A Bispecific Enediyne-Energized Fusion Protein Containing Ligand-Based and Antibody-Based Oligopeptides against Epidermal Growth Factor Receptor and Human Epidermal Growth Factor Receptor 2 Shows Potent Antitumor Activity

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Abstract

Purpose: The cooverexpression of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) observed in many human tumors and their synergistic interaction in the transformation of cells make these receptors important targets for the development of new targeted therapeutics. Targeting of EGFR and HER2 simultaneously has been pursued as a strategy with which to potentially increase efficiency and selectivity in therapy of certain cancers. This study was set to construct a bispecific energized fusion protein (Ec-LDP-Hr-AE) consisting of two oligopeptides against EGFR and HER2, and lidamycin, and investigate its antitumor efficacy.

Experimental Design: In vitro experiments measured the binding and internalization of bispecific Ec-LDP-Hr fusion protein. The potency of energized fusion proteins was also done in which the bispecific Ec-LDP-Hr-AE was compared with lidamycin (LDM) and its monospecific counterparts, Ec-LDP-AE and LDP-Hr-AE. In vivo, Ec-LDP-Hr-AE was given i.v. to nude mice bearing human ovarian carcinoma SK-OV-3 xenografts.

Results: Binding and internalization studies showed that bispecific fusion protein Ec-LDP-Hr bound to carcinoma cells specifically and then were internalized into the cytoplasm. Bispecific Ec-LDP-Hr-AE was more potent and selective in its cytotoxicity against different carcinoma cell lines than corresponding monospecific agents and LDM in vitro. In addition, Ec-LDP-Hr-AE significantly inhibited the growth of SK-OV-3 xenografts in nude mouse model. In vivo imaging study showed that FITC-labeled Ec-LDP-Hr was targeted and accumulated in the tumors.

Conclusion: A ligand-based and an antibody-based oligopeptide fused to the enediyne antibiotic LDM created a new bispecific fusion protein with low molecular weight and more potent in vitro and in vivo antitumor activity (than monospecific fusion proteins).

The ErbB family (ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4) of receptor tyrosine kinases is known to drive both formation and progression of several commonly occurring cancers (1). Ligand binding to the receptors results in receptor homodimerization and heterodimerization, then initiated a series of intracellular events that ultimately promote cell growth, proliferation, differentiation, and migration (2–4). Human epidermal growth factor receptor 2 (HER2) has no identified ligand but it is the preferred binding partner for the other three family members (5). The cooverexpression of epidermal growth factor receptor (EGFR) and HER2 observed in many human tumors and their synergistic interaction in the transformation of cells make these receptors important targets for the development of new targeted therapeutics (6–8). Several monoclonal antibodies (e.g., cetuximab, trastuzumab, and pertuzumab) and tyrosine kinase inhibitors (e.g., gefitinib, erlotinib, and lapatinib) targeting EGFR and HER2 or both have been approved by the U.S. Food and Drug Administration for therapeutic use and several more are in preclinical and clinical development (4, 9–12). Immunotoxins taking advantage of the specificity of antibodies or ligands and the potent cytotoxicity of toxins are highly effective in cancer therapy (13). However, in many cases, immunotoxin with high molecular weight are hard to penetrate into solid tumors and they have been associated with significant toxicities to normal tissues and...
effective potent antitumor effect.

**Materials and Methods**

**Construction of expression vector pET-Ec-ldp-Hr.** DNA fragments encoding for Ec-LDP-Hr were obtained by PCR and DNA cloning techniques. As shown in Fig. 1A, the full gene of fusion protein Ec-LDP-Hr (from 5' to 3') consisted of pelB signal peptide (22 amino acids; ref. 21), C-loop of EGF (the 22 amino acids of EGF COOH terminal), apoprotein LDP (110 amino acids; ref. 22) and V_{H} CDR3 region of C6.5 antibody (20 amino acids; refs. 23, 24). Two (GGGGS)_{2} linkers were inserted to the space the coding sequences of EGF C-loop, LDP, and V_{H} CDR3 region of C6.5 antibody. After four rounds of PCR and DNA cloning process, the resultant 612-bp fragment was digested by NdeI/XhoI and was inserted into pET30a expression vector to generate plasmid pET-Ec-ldp-Hr. Coding sequences for monospecific fusion protein Ec-LDP and LDP-Hr were created by the same way. DNA sequencing analysis (Invitrogen Corp.) was used to verify that the gene was correct in sequence and had been cloned in the frame.

**Expression and purification of fusion proteins.** Expression plasmids pET-Ec-ldp-Hr, pET-Ec-ldp, and pET-LDP-Hr were transformed into *Escherichia coli* strain BL21(DE3) star (Novagen). After overnight culture, bacteria were diluted 100-fold in Luria-Bertani medium containing 50 μg/mL kanamycin and grown at 37°C until the OD600 reaches 1.0. Gene expression was induced with the addition of isopropyl-β-D-thiogalactopyranoside at 0.1-mmol/L concentration. Eight hours after induction, bacteria were harvested by centrifugation and the periplasmic fraction was prepared by osmotic shock method (pET system manual 9th edition; Novagen). The fusion proteins were purified by affinity chromatography (HisTrap HP, GE Healthcare) according the manufacturer's instruction and the purity of fusion proteins were analyzed by high-performance liquid chromatography (HPLC) on a TSK G2000 SWXL column (Tosoh). 

**Preparation of energized fusion proteins.** The active enediyne chromophore (AE) of LDM was separated by using C4 column (GE Healthcare) with a 22% acetonitrile in 0.05% trifluoroacetic acid mobile phase. The AE-containing solution was added to Ec-LDP-Hr/PBS (10 mmol/L; pH 7.4), Ec-LDP/PBS, and LDP-Hr/PBS, respectively, with the molecular ratio of 3:1, and was incubated at 4°C for 12 h while rocking. Free AE was removed by using a Sephadex G-75 column (GE Healthcare) and assembled energized fusion proteins were confirmed by reverse-phase HPLC using a Vydac C4 300A column (Grace). Absorbance at 350 nm was measured.

**Cell culture.** The human breast cancer cell lines SK-BR-3 and MCF-7, the ovarian cancer cell line SK-OV-3 and OV-CAR-3, and the epidermoid carcinoma cell line A431 were...
grown as monolayers in culture flasks and maintained in exponential growth to ∼90% confluence for experiments. Cells were cultured in either DMEM (SK-BR-3, SK-OV-3, OVCAR3, and A431) or RPMI 1640 (MCF-7) supplement with 10% fetal bovine serum (Life Technologies), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Western blot and coimmunoprecipitation analysis. Cells were lysed for 30 min in radioimmunoprecipitation assay buffer supplement with 1 μg/mL Aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L NaVO₄, 50 mmol/L NaF, and 1% Triton X-100 for 30 min at 4°C. One milligram of total protein from cell lysates was incubated with 100 μg Ec-LDP-Hr protein at 4°C then the mixtures were incubated overnight with 1 μg anti-EGFR or anti-HER2 antibody at 4°C; complexes were collected with protein A+G agarose (Beyotime) and the precipitates were washed four times with ice-cold PBS. Then, proteins were released by boiling in sample buffer, followed by Western blot analysis as described above. The fusion protein was detected by incubation of the membranes with anti–His tag monoclonal antibody.

**MTT assay.** The MTT assay was used for measuring in vitro cytotoxicity of energized fusion proteins as described by Miao QF et al. (22). Cells were seeded in 96-well plates and incubated in 37°C for 24 h and then exposed to different concentrations of LDM and energized fusion proteins (Ec-LDP-Hr-AE, Ec-LDP-AE, and LDP-Hr-AE) for 48 h. MTT (Sigma) solution (5 mg/mL, 20 μL) was added to each well and incubated for another 4 h at 37°C. The supernatant was removed and 150 μL DMSO was added to each well. The absorbance at 570 nm was measured using an ELISA reader (Thermo Fisher). Growth inhibition was calculated as a percentage of the nontreated controls.
Binding affinity assay. SK-OV-3 cells were grown on coverslides and incubated for 24 h, fixed with methanol, blocked with normal goat serum, then incubated with Ec-LDP-Hr protein (50 μg/mL) for 1 h. After getting washed with PBS, cells were incubated with mouse anti-His-tag monoclonal antibody (Novagen; diluted 1:200), followed with FITC-conjugated goat anti-mouse antibody (Zhongshan Golden Bridge Biotechnology; diluted 1:500). The images were observed under a fluorescence microscope and collected by camera (Nikon TE 2000u).

Internalization assay. SK-OV-3 cells were seeded on coverslides at a density of 3 × 10⁴ cells per well. After 24 h of incubation, Ec-LDP-Hr was added at final concentration of 1 μmol/L and incubated for another 6 h. The following steps were carried out according to Moulder's method with some modifications (26). Cells were fixed with methanol, blocked with normal goat serum, and permeabilized with 0.2% Triton X-100/PBS for 15 min. Then, the cells were incubated with mouse anti-His-tag monoclonal antibody diluted 1:200 in 0.05% Triton X-100/PBS for 2 h (Novagen), followed by incubation with FITC-labeled anti-mouse IgG diluted 1:500 in 0.05% Triton X-100/PBS for 1 h, and counterstained with 1 mg/mL Hoechst 33342 (Beyotime) for 15 min. The images were observed under a laser scanning confocal microscope (Leica TCS SP2, Germany).

FACS-based internalization assays were also done to compare the internalization efficiency of three fusion proteins as described by Stish et al. (27).

In vitro selectivity assay. OVCAR-3 cells (1 × 10⁶) were plated and allowed to adhere overnight. Once adhered,
cells were incubated in serum-free DMEM containing 10 μmol/L Dil (Beyotime) for 15 min then rinsed with PBS to remove excess dye. Cells were allowed to recover in DMEM containing 10% fetal bovine serum for 30 min (15). Following recovery, cells were trypsinized and counted. SK-OV-3 cells were left unattained. OVCAR-3 and SK-OV-3 cells were mixed at 1:1 ratios and were incubated with FITC-labeled Ec-LDP-Hr at concentrations of 10, 1 μmol/L, 100, 10, 1, or 0.1 nmol/L for 1 h at room temperature; washed with PBS; and then subjected to flow cytometric analysis. To mimic the in vivo situation, SK-OV-3 cells were mixed with OVCAR-3 cells at 1:5 and 1:10 ratios, incubated with FITC-labeled Ec-LDP-Hr (100 nmol/L), and analyzed by flow cytometry as described above.

In vivo efficacy studies. Female BALB/c nude mice (18-22 g) were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences. SK-OV-3 cells (1 × 10⁶) suspended in 200 μL sterile saline were inoculated s.c. in the right armpit of nude mouse. After 3 wk, the tumors were aseptically dissected and pieces of tumor tissue (2 mm³ in size) were transplanted s.c. by a trocar into mice. When the tumor size was over 100 mm³, mice were divided into groups (n = 6) and treated with lidamycin, Ec-LDP-Hr-AE, Ec-LDP-AE, and LDP-Hr-AE, respectively, at different doses. All fusion proteins were injected i.v. in the tail vein and given in a 200 μL volume of sterile saline. Ten days after the first treatment, all nude mice were injected with the fusion proteins again at same doses. Tumor size was measured every 3 d and tumor size was calculated by the formula: tumor weight (control) × 100%. To determine the inhibition rate was calculated by (1 − tumor weight (treated)/tumor weight (control)) × 100%.

In vivo fluorescence imaging. SK-OV-3 cells were inoculated to the right armpit of nude mice. Three hundred micrograms of FITC-labeled Ec-LDP-Hr were injected i.v. 25 d after tumor inoculation (n = 3). The mice were placed under anesthesia by inhalation of isoflurane and the images were observed with the Xenogen Ivis 200 system and recorded by built-in camera (Caliper Life Sciences).

Results

Construction and preparation of fusion proteins. After four rounds of PCR and DNA cloning process, the DNA fragments encoding for Ec-LDP-Hr protein was cloned and inserted into the pET30a expression vector under control of an isopropyl-β-d-thiogalactopyranoside-inducible T7 promoter. A (His)₆ tag was also introduced at the COOH terminal of the constructs to facilitate purification of the recombinant proteins through Ni²⁺ affinity chromatography (Fig. 1A). The purity of fusion proteins were all over 95% when analyzed by SDS-PAGE and HPLC (Fig. 1B and C), and the production of Ec-LDP-Hr, Ec-LDP, and LDP-Hr was 9, 18, and 60 mg/L fermentation broth, respectively. The energized fusion proteins were prepared by integrating AE molecule of LDM into Ec-LDP-Hr, Ec-LDP, and LDP-Hr, respectively. Data from reverse-phase HPLC showed that AE molecule integrated into fusion proteins successfully (Fig. 1D).

Binding and internalization of fusion proteins. The results of immunofluorescence assay showed that Ec-LDP-Hr protein bound specifically to the membrane of SK-OV-3 cells and then internalized into the cytoplasm through receptor-mediated endocytosis (Fig. 2A and B). To further confirm the ability of Ec-LDP-Hr binding to EGFR and HER2, coimmunoprecipitation assay was also done. As shown in Fig. 2C, the bispecific fusion protein Ec-LDP-Hr was precipitated as part of the complex with EGFR (lane 2) and as part of the complex with HER2 (lane 5). Preimmune serum did not precipitate Ec-LDP-Hr protein (lane 3 and 6).

In vitro efficacy of energized fusion proteins. We measured the cytotoxicity of the bispecific energized fusion protein Ec-LDP-Hr-AE on five human carcinoma cell lines expressing different levels of EGFR and HER2 (Fig. 3A) by using MTT assay. LDM and monospecific energized fusion proteins (Ec-LDP-AE and LDP-Hr-AE) were also tested for comparison. As shown in Fig. 3B, the bispecific energized fusion protein was more potent than the monospecific energized fusion proteins and lidamycin. Against EGFR- and HER2-overexpressed SK-OV-3 cells, the bispecific molecule Ec-LDP-Hr-AE was 9 and 40 times more potent than the Ec-LDP-AE and LDP-Hr-AE, respectively. In addition, for the EGFR-overexpressed A431 cells, Ec-LDP-Hr-AE had at least 8-fold increase compared with either monospecific energized fusion protein. However, for EGFR and HER2, both negative OVCAR-3 cells, the bispecific Ec-LDP-Hr-AE was less cytotoxic to those of the corresponding monospecific energized fusion proteins and lidamycin. The results revealed that Ec-LDP-Hr-AE is able to selectively kill a variety of human carcinoma cell lines and is generally more active than the monospecific energized fusion proteins. Furthermore, the increased activity observed with Ec-LDP-Hr-AE is due to the presence of the two different ligands on a single-chain molecule (shown in Supplementary Fig. S1). Lapatinib is a small-molecule inhibitor of both EGFR and HER2 tyrosine kinases. We also compare the cytotoxicity of Ec-LDP-Hr-AE with that of lapatinib on EGFR- and HER2-overexpressed SK-OV-3 cells and EGFR- and HER2-negative OVCAR-3 cells. The results showed that Ec-LDP-Hr-AE is 10,000 times more potent than lapatinib in cytotoxicity to both ovarian cancer cell lines (shown in Supplementary Fig. S2).

Increased cytotoxicity of bispecific Ec-LDP-Hr-AE irrelevant to binding affinity and internalization efficiency. A study has shown that immunotoxin activity is related to binding ability (28). To determine the relationship between cytotoxicity and binding affinity, a FACS-based assay was used to compare the binding of Ec-LDP-Hr, Ec-LDP, and LDP-Hr to SK-OV-3 cells. The binding affinities (K_d) for Ec-LDP-Hr, Ec-LDP, and LDP-Hr were 0.831, 0.634, and 0.582 μmol/L, respectively. However, the maximum number of binding site (B_max) of Ec-LDP-Hr (519.6) was higher than that of Ec-LDP (363.3) and LDP-Hr (291.7), indicating that Ec-LDP-Hr reacted with the greatest number of SK-OV-3 cell surface receptors.
These data indicated that increased cytotoxicity was not associated with binding affinity.

The internalization efficiency of all fusion proteins was also measured using FITC-labeled agents. Figure 3D showed that more Ec-LDP-Hr was internalized than Ec-LDP and LDP-Hr, but they did not show statistical differences (P > 0.5). This fact proved that cytotoxicity was also independent of internalization efficiency.

In vitro selectivity of Ec-LDP-Hr protein. The ability of bispecific Ec-LDP-Hr protein to selectively target tumors was analyzed by using FACS-based assays in vitro. OVCAR-3 cells were used to approximate normal tissues that express low levels of EGFR and HER2, and SK-OV-3 cells represented EGFR- and HER2-positive tumors. Dil-labeled OVCAR-3 cells and unlabeled SK-OV-3 cells were readily distinguishable by flow cytometry when mixed at a 1:1 ratio (Fig. 4A). At a concentration of 100 nmol/L, Ec-LDP-Hr was detected in 21.4% of the SK-OV-3 cells whereas OVCAR-3 cells failed to exhibit significant binding (data are not shown). However, EGFR- and HER2-low expressed normal cells are predicted to be in vast excess compared with tumor cells in vivo. To mimic this situation, we incubated Ec-LDP-Hr with SK-OV-3 cells that were mixed with increasing numbers of OVCAR-3 cells. At OVCAR-3/SK-OV-3 ratios as high as 5:1 and 10:1, Ec-LDP-Hr still specifically bind to EGFR and HER2 overexpressed tumor cells. The Ec-LDP-Hr was detected on 12.3% and 6.11% SK-OV-3 cells present in the assay without significant binding to OVCAR-3 cells despite the 5- and 10-fold higher number of these cells (Fig. 4C and D). Together, these data show that Ec-LDP-Hr is capable of selectively targeting tumor cells that express high levels of both receptors over normal cells that express low levels of receptors.

In vivo efficacy of energized fusion proteins. The in vivo efficacy of bispecific Ec-LDP-Hr-AE and monospecific Ec-LDP-AE, LDP-Hr-AE were tested in a SK-OV-3 xenografts nude mouse model in two separate experiments. In first experiment, the mice bearing SK-OV-3 xenografts were divided into six groups and were treated with i.v. injections of Ec-LDP-Hr, LDM, or Ec-LDP-Hr-AE. LDM was given at dose of 0.05 mg/kg; Ec-LDP-Hr was given at dose of 1 mg/kg; and Ec-LDP-Hr-AE was given at doses of 0.3, 0.4, and 0.5 mg/kg, respectively. Control mice received no treatment. Figure 5A shows that Ec-LDP-Hr-AE significantly inhibited the growth of SK-OV-3 xenografts. At the dose of 0.3, 0.4, and 0.5 mg/kg Ec-LDP-Hr was given at dose of 1 mg/kg; and Ec-LDP-Hr-AE was given at doses of 0.3, 0.4, and 0.5 mg/kg, respectively. Control mice received no treatment. Figure 5A shows that Ec-LDP-Hr-AE significantly inhibited the growth of SK-OV-3 xenografts. At the dose of 0.3, 0.4, and 0.5 mg/kg Ec-LDP-Hr-AE suppressed the tumor growth by 64.7%, 79.5%, and 91.3%, respectively. Furthermore, the Ec-LDP-Hr-AE–treated group at 0.5 mg/kg showed statistically significant differences (P < 0.01) compared with lidamycin-treated group at 0.05 mg/kg tolerated dose (inhibition rate, 65.6%). Almost no therapeutic benefit was achieved in the group treated with fusion protein Ec-LDP-Hr at 1 mg/kg (inhibition rate, 34.9%) compared with control group. Figure 5B shows that weight loss resulting from the Ec-LDP-Hr-AE treatment at the termination of the
In experiment at different doses did not exceed 10% of the pretreatment weight. Thus, the dosages of Ec-LDP-Hr-AE were tolerated.

In second experiment, SK-OV-3 xenografts were treated with lidamycin, Ec-LDP-Hr-AE, Ec-LDP-AE, and LDP-Hr-AE. LDM was still given at tolerate dose of 0.05 mg/kg; Ec-LDP-Hr-AE, Ec-LDP-AE, and LDP-Hr-AE were given at doses of 0.5, 0.416, and 0.404 mg/kg, respectively. Figure 5C showed that both bispecific and monospecific energized fusion proteins had remarkable inhibitory effect on the growth of SK-OV-3 xenografts. Mice receiving LDM at 0.05 mg/kg showed an inhibition rate of 66.3%. Ec-LDP-Hr-AE, Ec-LDP-AE, and LDP-Hr-AE at equimolar doses suppressed the tumor growth by 90.2%, 83.9%, and 80.3%, respectively. However, the fact that two mice in Ec-LDP-AE group died 3 days after the second injection and one mouse in the LDP-Hr-AE group died 4 days after the second injection, whereas no mouse died in the Ec-LDP-Hr-AE–treated group revealed that monospecific energized fusion proteins were more toxic than that of bispecific fusion protein, indicating that bispecific fusion protein has greater selectivity.

In vivo targeting of Ec-LDP-Hr protein. In vivo distribution of FITC-labeled Ec-LDP-Hr protein was observed in nude mice bearing human ovarian carcinoma xenografts. As shown in Fig. 6,Ec-LDP-Hr protein penetrated into tumors within 1 hour after i.v. injection and then gradually accumulated in the tumors. Such accumulation reached highest within 4 hours after injection and then gradually cleared from the tumor area.

Discussion

Targeting two receptors has been proven to be a promising therapeutic strategy. One possible application for bispecific molecules would be to bind surface receptors on tumor cells in such a way that active dimerization and, thus, cell signaling, is sterically inhibited (29). Another attractive property for bispecific molecules is the capacity for binding two tumor-associated receptors to increase the selectivity by preferably directing the agents to cells expressing both receptors instead of binding to cells expressing only one of these receptors. Bispecific agents would also extend the patient coverage, which is economically
advantageous. Many antitumor molecules targeting dual receptors have been reported (14). DT2219, simultaneously targeting CD19 and CD22 on B cell, was effective in the therapy of systemic B-cell malignancy in a mouse model (30). The antiglioblastoma bispecific cytotoxin DTAT13 was constructed with human interleukin-13 and the NH2-terminal fragment of urokinase-type plasminogen activator fused to DT390 toxin. This cytotoxin did not enhance the activity against MG U873 cells but did show broader spectrum of reactivity to glioblastoma cell lines and unique pharmacokinetics (31). Another bispecific immunotoxin DTEGF13 targeting EGFR and interleukin-13R simultaneously yielded 32- to 2,860-fold greater cytotoxicity to a variety of epithelial cancer cell lines than corresponding monospecific immunotoxins (25). Similar to DTEGF13, the bispecific DTEpCAM23 targeting EpCAM and erbB2, showed significantly greater activity against the two monomeric immunotoxins (27).

These above-mentioned bispecific agents used receptor-specific ligands or single-chain antibodies for targeting. In this study, we tried to use a ligand-based and an antibody-based oligopeptide for receptor binding and an enediyne antibiotic LDM for cell cytotoxicity, which created a new bispecific fusion protein with low molecular weight and potent antitumor activity. Data from immunofluorescence and immunoprecipitation assay revealed that Ec-LDP-Hr protein was able to bind EGFR and HER2 specifically. In cell viability assays, the EGFR-specific Ec-LDP-PE, HER2-specific LDP-Hr-PE, and bispecific Ec-LDP-Hr-PE all displayed potent antitumor activity against a variety of tumor cell lines with IC50 values in the picomolar level. Bispecific Ec-LDP-Hr-PE showed the most potent cytotoxicity against all cell lines, which express both EGFR and HER2 or each receptor alone. However, for EGFR- and HER2-negative OVCAR-3 cells, Ec-LDP-Hr-PE is less cytotoxic than LDM and monospecific fusion proteins, indicating Ec-LDP-Hr-PE was more selective against cancer cells. The in vitro selectivity study also provided evidence for our estimation. In vivo, Ec-LDP-Hr-PE was able to significantly suppress the growth of ovarian cancer cell SK-OV-3 xenografts. At the dose of 0.5 mg/kg, the inhibition rate reaches 91.3%
compared with control group and showed significant difference ($P < 0.01$) to the group treated with LDM at tolerated dose of 0.05 mg/kg. Furthermore, bispecific Ec-LDP-Hr-AE is less toxic than that of monospecific Ec-LDP-AE and LDP-Hr-AE at the equimolar doses.

FACS-based binding study did not reveal a higher affinity for bispecific Ec-LDP-Hr than monospecific agents. Results from the assay SK-OV-3 cells treated with a mixture of equimolar concentration of monospecific Ec-LDP-AE and LDP-Hr-AE did not show additive cytotoxicity, which also approved that binding affinity is irrelevant to cytotoxicity (shown in Supplementary Fig. S1). The internalization efficiency assay revealed no significant difference between bispecific Ec-LDP-Hr and monospecific agents, indicating that enhanced cytotoxicity of bispecific Ec-LDP-Hr-AE cannot be attribute to enhance internalization.

To clarify the function of bispecific Ec-LDP-Hr-AE, propidium iodide staining and Annexin V/PI staining studies were done to examine the cell cycle arrest and apoptosis. The results showed that Ec-LDP-Hr-AE induced G2-M arrest and apoptosis of SK-OV-3 cells dose dependently. The apoptotic SK-OV-3 cells after exposure to 0.1, 0.5, and 1 nmol/L Ec-LDP-Hr-AE were 24.73 ± 3.67%, 38.97 ± 2.55%, and 50.13 ± 4.32%, respectively (shown in Supplementary Figs. S3 and S4). Ec-LDP-Hr-AE induces cell cycle arrest and apoptosis by regulating the activities of EGFR and HER2 signaling pathways. It inhibits the proliferation of SK-OV-3 cells by increasing the phosphorylation of EGFR and HER2 and then activating extracellular signal-regulated kinase and c-Jun-NH2-kinase, which is quite different from lapatinib. Lapatinib has been reported to reduce the activation of EGFR/HER2 and extracellular signal-regulated kinase, which result in growth inhibition (32). The decreased activity of Akt and upregulated p27 expression after being treated with Ec-LDP-Hr-AE were also responsible for cell apoptosis and cell cycle arrest (shown in Supplementary Fig. S5). Binding of Ec-LDP-Hr-AE led to the activation of both EGFR and HER2 tyrosine kinases, which may facilitate receptor dimerization and internalization, accelerating the intrinsic turnover rate of targeted receptors, and might account for the increased cytotoxic activity of Ec-LDP-Hr-AE compared with the monospecific agents (33). In addition, the ability of the bispecific Ec-LDP-Hr binding to EGFR or HER2, or both, might make it less likely to dissociate from the cell surface, thereby increasing the chances for its internalization.

The bispecific Ec-LDP-Hr-AE has a molecular weight of 18.4 kDa, which is the smallest bispecific fusion protein that have antitumor efficacy in vivo ever reported. Recognition and targeting moiety of this fusion protein was composed of two oligopeptides. The “warhead” moiety LDM (~11 kDa) was much smaller than conventional toxins used in the construction of immunotoxins, such as truncated Pseudomonas exotoxin (PE40, 40 kDa), ricin A (32 kDa), and diphtheria toxin (DT, 38 kDa; refs. 34–36). The smaller size would offer Ec-LDP-Hr-AE better solid tumor penetration and lower immunogenicity.

In summary, our research shows for the first time that a ligand-based and antibody-based bispecific fusion protein energized with enediyne antibiotic not only showed potent cytotoxicity to a variety of carcinoma cells in vitro but also was highly effective in inhibiting the growth of SK-OV-3 xenografts in vivo. These properties, together with its much smaller molecular size, suggested that Ec-LDP-Hr-AE would be a promising candidate for targeted cancer therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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