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ORIGINAL ARTICLE

Site-specific PEGylation of lidamycin and its antitumor activity

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KEY WORDS

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Abstract In this study, N-terminal site-specific mono-PEGylation of the recombinant lidamycin apoprotein (rLDP) of lidamycin (LDM) was prepared using a polyethyleneglycol (PEG) derivative (M_w 20 kDa) through a reactive terminal aldehyde group under weak acidic conditions (pH 5.5). The biochemical properties of mPEG-rLDP-AE, an enediyne-integrated conjugate, were analyzed by SDS-PAGE, RP-HPLC, SEC-HPLC and MALDI-TOF. Meanwhile, *in vitro* and *in vivo* antitumor activity of mPEG-rLDP-AE was evaluated by MTT assays and in xenograft model. The results indicated that mPEG-rLDP-AE showed significant antitumor activity both *in vitro* and *in vivo*. After PEGylation, mPEG-rLDP still retained the binding capability to the enediyne AE and presented the physicochemical characteristics similar to that of native LDP. It is of interest that the PEGylation did not diminish the antitumor efficacy of LDM, implying the possibility that this derivative may function as a payload to deliver novel tumor-targeted drugs.

Abbreviations: ADCs, antibody drug conjugates; AE, active enediyne; Anti-TNF Fab′, anti-tumor necrosis factor Fab′; DMSO, dimethyl sulfoxide; G-CSF, granulocyte colony stimulating factor; IC_{50} values, half-inhibitory concentrations; IFN, interferon; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; LDM, lidamycin; mPEG-ALD, methoxy-PEG-propionaldehyde; PEG, polyethyleneglycol; rhG-CSF, recombinant human granulocyte colony stimulating factor; rLDP, recombinant lidamycin apoprotein; SEC-HPLC, size-exclusion high-performance liquid chromatography

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1. Introduction

Lidamycin (LDM, also called C-1027) is a chromoprotein enediyne antitumor antibiotic produced by *Streptomyces globisporus* C-1027 which was found by screening with spermatogonial assay. This compound consists of a nine-membered cyclic active enediyne (AE) chromophore and a noncovalent bound apoprotein (LDP)[1–3]. Meanwhile, AE and LDP can be dissociated and reconstituted *in vitro*[4]. LDM can induce DNA single-strand breaks and double-strand breaks[5,6], generating free radicals that abstract hydrogen atoms from the backbone of DNA while undergoing a Bergman cycloaromatization reaction[7]. LDM shows extremely potent cytotoxicity toward cultured cancer cells with nanomolar IC_{50} values and displays high efficacy against a variety of murine transplantable tumors and human cancer xenografts, including glioma, multiple myeloma, and the cancers of breast, pancreas, colon, lung, stomach and liver[8]. Furthermore, LDM is highly active against multidrug-resistant cancer cells[9]. With potencies several orders of magnitude higher than conventional chemotherapeutics, LDM showed exceptional promise as new payload in the study of antitumor therapeutic agents.

PEGylation, representing the covalent linkage of polyethyleneglycol (PEG) to accessible amino residues of bioactive substances, has become a major approach for overcoming most of the biologics' limits, such as fast degradation by proteases, glomerular filtration in the kidney and antigenic response. The efficacy of PEGylation is evidenced by the clinical efficacy of many PEGylated proteins that are on the market or in late-stage clinical trials[10–12]. The pharmacological properties of many protein therapeutics, including interferon (IFN), granulocyte colony stimulating factor (G-CSF), anti-tumor necrosis factor Fab′ (anti-TNF Fab′) and uricase, can be improved by modification of the proteins with PEG derivatives[12]. Considering the versatile PEGylated strategies in development, N-terminal site-specific PEGylation is increasingly dominating the development of mono-PEGylated protein[13], based on the fact that primary amine residues in protein have different pK_a 7.8 for the N-terminal α-amino group and 10.1 for the ε-amino group in lysine residues[14]. For example, Neulasta®, produced by the attachment of a 20 kDa mPEG ALD molecule to the α-amino group of the N-terminal methionine residue of rhG-CSF, has already been successful in clinical use[15].

In our previous work, recombinant LDP (rLDP) has been successfully constructed and expressed in *Escherichia coli* through recombinant DNA technology, which has similar activity to that of native LDP. In this study, we PEGylated the N-terminus of rLDP in a site-specific manner using mPEG ALD derivative (M_w 20 kDa). The site-specific PEGylated rLDP was characterized by various analytical methods and then assembled with AE, which was separated from LDM, to form an enediyne-energized analog, PEG-rLDP-AE. *In vitro*, the cytotoxicity of mPEG-rLDP-AE against several cancer cell lines was evaluated by MTT assays, and *in vivo* antitumor efficacy was assessed using the nude mouse xenograft model.

2. Materials and methods

2.1. Chemicals and cell culture

LDM, preserved by our laboratory, was isolated from *S. globisporus* C-1027. The mPEG-propionaldehyde (20 kDa) was purchased from Beijing Kaizheng Biotech Development Co., Ltd., and HPLC grade acetonitrile and formic acid were purchased from Thermo Fisher Scientific, Inc. Other chemicals were purchased from J & K Scientific Ltd.

MCF-7 (human mammary adenocarcinoma) cells, PG-BE1 (human lung giant cell carcinoma) cells, SW1990 (human pancreas adenocarcinoma) cells and HepG2 (human liver carcinoma) cells were grown in RPMI 1640 medium supplemented with 10% fetal newborn bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, and cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Expression of rLDP

As previously reported[16], 1 L Luria-Bertani (LB) medium containing 50 mg/L kanamycin was inoculated with 50 mL of a starter culture that contained an overnight culture of the rLDP-expressing strain. The inoculated medium was then maintained at 37 °C with shaking at 220 rpm, and recombinant protein expression was induced with isopropyl-β-D-thiogalactoside (IPTG) at an optical density at 600 nm of 1.0. After 8 h of induction, the culture was harvested by centrifugation, and the periplasmic fractions of the collected culture were prepared by the osmotic shock method and purified with a Ni^{2+} affinity column.

2.3. Preparation of mPEG-rLDP

The mPEG-propionaldehyde derivative (M_w 20 kDa) and rLDP (molar ratio = 5:1) were dissolved in sodium acetate buffer (50 mmol/L, pH 5.5) in the presence of sodium borohydride (2.5 mmol/L) as a reducing agent. The PEGylation reaction was conducted for 24 h at room temperature and the excess reducing agent was removed by repeated ultrafiltration using a centrifugal filter with MWCO 10 kDa (Merck Millipore, USA), followed by lyophilization with an Alpha 1-4LD Plus freeze dryer (Martin Christ, Germany) to give crude mPEG-rLDP.

2.4. Purification and characterization of mPEG-rLDP and its enediyne-energized analog

The crude mixture of mPEG-rLDP and excess mPEG-propionaldehyde derivative was loaded onto a G75 sephadex gel filtration column pre-equilibrated with 20 mmol/L sodium acetate, pH 5.5, at a flow rate of 1.0 mL/min. The fractions were analyzed by size-exclusion high-performance liquid chromatography (SEC-HPLC, Agilent 1200 series) equipped with a manual injector on a TSK G2000SW_{XL} gel filtration column (300 mm × 7.8 mm, 5 mm particle size) (Tosoh, Tokyo, Japan); 20 μL of the sample was loaded into the column; an isocratic mobile phase with 20 mmol/L PBS, pH 7.4; a flow rate of 0.8 mL/min; UV detection at 280 nm. The fractions containing mPEG-rLDP were collected, pooled, desalted and lyophilized to produce the aimed mPEG-rLDP and then the samples were stored at −20 °C.

The active AE was separated from LDM by using C4 pre-column (250 mm × 10 mm, 5 mm particle size) (Phenomenex, Torrance, USA) with a 22% acetonitrile in 0.025% trifluoroactic acid mobile phase. The AE solution was added to PBS (10 mmol/L, pH 7.4) containing mPEG-rLDP, with the molecular ratio of 3:1, and was incubated at 4 °C for 12 h while shaking. Free AE was removed by repeated ultrafiltration using a centrifugal filter with MWCO (1000 Da). The assembled enediyne-energized mPEG-rLDP-AE was confirmed by HPLC equipped with a Jupiter C4 column

(250 mm × 4.6 mm, 5 mm particle size) (Phenomenex, Torrance, USA). Absorbance at 340 nm and 214 nm was measured.

2.5. *Cytotoxicity assay*

Antiproliferative effect of mPEG-rLDP-AE was evaluated using MTT assay. Adherent cells were seeded into 96-well plates (Costar, Cambridge, Mass.) at (4–5) × 10^3 cells/well, depending on the cell lines, in 180 μL of medium and incubated for 24 h. Cells were treated with various concentrations of mPEG-rLDP-AE in 200 μL of medium for 48 h. After the treatment periods, the supernatant medium was decanted and 20 μL of 5 mg/mL MTT in PBS was added. Then cells were incubated for another 4 h. Finally, culture medium was removed from each well, and formazan was dissolved with 150 μL of dimethyl sulfoxide (DMSO). The absorbance was quantitated by a Microplate Reader (Multiskan MK3, Thermo Labsystem, USA) at 570 nm. Survival ratio was calculated according to the following formula:
Survival ratio (%) = $(A_{test} - A_{blank})/(A_{control} - A_{blank}) \times 100$.

2.6. *In vivo antitumor efficacy*

The PG-BE1 human lung cancer cell line was grown as a subcutaneous xenograft in female athymic nude mice (*nu/nu*), 6–8 weeks old, by injecting 5 × 10^6 cells in each flank. Mice with established tumors corresponding to a mean volume of 2–3 mm^3 in diameter were randomized into groups (6 animals each) in a manner that minimized the difference in tumor size between the groups. Each animal received vehicle control, LDM, mPEG-rLDP and mPEG-rLDP-AE, respectively by intravenous injection once every two weeks. The doses of mPEG-rLDP-AE were 0.075, 0.15 and 0.3 mg/kg, respectively. The mice were weighed and tumor sizes were measured with a vernier caliper and recorded twice a week. Tumor volume was calculated using the formula: $a \times b^2/2$, where a was the longer diameter and b was the perpendicular shorter diameter.

2.7. *Statistical analysis*

Data were expressed as mean ± standard error ($\overline{X} \pm SE$). Statistical analysis was performed using SPSS. Statistical significance was assessed by the Student's *t*-test, and *P* values less than 0.05 were considered to be statistically significant.

3. Result

3.1. *Preparation and characterization of rLDP*

The DNA fragment encoding rLDP was cloned and inserted into the pET30a expression vector. SDS-PAGE was used to detect the expression of fusion proteins. The results showed that fusion protein rLDP was expressed (Fig. 1A). The rLDP was purified using immobilized metal-affinity chromatography resin under denaturing conditions and the target rLDP was obtained in over 95% purity (Fig. 1B). The rLDP, an *E. coli*–expressed $(His)_6$-tagged protein, with a molecular mass of approximately 11.7 kDa is determined by MALDI-TOF mass spectrometry (Fig. 1C). The rLDP with confirmed physicochemical characteristics and the

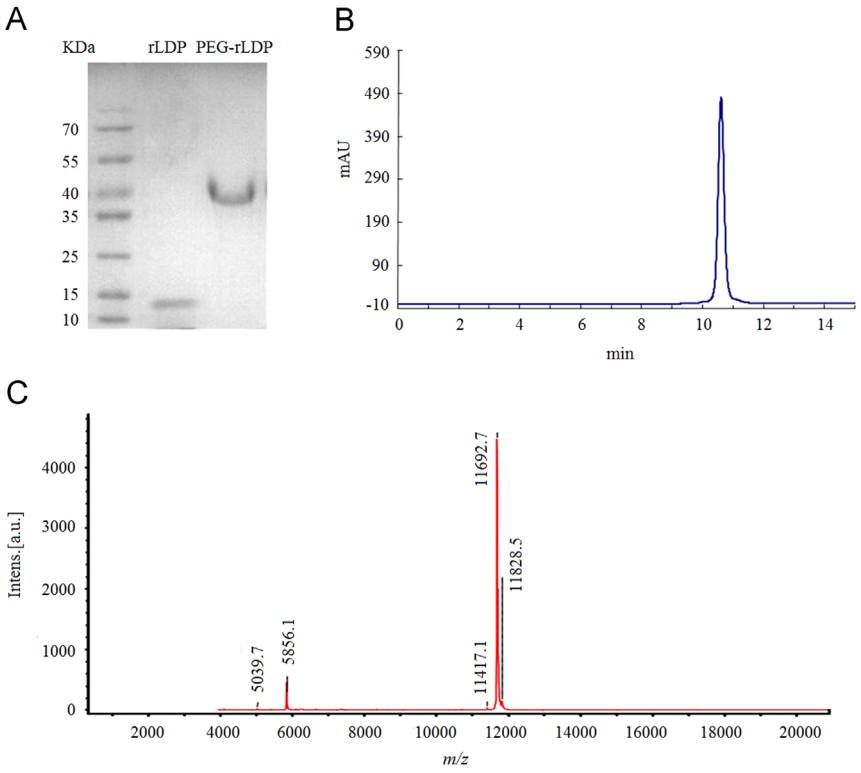

Figure 1 The characterization of rLDP. (A) SDS-PAGE analysis of the mPEG-rLDP and rLDP samples stained with Coomassie Blue. (B) SEC-HPLC analysis of rLDP was performed on a TSK G2000SW_{XL} column at ambient temperature. The mobile phase was 20 mmol/L sodium phosphate (pH 7.4) with a flow rate of 0.8 mL/min and a detection wavelength of 280 nm. (C) MALDI-TOF spectra of rLDP. The molecular weight of rLDP was 11,692 Da.

affinity binding to AE had similar activity to that of native LDP (Fig. 2A).

3.2. PEGylated rLDP and its enediyne-energized analog

The native LDP has 110 amino acids and two disulfide bonds. There are many potential PEGylation sites in rLDP including several ε-amine groups of lysine and one α-amino group at the N-terminus. The methoxy-PEG-ALD (M_w 20 kDa) was conjugated under acidic conditions (pH 5.5) for 24 h, and the conjugated mixture was analyzed by SEC-HPLC on a TSK G2000SW$_{XL}$ gel filtration column (Fig. 2A). Chromatography results showed that mono-PEGylated rLDP was predominantly produced when using PEG-propionaldehyde derivative. It was also noted that mono-PEGylated rLDP could be readily separated from the unPEGylated rLDP fraction by a SEC gel filtration column (Fig. 2B). Use of the G75 sephadex gel filtration column was sufficient to successfully purify the mono-PEGylated rLDP fraction. The unconjugated, activated mPEG-ALD and rLDP were removed by ongoing chromatographic separation processes.

To estimate the molecular weight distribution of PEGylated rLDP, MALDI-TOF mass spectrometry was used to further confirm the SEC-HPLC results (Fig. 2C). Mass value is expressed as M_p (the most probable mass peak) for the mPEG-rLDP. The measured mass-to-charge ratio was 33,231 m/z (16,607 $m/2z$) for mPEG-rLDP without showing any other mass peaks. Considering the molecular weights of rLDP (11.7 kDa) and PEGs (20 kDa), the obtained mass of the conjugate was very consistent with the calculated mass sum of individual molecules.

The energized mPEG-rLDP-AE was prepared by integrating AE molecule of LDM into mPEG-rLDP conjugate. Data from reverse-phase HPLC showed that AE molecule was successfully integrated into mPEG-rLDP (Fig. 2D), which implies that mPEG-rLDP keeps native LDP structure in the PEGylation modified product.

3.3. In vitro cytotoxicity of mPEG-rLDP-AE

The cytotoxicity of mPEG-rLDP-AE and LDM against four different cell lines was determined using MTT assays. As shown in Table 1, both unmodified LDM and PEGylated LDM (mPEG-rLDP-AE) showed highly potent growth inhibitory effects, judging from their half-inhibitory concentrations (IC_{50} values). The unmodified LDM showed IC_{50} values ranging from 0.1 nmol/L to 4.2 nmol/L, while the IC_{50} values ranged from 0.03 nmol/L to 1 nmol/L for the PEGylated LDM. As shown in Figs. 1A and B, and 2A, mPEG-rLDP still retained the binding capability to AE and presented similar physicochemical characteristics to that of native LDP. Furthermore, the PEGylation did not diminish the antitumor efficacy of LDM.

3.4. Therapeutic efficacy of mPEG-rLDP-AE

The antitumor activity of mPEG-rLDP-AE was investigated in nude mice with an established human lung cancer PG-BE1 xenograft. Tumor-bearing athymic mice were treated with LDM, mPEG-rLDP-AE and mPEG-rLDP. LDM at the tolerated dose of 0.05 mg/kg showed a tumor inhibitory rate of 76.9%. The mPEG-rLDP-AE at 0.3 mg/kg suppressed the tumor growth by 82.8%, while mPEG-rLDP at 20 mg/kg did not show significant therapeutic efficacy (Fig. 3A). No obvious toxic effects were observed in all groups during the experiment. These results suggested that both mPEG-rLDP-AE and LDM could retard the growth of PG-BE1 xenografts significantly in nude mice. Body weight loss resulted from the enediyne energized mPEG-rLDP-AE

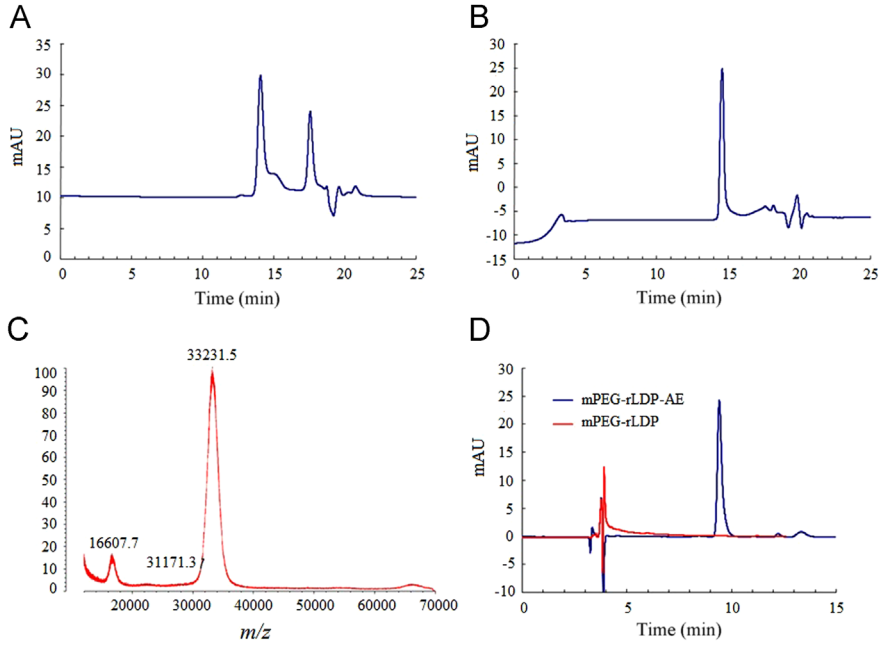

Figure 2 The physicochemical characters of mPEG-rLDP. (A) SEC-HPLC analysis of mPEG and rLDP conjugated mixture was performed on a TSK G2000SW$_{XL}$ column at ambient temperature. The mobile phase was 20 mmol/L sodium phosphate (pH 7.4) with a flow rate of 0.8 mL/min and a detection wavelength of 280 nm. (B) SEC-HPLC analysis of mPEG-rLDP after purification. (C) MALDI-TOF spectra of mPEG-rLDP. The molecular weight of mPEG-rLDP was 33,231 Da. (D) Analysis of mPEG-rLDP and mPEG-rLDP-AE determined by RP-HPLC on C4 300A column at 340 nm.

Table 1 Determined IC_{50} (nmol/L)[a] values on different cancer cell lines.

Cell line	HT-1080	SW1990	MCF-7	PG-BE1	HepG2
LDM	0.98±0.03	0.54±0.05	0.42±0.05	0.56±0.05	0.09±0.04
mPEG-rLDP-AE	0.55±0.08	0.39±0.06	0.11±0.02	0.25±0.07	0.03±0.01

[a]Data are expressed as mean±SD, $n=6$.

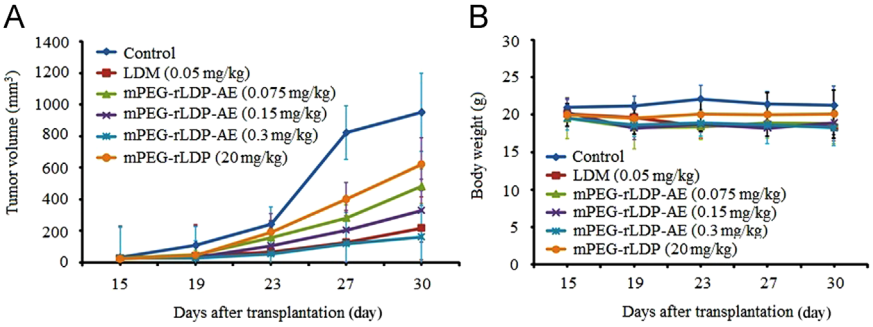

Figure 3 *In vivo* antitumor effect of mPEG-rLDP-AE on transplanted tumor. (A) Changes in tumor volume ($P<0.05$ *vs.* Control); (B) changes in body weight of human lung cancer cell PG-BE1 xenograft-bearing mice.

treatment in each group was also measured at the termination of the experiment, in which all groups except mPEG-rLDP-AE treated group did not exceed 10% of the pretreatment weights (Fig. 3B). No deaths were found in any of the treated groups.

4. Discussion

Based on our previous reports that the rLDP has been successfully expressed in *E. coli* and shows the binding activity similar to that of native LDP, the present study developed a simple preparation method for N-terminal site-specific mono-PEGylated LDM which still retains its highly potent cytotoxicity. In this study, the PEG derivative was a methoxy-PEG-ALD having the average M_w of 20 kDa. The RP-HPLC result in Fig. 2 and antiproliferative effect of mPEG-rLDP-AE suggest that N-terminal PEGylation did not hinder the binding capacity of AE to rLDP, because the N-terminal residue is located far from the AE binding pocket in rLDP. The conjugation of mPEG-ALD to the primary amine groups of rLDP depends on the nucleophilic substitution reaction, with the attacking of unprotonated amine group to the carbonyl groups of aldehyde. Under basic conditions, all α- and ε-primary amines in rLDP were equally reactive to the aldehyde. A heterogeneous mixture of multi-site PEGylated rLDP may also be produced. In contrast, under acidic conditions, unprotonated N-terminal α-amine group (pK_a value: 7–8) is more reactive than ε-amine groups (pK_a value: 10–11) in lysine residues, which possibly resulted in the site-specific mono-PEGylated rLDP. This reductive amination reaction with the N-terminal α-amine group, in the presence of reducing agent, has also been reported in other therapeutic protein PEGylated modification[17,18]. After the simple purification steps of G75 sephadex gel filtration column, the purified mPEG-rLDP could be obtained by removing free mPEG-ALD and unmodified rLDP as evidenced by the SDS-PAGE, SEC-HPLC and MALDI-TOF analysis. The content of rLDP protein in the mPEG-rLDP was measured by UV 280 nm. These results not only indicated that mPEG-rLDP was pure, but also adequately revealed that the molecular weights were in good agreement with the rLDP conjugate containing a single 20 kDa PEG derivative.

LDM is a highly potent antitumor antibiotic composed of LDP and AE. Considering the extremely potent DNA-breaking activity, LDM could be the attractive payload molecule for preparation of tumor-targeted therapeutics, such as antibody drug conjugates (ADCs), nanomedicines and target-active fusion proteins. Indeed, several antibody-LDM conjugates, polymer conjugates, fusion proteins and ligand oligopeptide-integrated fusion proteins have been manufactured in previous reports, such as monoclonal antibody-LDM conjugate (3G11-LDM), dextran-LDM conjugate (Dex-rLDP-AE), bispecific enediyne-energized fusion protein (Ec-LDP-Hr-AE) and tandem scFv-based enediyne-energized fusion protein (dFv-LDP-AE)[16,19–21]. All these LDM-based tumor-targeted drugs possessed highly potent cytotoxicity to cancer cells and significant antitumor efficacy *in vivo*. The present results indicated that mPEG-rLDP-AE (namely, mPEG-LDM) shows significant *in vitro* and *in vivo* antitumor activity. Interestingly, unlike other proteins that are generally inactivated after PEGylation, the conjugate mPEG-rLDP still retains the binding capability to AE and demonstrates the physicochemical characters similar to that of native LDP. Therefore, the PEGylation did not diminish the antitumor efficacy of LDM and also showed the possibility as payload to develop novel tumor-targeted drugs.

5. Conclusions

In this study, mPEG-ALD was specifically conjugated at the N-terminal α-amine group of rLDP in a weakly acidic environment. The mPEG-rLDP still retained the AE-binding capacity and mPEG-rLDP-AE showed highly potent cancer cells growth inhibitory effects at nanomolar level. Meanwhile, the *in vivo* antitumor activity of mPEG-rLDP-AE, investigated in nude mice with established human lung cancer PG-BE1 xenograft, also remained after modification of the LDP with PEG. The results

suggested that PEGylation of rLDP could be a useful approach to develop novel tumor-targeted drugs based on lidamycin.

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