGTC-3'; *ldp* and *ldp-ec* forward (P3), 5'-CAGAATTCGCGCCGCTTCTCCGTC-3'; *ldp* reverse (P4), 5'-CCGCTCGAGGCCGAACGTCAGTGCGAC-3'; and *ldp-ec* reverse (P5), 5'-CCGCTCGAGCGCAGTTCCACCATTTCAGATCGCGATACTGACA

GCGTTCGCCAATATAGCCCACCACACAGTTTGAGCCACCTCCGCCTGAGCCACCTCCGCCGCCGAAGGTCAGAGC-3'. The fragments were digested with *NdeI*, *BglII* and *XhoI* restriction enzymes (Takara Biotechnology Co., Ltd.) and ligated into the *pET30(a)* plasmid to construct expression plasmids *pET30(a)-abd-ldp* and *pET30(a)-abd-ldp-ec*.

The positive plasmids were confirmed by Invitrogen; Thermo Fisher Scientific, Inc. and transformed into the expression strain *Escherichia coli* BL21(DE3) Star (Novagen; Merck KGaA) at 42°C for 45 sec. Following culture in Luria-Bertani medium (1% NaCl, 1% peptone, 0.5% yeast extract; pH 7.4) at 37°C and induction with 0.3 mM isopropyl β -D-thiogalactopyranoside (IPTG) at 30°C for 12 h, the bacterial cell pellets were harvested, resuspended in 20 mM Tris-HCl (pH 8.0) and sonicated on ice to collect the inclusion bodies. The purification and refolding of the recombinant proteins were performed as described by Sheng *et al.* (27). The proteins were concentrated using a centrifugal filter unit (EMD Millipore), and the concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc.).

To prepare the analogues of the recombinant proteins (Fig. 1A), the active enediynes was separated from lidamycin using a C4 column (GE Healthcare Life Sciences) at 4°C overnight. The recombinant proteins reacted with AE at room temperature for 12 h at a 1:3 molar ratio, and the free AE was removed by ultrafiltration. The reconstituted proteins were confirmed by reverse-phase high performance liquid chromatography (HPLC) using a DELTA PAK C4 column (8x100 mm; Waters Corporation) with an Alliance HPLC system (Waters Corporation) at 25°C. The sample quantity was 40 μ l, and the mobile phase consisted of 75% solvent A (H_2O with 0.05% v/v TFA) and 25% solvent B (100% acetonitrile). The flow rate was 0.5 ml/min.

Western blot analysis. The identification of proteins with His-tag was performed by western blot analysis. Proteins (30 μ l concentrated solution per lane) were separated by 10% SDS-PAGE (5% stacking gel; 10% separating gel) and electrophoretically transferred onto PVDF membranes (EMD Millipore). The membranes were blocked with 5% w/v dry milk in TBS + 0.5% Tween-20 at 4°C overnight, incubated with a horseradish peroxidase (HRP)-conjugated His-tag antibody (1:1,000; cat. no. HRP-66005; Proteintech Group, Inc) at room temperature for 2 h, and the bands were visualized with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore).

Cell culture. EGFR-positive human pancreatic cancer cell lines AsPC-1, BxPC-3 and MIA PaCa-2 were obtained from American Type Culture Collection. AsPC-1 and BxPC-3 cells were cultured in modified RPMI medium (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). MIA PaCa-2 cells were cultured in high glucose DMEM (HyClone; GE Healthcare Life Sciences) supplemented with the same additives. The cells were cultured in a humidified incubator (Thermo Fisher Scientific, Inc.) at 37°C with 95% air and 5% CO_2 .

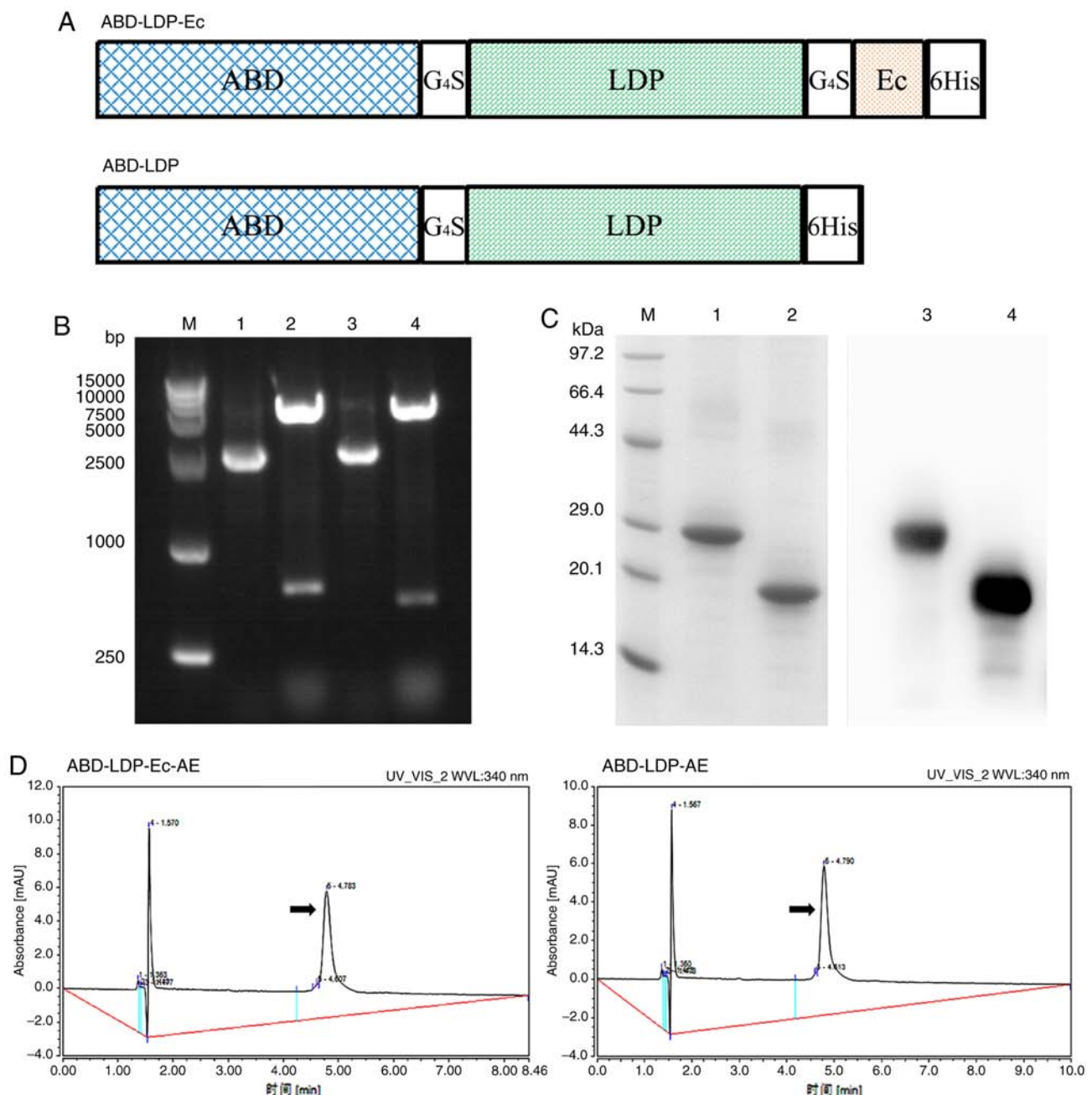


Figure 1. Preparation of recombinant proteins ABD-LDP-Ec, ABD-LDP and their enediynes-integrated analogues ABD-LDP-Ec-AE, ABD-LDP-AE. (A) Schematic presentation of the components of ABD-LDP-Ec and ABD-LDP. (B) Restriction enzyme analysis of recombinant plasmids. Lane 1, pET30(a)-*abd-ldp-ec*; lane 2, pET30(a)-*abd-ldp-ec* digested with *NdeI*/*XhoI*; lane 3, pET30(a)-*abd-ldp*; lane 4, pET30(a)-*abd-ldp* digested with *NdeI*/*XhoI*. (C) SDS-PAGE and western blot analysis of purified recombinant proteins. Lane 1 and 3, purified ABD-LDP-Ec; lane 2 and 4, purified ABD-LDP. (D) Enediynes-integrated analogues ABD-LDP-Ec-AE and ABD-LDP-AE determined by reverse-phase HPLC at 340 nm. The absorption peaks of AE are indicated by arrows. ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, epidermal growth factor receptor-targeting oligopeptide; AE, enediynes chromophore; HPLC, high performance liquid chromatography.

Co-immunoprecipitation (Co-IP) analysis. Co-IP analysis was performed using a Co-immunoprecipitation kit (Pierce; Thermo Fisher Scientific, Inc.) to verify the binding capacity of recombinant proteins against albumin and EGFR, and mouse peripheral blood serum and cell lysate of EGFR-positive AsPC-1 cells were used as the baits. The peripheral blood was incubated for 0.5 h at room temperature and centrifuged at 1,000 x g for 5 min at room temperature to collect serum for further analysis. Adherent cultured AsPC-1 cells were lysed in cell lysis buffer (Beyotime Institute of Biotechnology) supplemented with

1 mM phenylmethylsulfonyl fluoride, and cellular protein was obtained by high-speed centrifugation at 10,000 x g for 10 min 4°C. The protein concentration was quantified using a BCA protein assay kit. The baits were re-cleared using the control agarose resin to decrease non-specific binding. Each bait/recombinant protein mixture at a 5:1 mass ratio was diluted in IP lysis/wash buffer, added to the resin coupling albumin (undiluted; cat. no. sc-271605) or EGFR antibody (undiluted; cat. no. sc-373746; both from Santa Cruz Biotechnology) and incubated with gentle agitation at 4°C overnight. Following

centrifugation at 10,000 \times g at 4°C for 5 min of the spin columns to remove unbound proteins and washing the sample three times with IP lysis/wash buffer, the flow-through of the elution buffer was collected for western blot analysis.

ELISA assay. ELISA was performed to determine the binding affinity of the recombinant proteins with HSA protein. The recombinant proteins at 0.01, 0.1, 1, 10, 100, 1,000 or 10,000 nM were added into 96-well plates coated with HSA (2 μ g/well) and incubated at 37°C for 2 h. Following incubation with an HRP-conjugated His-tag antibody (1:1,000; cat. no. HRP-66005; Proteintech Group, Inc) at room temperature for 2 h, 0.01% 3,3',5,5'-tetramethylbenzidine (Tiangen Biotech Co., Ltd.) was added as a substrate solution, and the reaction was terminated with 2 M H₂SO₄. The absorbance at 450 nm was measured by a microplate reader (Thermo Fisher Scientific, Inc.).

Transmission electron microscopy (TEM). TEM was used to observe the complex of ABD-LDP-Ec and HSA. ABD-LDP-Ec was mixed with HSA at equimolar concentrations and incubated at room temperature for 1 h. The imaging of protein particles under TEM at x400,000 magnification was performed by Qingdao Sci-tech Innovation Co., Ltd.

Immunofluorescence assay. Immunofluorescence assay was performed to determine the binding activity of ABD-LDP-Ec to pancreatic cancer cells overexpressing EGFR. The recombinant proteins were pretreated with DyLight 488 Antibody Labeling kit (Thermo Fisher Scientific, Inc.). The proteins diluted in 0.05 M borate buffer were incubated with the DyLight reagent at room temperature for 1 h protected from light. The labeling reaction mixtures were added into the spin columns preloaded with purification resin and mixed with the resin by briefly vortexing, and the columns were centrifuged to collect the labeled proteins. AsPC-1, MIA PaCa-2 and BxPC-3 cells were seeded on coverslips, incubated at 37°C overnight and fixed with 100% methanol at -20°C for 10 min. The cells were incubated with 50 μ M (~1 mg/ml) DyLight 488-labeled ABD-LDP-Ec at room temperature for 1 h. Fluorescence was observed under a fluorescence microscope (Nikon Corporation) at x400 magnification and images were captured in four random fields of view.

Flow cytometry. To compare the binding activity of the recombinant proteins ABD-LDP-Ec, ABD-LDP and LDP to cancer cells, flow cytometry was used. A total of 5 \times 10⁵ cells/tube of AsPC-1 cells in the logarithmic phase were incubated with labeled proteins in reaction buffer (PBS + 2% FBS) at 4°C for 1 h avoiding internalization. Following washing with cold PBS, the cells were resuspended and analyzed with a FACSCalibur™ cell analyzer (BD Biosciences).

Internalization of the recombinant proteins. Internalization of the recombinant proteins was observed using laser scanning confocal microscopy. AsPC-1 (carrying a K-ras mutation) and BxPC-3 (K-ras wild-type) cells were seeded in Nunc™ Lab-Tek chambered coverglass (Thermo Fisher Scientific, Inc.) at 2 \times 10⁴ cells/well and cultured overnight. The cells were incubated with 50 μ M DyLight 488-labeled ABD-LDP-Ec,

ABD-LDP or LDP and 50 μ M HSA at room temperature for 1 h with or without the specific macropinocytosis inhibitor ethyl-isopropyl amiloride (EIPA). Fluoroshield mounting medium with DAPI (Abcam) was added to stain the nuclei, and fluorescence images at x400 magnification in four random fields of view were captured by a confocal microscope (Zeiss GmbH).

Flow cytometry was used to examine the association between the internalization efficiency and HSA concentration or reaction time. Following treatment with 50 μ M DyLight 488-labeled recombinant proteins and HSA, AsPC-1 cells were washed with PBS and incubated in 0.4% trypan blue at room temperature for 10 min to quench the fluorescence signal on the cell surface. The cells were analyzed by flow cytometry using a FACSCalibur cell analyzer (BD Biosciences) with FlowJo software (BD Biosciences).

In vitro cytotoxicity assay. To assess the cytotoxicity of the recombinant proteins and their analogues to pancreatic cancer cells, clonogenic and Cell Counting Kit-8 (CCK-8) viability assays were used, respectively. For the recombinant proteins, AsPC-1, MIA PaCa-2 and BxPC-3 cells were seeded in a 24-well plate at 100 cells/well and cultured overnight for adhesion. The recombinant proteins and HSA, at the molar ratio of 3:1, were added to treat tumor cells for 120 h. A colony was regarded as >30 cells, and the number of cell colonies was counted under an optical microscope (Olympus Corporation).

For the analogues of recombinant proteins, AsPC-1, MIA PaCa-2 and BxPC-3 cells were seeded in a 96-well plate at 5,000 cells/well. Following treatment with the analogues and HSA for 48 h, CCK-8 reagent (Beyotime Institute of Biotechnology) was added and incubated for 4 h. Absorbance at 570 nm was evaluated using a microplate reader (Thermo Fisher Scientific, Inc.).

In vivo imaging of the recombinant proteins. The animal studies were approved by the Ethics Committee for Animal Experiments of The Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences (approval no. IMBF20060302). All animal experiments were performed in accordance with the Good Laboratory Practice for Nonclinical Laboratory Studies guidelines published by The Ministry of Science and Technology of China. Female BALB/c (*nu/nu*) mice (4–6 weeks old; n=41) were purchased from SPF (Beijing) Lab Animal Technology Co., Ltd.

The recombinant proteins were labeled using a DyLight 680 Antibody Labeling kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Each mouse (n=5) was subcutaneously inoculated with 1 \times 10⁷ human pancreatic cancer AsPC-1, MIA PaCa-2, or BxPC-3 cells suspended in 0.2 ml PBS in the right armpit. When the tumor volume was ~400 mm³, 0.4 mg DyLight 680-labeled recombinant proteins were administered intravenously through the tail vein. Images of fluorescence distribution in the mice were observed and recorded by IVIS Spectrum System (PerkinElmer, Inc.), and fluorescence signals were measured and analyzed using Living Image software (PerkinElmer, Inc.).

In vivo efficacy study. Due to EGFR overexpression and the K-ras mutation, human pancreatic cancer AsPC-1

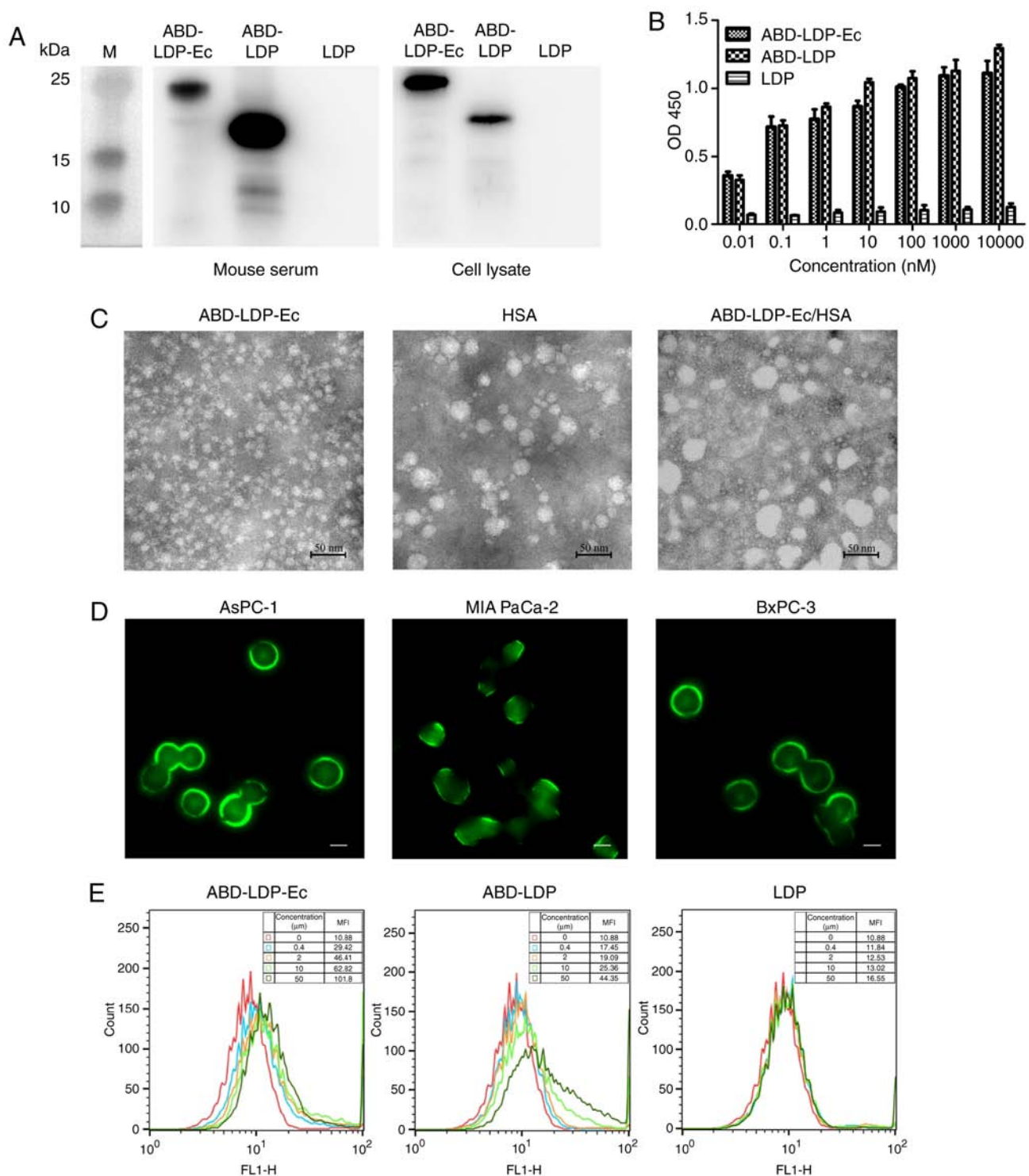


Figure 2. Recombinant proteins bind to target proteins and pancreatic cancer cells. (A) Co-immunoprecipitation assay of the recombinant protein binding specificity to HSA and EGFR. Mouse peripheral blood serum and AsPC-1 cell lysate were incubated with ABD-LDP-Ec, ABD-LDP or LDP. The complexes were collected using a co-immunoprecipitation kit and analyzed by SDS-PAGE and immunoblotting with a His-tag monoclonal antibody. (B) ELISA analysis of the recombinant protein binding with HSA protein. (C) Transmission electron microscopy observation of ABD-LDP-Ec, HSA and ABD-LDP-Ec/HSA complex particles. (D) Immunofluorescence assay of ABD-LDP-Ec bound to AsPC-1, MIA PaCa-2 and BxPC-3 cells. Green fluorescence located around the cells indicated ABD-LDP-Ec bound to EGFR on the cell membrane. Magnification, x400; scale bar, 5 μ m. (E) Flow cytometry analysis of the binding activity of DyLight 488-labeled ABD-LDP-Ec, ABD-LDP and LDP to AsPC-1 cells. MFI, mean fluorescence intensity; OD, optical density; HSA, human serum albumin; EGFR, epidermal growth factor receptor; ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, EGFR-targeting oligopeptide; AE, enediyne chromophore.

cell line was used in a xenograft model in athymic mice to determine the therapeutic efficacy of the recombinant proteins and their analogues. Mice were subcutaneously

inoculated with 1×10^7 AsPC-1 tumor cells in the right flank and randomly divided into groups of 6 mice when the tumor volumes were $>100 \text{ mm}^2$. The recombinant proteins and their

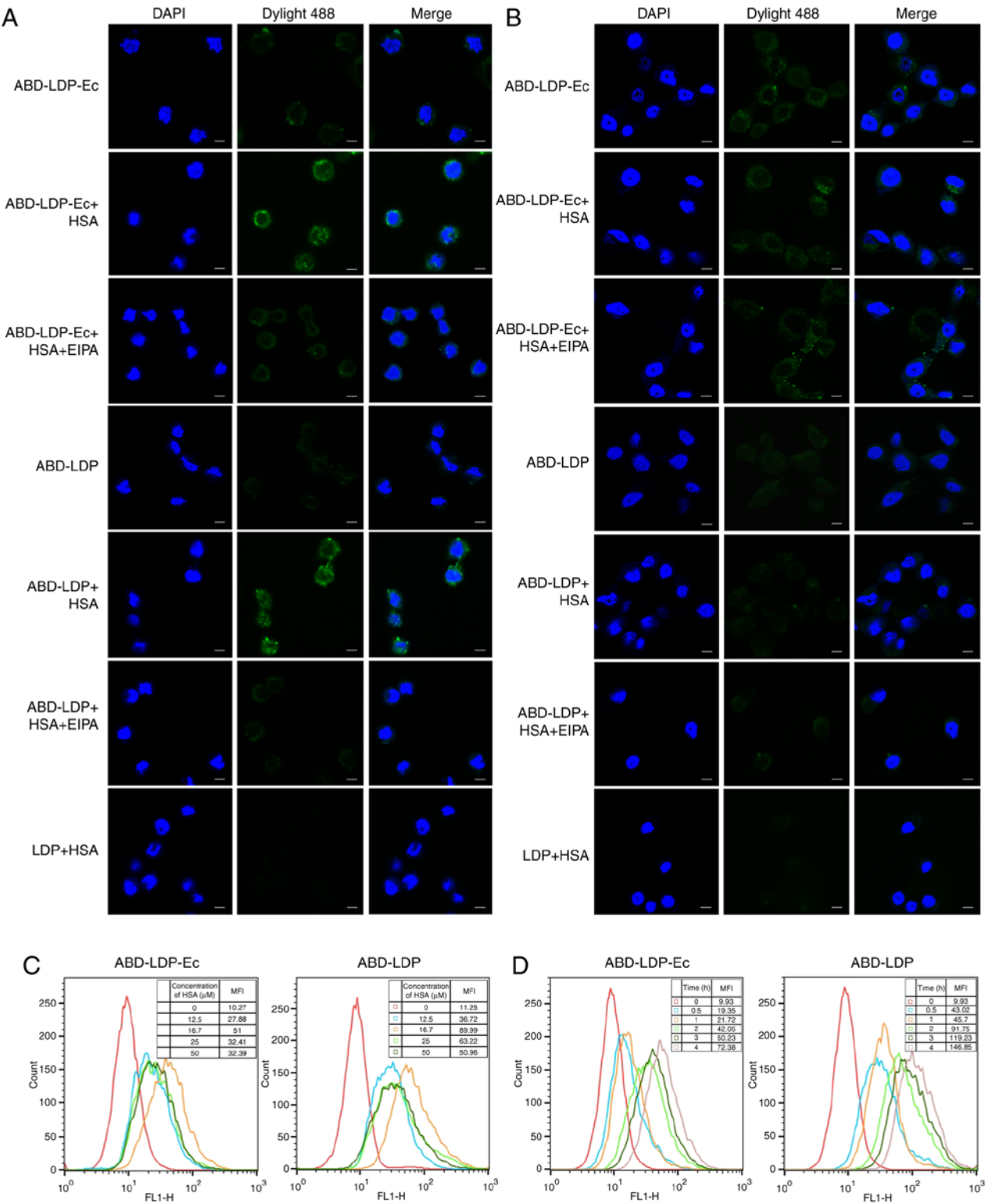


Figure 3. Recombinant proteins are internalized into pancreatic cancer cells. (A) Confocal microscopic observation of K-ras mutant AsPC-1 cells treated with the recombinant proteins with or without HSA and EIPA. (B) Confocal microscopic observation of K-ras wild-type BxPC-3 cells treated with the recombinant proteins with or without HSA and EIPA. Cells were observed under a confocal microscope at x400 magnification. Green fluorescence, DyLight 488-labeled recombinant proteins; blue fluorescence, DAPI-stained nuclei; scale bar, 5 μ m. (C) Flow cytometry analysis of the association between 50 μ M recombinant protein uptake and HSA concentration (0, 12.5, 16.7, 25, 50 μ M) in AsPC-1 cells. (D) With 50 μ M recombinant proteins and 16.7 μ M HSA, flow cytometry analysis of the association between protein uptake and incubation time (0, 0.5, 1, 2, 3, 4 h) in AsPC-1 cells. MFI, mean fluorescence intensity. HSA, human serum albumin; EIPA, ethyl-isopropyl amiloride; ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, epidermal growth factor receptor-targeting oligopeptide; AE, enediyne chromophore.

analogues (20 mg/kg ABD-LDP, 20 mg/kg ABD-LDP-Ec, 0.05 mg/kg LDM, 0.1 mg/kg ABD-LDP-AE or 0.1 mg/kg

ABD-LDP-Ec-AE) were administered intravenously through the tail vein twice with a 7-day interval. Tumor size and mouse

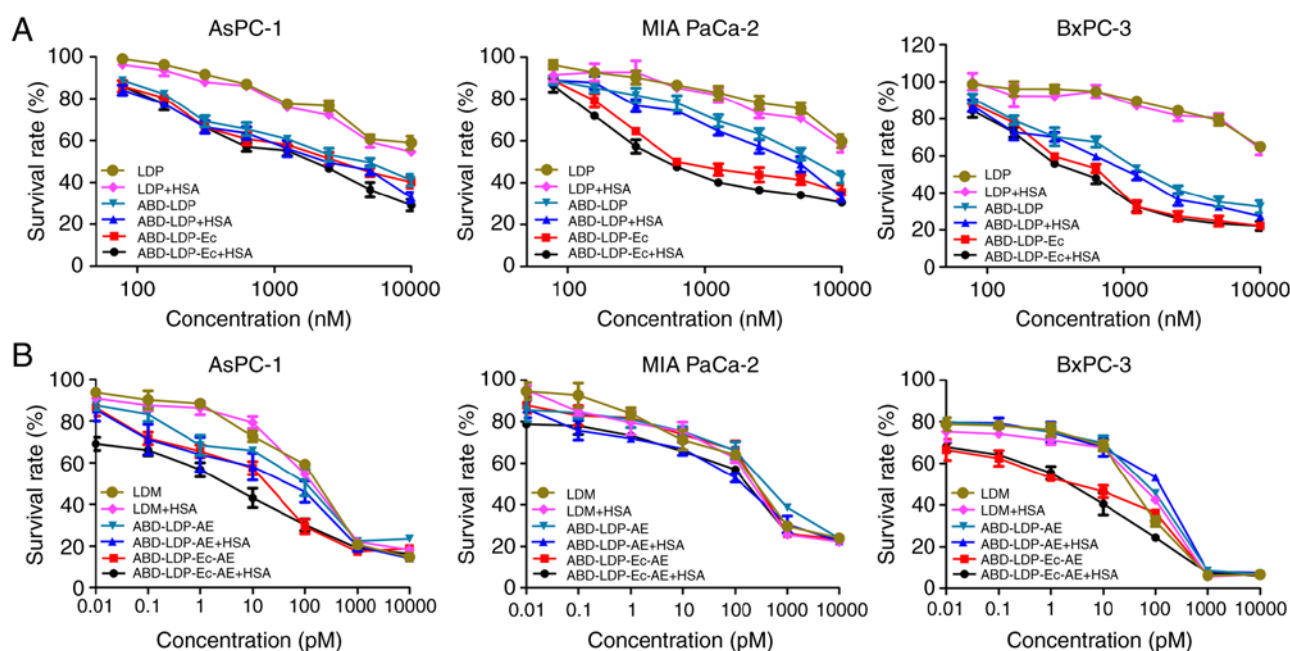


Figure 4. Recombinant proteins and their enediyne-integrated analogues inhibit the proliferation of pancreatic cancer cells *in vitro*. (A) Proliferation inhibition of the recombinant proteins on AsPC-1, MIA PaCa-2 and BxPC-3 cells measured by clonogenic assay. (B) Proliferation inhibition of the enediyne-integrated analogues on AsPC-1, MIA PaCa-2 and BxPC-3 cells measured by Cell Counting Kit-8 viability assay. ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, epidermal growth factor receptor-targeting oligopeptide; AE, enediyne chromophore; HSA, human serum albumin; LDM, lidamycin.

weight were measured every three days, and tumor volume was calculated using the following formula: Tumor volume = $0.5 \times \text{length} \times (\text{width})^2$. The maximum allowed tumor volume was 1,500 mm³. On day 30, the mice were euthanized by cervical dislocation and aseptically dissected, and solid tumors were collected. The tumors were weighed and the tumor growth inhibition (TGI) was calculated as follows: $\text{TGI} = (1 - T/C) \times 100\%$, where T is the mean tumor weight of the therapy group and C is the mean tumor weight of the control group.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc.). Data are presented as the mean \pm SEM. Student's t-test was used to compare two groups, whereas Tukey's test with one-way ANOVA was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Preparation of the recombinant proteins and their analogues. Recombinant plasmids *pET30(a)-abd-ldp-ec* and *pET30(a)-abd-ldp* including gene fragments encoding albumin binding domains, apoprotein of lidamycin, EGFR-directed ligand peptide and glycine-serine (G₄S) linkers were constructed and identified with *NdeI/XhoI* digestion (Fig. 1B). Recombinant proteins ABD-LDP-Ec and ABD-LDP with a His-tag at C-terminus were produced in the form of inclusion bodies by *E. coli* BL21(DE3) Star following IPTG induction, purified with Ni²⁺ affinity chromatography and refolded by stepwise dialysis. A total of ~20 mg ABD-LDP-Ec and ~30 mg ABD-LDP were yielded from 1 l fermentation broth and migrated as a band of 21.1 and 19.8 kDa in SDS-PAGE under reducing conditions, respectively (Fig. 1C). The analogues of

recombinant proteins ABD-LDP-Ec-AE and ABD-LDP-AE were prepared by integrating the recombinant proteins with active enediyne chromophore *in vitro*, and the absorption peak of AE at 340 nm was detected by reverse-phase HPLC, indicating successful assembly of enediyne analogues (Fig. 1D).

Affinity of the recombinant proteins. To confirm the binding capacity of the recombinant proteins ABD-LDP-Ec, ABD-LDP and LDP against albumin and EGFR, co-immunoprecipitation assay and western blot analysis were used. As demonstrated by the western blot analysis, ABD-LDP-Ec and ABD-LDP formed complexes with albumin sourced from mouse serum (Fig. 2A). ABD-LDP-Ec and ABD-LDP exhibited binding capacity in AsPC-1 cell lysate. No binding was observed between LDP and the target proteins.

Comparison of binding to HSA protein among the three proteins was examined by ELISA. ABD-LDP-Ec and ABD-LDP exhibited high affinity for HSA, whereas that of LDP was weak (Fig. 2B). ABD-LDP presented slightly stronger binding efficiency compared with ABD-LDP-Ec, which may have been due to the steric effects of Ec. The particles of ABD-LDP-Ec, HSA and the ABD-LDP-Ec/HSA complex were observed by TEM; as demonstrated in Fig. 2C, ABD-LDP-Ec/HSA particles were larger compared with the individual proteins. The diameter of the complex was ~50 nm.

EGFR-positive AsPC-1, MIA PaCa-2 and BxPC-3 cells were selected for the detection of the recombinant protein cell-binding activity. Green fluorescence was observed on the surface of cancer cells under a fluorescence microscope, indicating that ABD-LDP-Ec possessed superior binding capacity with pancreatic cancer cells compared with ABD-LDP and LDP (Fig. 2D). The slight fluorescence observed in the cells may be explained by internalization of the recombinant

Table I. Proliferation inhibition by the recombinant proteins in pancreatic cancer cells determined by the clonogenic assay.

Recombinant protein	IC ₅₀ , M (mean ± SEM)		
	AsPC-1	MIA PaCa-2	BxPC-3
ABD-LDP-Ec + HSA	1.44±0.03×10 ^{-6a}	1.05±0.18×10 ^{-6a}	7.10±0.71×10 ⁻⁷
ABD-LDP-Ec	2.16±0.11×10 ⁻⁶	1.30±0.11×10 ⁻⁶	8.12±0.65×10 ⁻⁷
ABD-LDP + HSA	1.80±0.08×10 ^{-6a}	3.03±0.09×10 ^{-6a}	1.43±0.18×10 ⁻⁶
ABD-LDP	2.44±0.14×10 ⁻⁶	4.21±0.24×10 ⁻⁶	1.75±0.09×10 ⁻⁶
LDP + HSA	7.23±0.31×10 ^{-6a}	8.54±0.80×10 ^{-6a}	1.36±0.17×10 ⁻⁵
LDP	8.10±0.48×10 ⁻⁶	1.04±0.10×10 ⁻⁵	1.50±0.11×10 ⁻⁵

^aP<0.05 vs. without HSA. ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, epidermal growth factor receptor-targeting oligopeptide; HSA, human serum albumin.

Table II. Proliferation inhibition by the enediyne-integrated analogues and LDM on pancreatic cancer cells determined by Cell Counting Kit-8 viability assay.

Recombinant protein analogue	IC ₅₀ , M (mean ± SEM)		
	AsPC-1	MIA PaCa-2	BxPC-3
ABD-LDP-Ec-AE + HSA	1.69±0.08×10 ^{-12a}	6.04±0.48×10 ^{-11a}	9.22±0.29×10 ⁻¹³
ABD-LDP-Ec + AE	7.91±0.84×10 ⁻¹²	1.61±0.10×10 ⁻¹⁰	1.21±0.23×10 ⁻¹²
ABD-LDP -AE + HSA	9.44±0.68×10 ^{-12a}	6.88±0.28×10 ^{-11a}	2.43±0.28×10 ⁻¹¹
ABD-LDP + AE	4.38±0.30×10 ⁻¹¹	3.20±0.15×10 ⁻¹⁰	2.13±0.29×10 ⁻¹¹
LDM + HSA	1.19±0.06×10 ⁻¹⁰	1.44±0.08×10 ⁻¹⁰	1.10±0.17×10 ⁻¹¹
LDM	1.06±0.04×10 ⁻¹⁰	1.78±0.11×10 ⁻¹⁰	1.12±0.24×10 ⁻¹¹

^aP<0.01 vs. without HSA. ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, epidermal growth factor receptor-targeting oligopeptide; AE, enediyne chromophore; LDM, lidamycin; HSA, human serum albumin.

proteins at room temperature. In flow cytometry experiments, ABD-LDP-Ec displayed a significantly stronger affinity to AsPC-1 cells compared with ABD-LDP and LDP (Fig. 2E) owing to the molecular recognition of EGFR by Ec. ABD-LDP at a high concentration also exhibited a certain affinity for AsPC-1 cells, whereas the affinity of LDP was low.

Internalization of the recombinant proteins in pancreatic cancer cells. AsPC-1 and BxPC-3 cells treated with recombinant proteins labeled with DyLight 488 were observed under a laser scanning confocal microscope. In the K-ras mutant AsPC-1 cells, HSA substantially improved the uptake of ABD-LDP-Ec and ABD-LDP, and the specific macropinocytosis inhibitor EIPA reversed the effects of HSA on endocytosis (Fig. 3A). In the K-ras wild-type BxPC-3 cells, HSA and EIPA exhibited limited effects on protein uptake (Fig. 3B). These results suggested that ABD-LDP-Ec and ABD-LDP benefited from HSA to internalize into K-ras mutant tumor cells by macropinocytosis-mediated uptake. In addition, the Ec component also promoted the internalization of ABD-LDP-Ec in EGFR-positive tumor cells.

In K-ras mutant AsPC-1 cells, flow cytometry detection demonstrated that the amount of protein uptake was associated with HSA concentration and incubation time. For HSA

concentration, the dependence had two phases. As presented in Fig. 3C, with 50 μ M recombinant proteins, the maximum amount of ABD-LDP-Ec and ABD-LDP were internalized into AsPC-1 cells when HSA was at 16.7 μ M. When the HSA concentration was <16.7 μ M, the internalization of recombinant proteins was improved with increasing HSA concentration, whereas when HSA concentration was >16.7 μ M, the internalization was reduced with increasing HSA concentration. The internalization of ABD-LDP-Ec and ABD-LDP was enhanced following a longer incubation (Fig. 3D), and the endocytosis of ABD-LDP was higher compared with that of ABD-LDP-Ec.

In vitro cytotoxicity of the recombinant proteins and their analogues. The anti-proliferative effect of the recombinant proteins and their analogues on pancreatic cancer cells was measured by a clonogenic and CCK-8 assays, respectively. As presented in Fig. 4A, the recombinant proteins at 100–10,000 nM moderately inhibited the proliferation of cancer cells, and the efficacy of ABD-LDP-Ec and ABD-LDP was stronger compared with LDP, especially for BxPC-3 cells. In addition, enhanced inhibitory activity of ABD-LDP-Ec and ABD-LDP with HSA on the K-ras mutant AsPC-1 and MIA PaCa-2 cells was observed (Table I). The enediyne-integrated

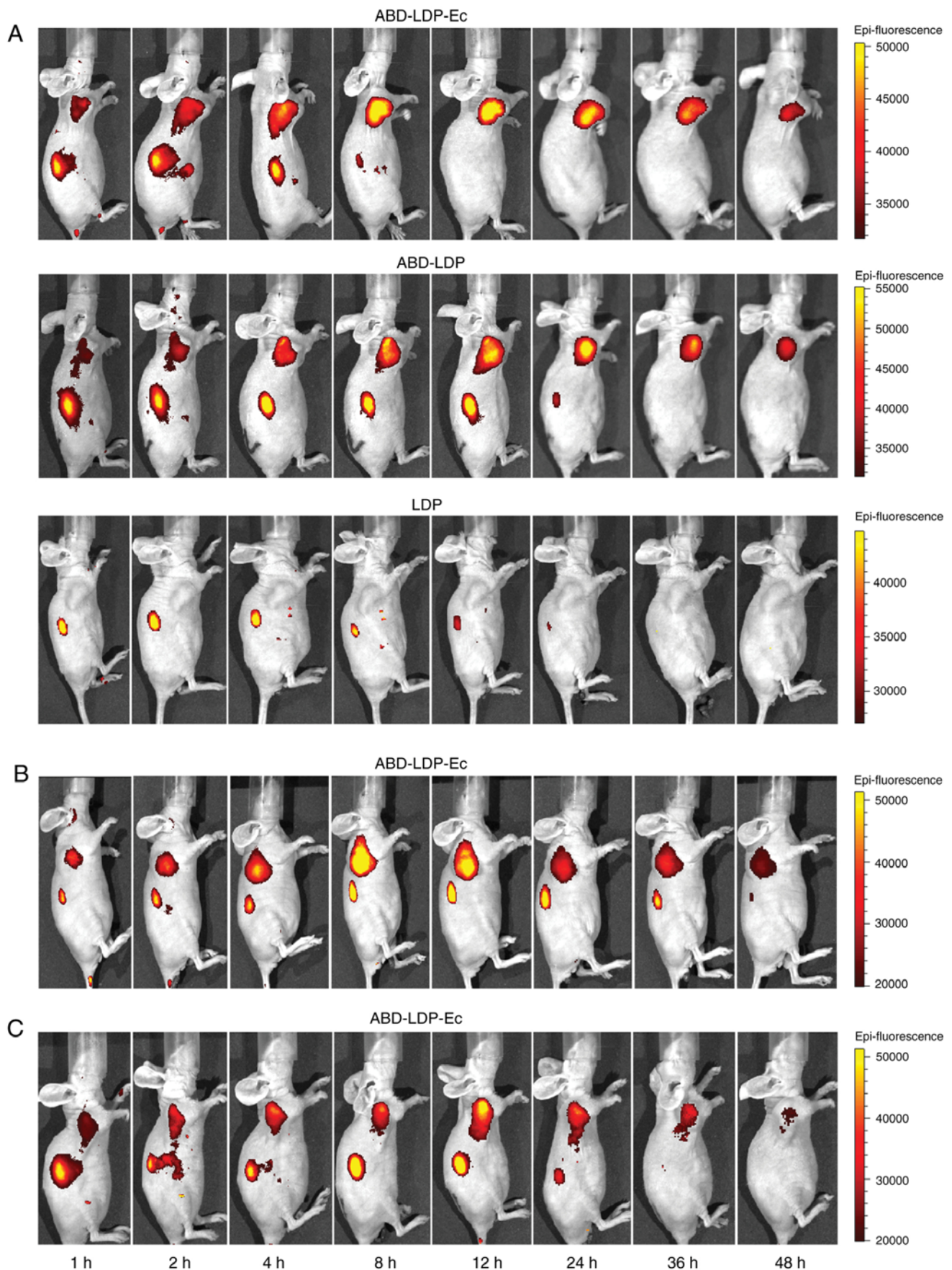


Figure 5. Distribution of the recombinant proteins in xenograft-bearing nude mice *in vivo*. (A) Representative fluorescence images at the indicated times (1, 2, 4, 8, 12, 24, 36 and 48 h) post-intravenous injection of 0.4 mg DyLight 680-labeled ABD-LDP-Ec, ABD-LDP or LDP in AsPC-1 xenograft-bearing nude mice. (B) Representative fluorescence images post-intravenous injection of DyLight 680-labeled ABD-LDP-Ec in MIA PaCa-2 xenograft-bearing nude mice. (C) Representative fluorescence images post-intravenous injection of DyLight 680-labeled ABD-LDP-Ec in BxPC-3 xenograft-bearing nude mice. ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, epidermal growth factor receptor-targeting oligopeptide.

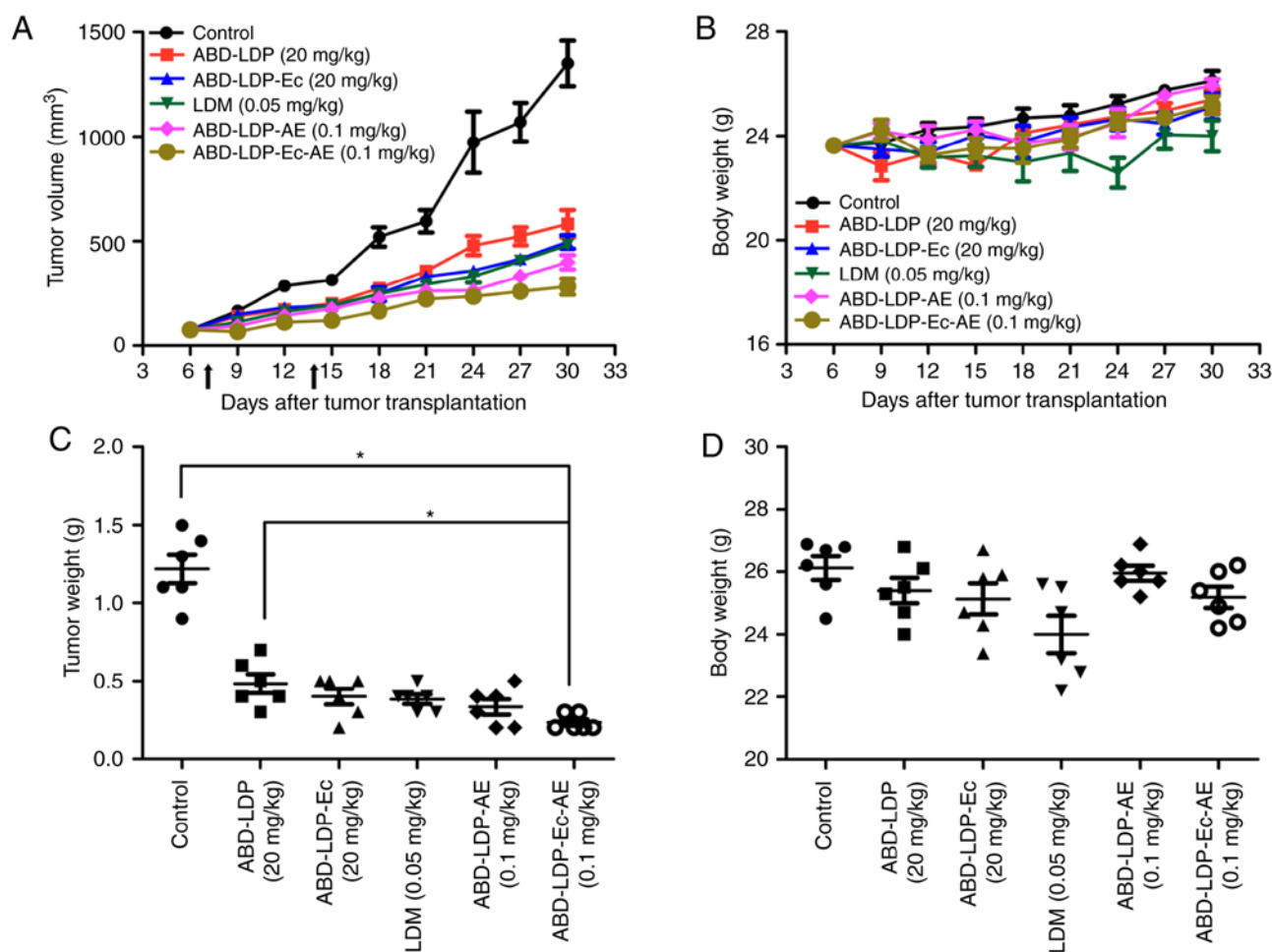


Figure 6. Recombinant proteins and their enediyne-integrated analogues inhibit the growth of pancreatic cancer xenografts in nude mice. (A) Tumor volume curves of AsPC-1 xenograft (n=6 mice/group). Arrows represent the days of injection. (B) Body weight curves of AsPC-1 xenograft-bearing nude mice. (C) Tumor weight of AsPC-1 xenograft on day 30. (D) Body weights of the mice on day 30. *P<0.05. ABD, albumin-binding domain; LDP, lidamycin apoprotein; Ec, epidermal growth factor receptor-targeting oligopeptide; AE, enediyne chromophore; LDM, lidamycin.

analogues ABD-LDP-Ec-AE and ABD-LDP-AE exhibited strong proliferation inhibition on pancreatic cancer cells with IC₅₀ values of 1-100 pM (Fig. 4B), and HSA significantly promoted the cytotoxicity of the analogues on K-ras mutant cells (Table II).

Optical imaging of the recombinant proteins in vivo. Female BALB/c (nu/nu) mice bearing human pancreatic cancer AsPC-1, MIA PaCa-2 and BxPC-3 xenografts were used to detect the biodistribution of the recombinant proteins *in vivo*. 0.4 mg DyLight 680-labeled recombinant proteins were injected intravenously, and images were captured and recorded by an IVIS Spectrum System at 1, 2, 4, 8, 12, 24, 36 and 48 h. No tumor site fluorescence was observed in the LDP group; by contrast, localized fluorescence was observed in ABD-LDP-Ec and ABD-LDP groups, which reflected the enhanced tumor-targeting ability of recombinant proteins *in vivo*. ABD-LDP-Ec benefited from the EGFR-targeting characteristic of Ec and exhibited an improved tumor-targeting capacity compared with ABD-LDP in the AsPC-1 xenograft that presented as protein enrichment in <1 h and maximum intensity at 8 h (Fig. 5A). Compared with the K-ras wild-type BxPC-3 xenograft (Fig. 5C), K-ras mutant AsPC-1 and MIA

PaCa-2 xenografts (Fig. 5A and B) were beneficial for the enrichment and reservation of ABD-LDP-Ec owing to the macropinocytosis of the ABD-LDP-Ec/HSA complex and its accumulation in the tumors.

In vivo efficacy of the recombinant proteins and their analogues. Human pancreatic cancer AsPC-1 xenograft in BALB/c (nu/nu) mice was used to evaluate the antitumor activity of the recombinant proteins and their analogues. When solid tumors were established following subcutaneous AsPC-1 cell inoculation, the reconstituted analogues were administrated at an equivalent molar dose of LDM (0.05 mg/kg), which was the tolerance dose described previously (28), through the tail vein. The recombinant proteins were used at the same concentration as in the optical imaging experiment (~20 mg/kg). In Fig. 6A, the curves of tumor volume over time demonstrated that the energized proteins presented stronger antitumor activity compared with the recombinant proteins and non-targeted LDM. At the end of experiment, the inhibitory rates based on tumor weight were 81.9% for ABD-LDP-Ec-AE and 75.2% for ABD-LDP-AE, respectively, which were higher compared with 68.6% for LDM (Fig. 6C). In addition, high-dose ABD-LDP-Ec and ABD-LDP also exhibited moderate inhibition in the AsPC-1 xenograft.

Body weights increased throughout the experiment among all experimental groups and the control, and no obvious differences were observed among the groups (Fig. 6B and D), which indicated that the administered doses were well tolerated.

Discussion

Targeted drug delivery serves an important role in improving the efficacy and reducing the side effects of drugs. A new form of recombinant protein ABD-LDP-Ec based on an albumin-binding domain and an EGFR-targeted oligopeptide was produced and used to prepare an anti-cancer drug capable of active and passive tumor targeting. Oligopeptide Ec, which comprises the 22 amino acids of the EGF COOH-terminal sufficient for high-affinity receptor binding (29), was used as an EGFR-targeting molecule to perform active targeting of EGFR-positive tumor cells. In contrast to conventional drug designs such as β -defensin 2-HSA (30), thioredoxin-albumin (31) and HSA-eTGFR2 (32), the ABD035 molecule instead of HSA was fused with drug molecules in the present study and exerted the properties of passive targeting and half-life extension, which resulted in the enrichment and retention of ABD-LDP-Ec and ABD-LDP in tumor tissue through capturing the functional albumin in plasma. In addition, ABD-LDP displayed a certain binding capacity to tumor cells, although the mechanism of action remains unclear. Compared with the proteins combining drugs with an antibody and HSA, Ec/ABD-fused proteins can be produced in engineered bacteria, and the small and efficient target molecules result in less interference with the function of fusion proteins and lower immune response (12).

Macropinocytosis is a highly conservative endocytic pathway observed in certain macrophages (33), dendritic (34) and tumor (35) cells that implements transmembrane transport of extracellular substances by the macropinosomes formed of plasma membrane folds. Macropinocytosis is significantly enhanced in tumor cells carrying EGFR or proto-oncogene mutations, such as Ras (36), Rac (37) and Src (38). As reported by Comisso *et al* (20), macropinocytosis serves an important role in the nutrient uptake of Ras mutant tumor cells, which enables HSA to be transported into tumor cells and further be decomposed into essential amino acids for sustained cell growth and proliferation, suggesting that HSA may be used as a macropinocytosis-targeted drug delivery carrier. For example, albumin-modified β -defensin DF-HSA displayed intensive macropinocytosis-mediated uptake in K-ras mutant MIA PaCa-2 cells and exerted high therapeutic efficacy against pancreatic xenograft tumors in athymic mice (29). The nanoparticle albumin-bound paclitaxel, which is the first-line treatment drug for pancreatic cancer combined with gemcitabine, had been demonstrated to be internalized into macrophages by macropinocytosis and to induce macrophage immunostimulatory cytokine expression (39). ABD-LDP-Ec and ABD-LDP combined with HSA achieved higher tumor uptake relative to LDP in AsPC-1 and MIA PaCa-2 cells, and the tumor uptake was associated with the content of HSA. The optimum molar ratio of ABD-LDP-Ec to HSA for the maximum uptake was 3:1, implying that excessive HSA monomers may hinder the endocytosis of the protein/HSA complex by occupying macropinocytosis sites.

Since LDP generally acts as the skeleton structure of recombinant proteins and the protective group for protecting and stabilizing the chromophore, ABD-LDP-Ec was assembled with the active enediyne of LDM by molecular reconstitution to produce its enediyne-integrated analogue ABD-LDP-Ec-AE, which demonstrated 1×10^6 -fold stronger inhibition on pancreatic cancer cells compared with ABD-LDP-Ec. Compared with LDM, ABD-LDP-Ec-AE at a lower concentration effectively inhibited the proliferation of AsPC-1, MIA PaCa-2 and BxPC-3 cells, whereas the TGI rate on AsPC-1 xenografts was improved by 20% at the same molar concentration. Benefiting from the EGFR-targeting Ec and the combination of ABD and HSA, ABD-LDP-Ec-AE was retained in tumor tissues for 48 h due to the enhanced permeability and retention effect, and was transported into K-ras mutant cancer cells, which significantly prolonged the duration of LDM activity and increased the cytotoxicity. In addition, free ABD-LDP-Ec-AE was decomposed and eliminated through biotransformation functions of liver within 8 h, and the damage of LDM on normal cells and tissues was weakened.

In summary, the results of the present study demonstrated that an ABD/oligopeptide-based cancer-targeted recombinant protein ABD-LDP-Ec and its enediyne-integrated analogue ABD-LDP-Ec-AE exhibited more potent antitumor efficacy compared with LDP and LDM in EGFR-positive and K-ras mutant pancreatic cancer. With the help of the albumin-binding ABD, ABD-LDP-Ec was enriched in solid tumors through the passive targeting of albumin, bound to EGFR on the cell membrane and was internalized into the cytoplasm via receptor-mediated endocytosis and albumin-induced macropinocytosis of K-ras mutant cells. These results suggested that the introduction of ABD-based multi-functional drug delivery may be an effective approach to improve the efficacy of anti-tumor drugs, especially for K-ras mutant cancers.

Acknowledgements

Not applicable.

Funding

This research was supported by the Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Sciences (grant nos. 2016-I2M-1-011 and 2019-I2M-1-005).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and MJ designed this study. WS and JG conducted main experiments and analyzed the data. LL performed molecular reconstitution. YS performed animal experiments. WS wrote the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal studies were approved by the Ethics Committee for Animal Experiments of The Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences (approval no. IMBF20060302). All animal experiments were performed in accordance with the Good Laboratory Practice for Nonclinical Laboratory Studies guidelines published by The Ministry of Science and Technology of China.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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