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Full Length Research Paper

PEGylation of α-momorcharin retained its anti-tumor activity with reduced potency *in vitro* and *in vivo*

Bo Leng and Yanfa Meng*

Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education/Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, College of Life Science, Sichuan University, Chengdu 610064, China.

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α-Momorcharin (α-MMC) is the ribosome inactivating protein (RIPs) found to possess antitumor activity. However, acute toxicity and short plasma half-life were the major side effects preventing further clinical trial. To overcome this, α-MMC was further coupled to polyethylene glycol with di-molecular size of 20 kDa (PEG) using the cysteine residues, namely α-MMC-PEG. The median effect concentrations (EC_{50}) were estimated as 100 and 130 µg, for α-MMC and α-MMC-PEG *in vitro*, respectively. Results show that the anti-tumor activity of α-MMC-PEG decreased by about 30% *in vitro*. This sensitivity increase of 50% from α-MMC to α-MMC-PEG reached 5 µg and was kept at 4°C for three months, and the thermal and pH-stability of the α-MMC-PEG was also strengthened. The *in vivo* study showed that the anti-tumor activity of pegylated α-MMC enhanced the activity of anti-tumor activity as compared to natural α-MMC. These results suggest that α-MMC-PEG may be useful for the therapy of tumor.

Key words: α-Momorcharin, acute toxicity, DNA glycosylase, N-glycosidase, stability.

INTRODUCTION

Pegylation was first described in the 1970s by Davies and Abuchowsky, and reported in two key papers on albumin and catalase modification (Veronese and Pasut, 2005). The effect of polyethylene glycol (PEG) in the stability of protein has been extensively studied during the last decade. The protective effect of PEG layers is believed to prevent protein adsorption on the RIPs. The covalent attachment of PEG molecules to pharmaceutical proteins can mitigate factors that adversely affect therapeutic effectiveness, including susceptibility to enzymatic degradation, short circulation time, low solubility and immunogenicity (Cisneros-Ruiz et al., 2009). So, chemical attachment of PEG to therapeutic proteins bestows several benefits such as, enhanced plasma half-life, decreased toxicity, increased drug stability and solubility (Roberts et al., 2002), despite a reduction in the *in vitro* activities (Wang et al., 2004). The importance of chemistry and quality of PEG reagents for peptide and protein modification has only been realized in the last several years as more PEG-conjugates have reached late phase clinical trials (Roberts et al., 2002).

α-Momorcharin is a type I ribosome-inactivating protein (RIPs) isolated from the bitter gourd, *Momordica charantia* (Leung et al., 1997). This protein possesses many biological and pharmacological activities, including antitumor, immunosuppression and antivirus (Xiong et al., 2009). Clinical trials have been performed to evaluate its activity and safety on patients, with human lymphocytes and human leukemic cells (Takemoto et al., 1982a, b). However, there were shortcomings that hindered further development of α-MMC into an antitumor therapeutic agent. Amongst the side effects are immunogenicity and short plasma half-life (Fontana et al., 2009). α-MMC is administered daily for most oncology indications. It has been speculated that a slow-releasing, long-acting formulation of interferon, with improved pharmacokinetics may provide improved tolerability and convenience and may demonstrate activity in patients with known α-MMC resistance. This is undesirable and frequent administration is required to maintain an effective therapeutic

*Corresponding author. E-mail: yanfameng506@yahoo.com.cn. Fax: 028-85412571.

concentration *in vivo* (Wang et al., 2004).

With the recent advance in structural genomics and pharmacoproteomics, many newly identified bioactive proteins are generally quite unstable *in vivo* (Wang et al., 2004). To overcome this problem, the most common polymer used is conjugated with some water-soluble polymers like polyethylene glycol. This was an important milestone because at that time, it was not conceivable to modify an enzyme so extensively and still maintain its activity (Veronese and Pasut, 2005).

α-Momorcharin was chosen as a model protein as it is well-characterized, and has potential therapeutic application as an antitumor agent. Studies have shown a much milder immunological reaction in rabbits when α-MMC is conjugated with PEG (Bian et al., 2010). The objective of this study was to test the antitumor activity, acute toxicity, cytotoxicity and stability of α-MMC-PEG.

MATERIALS AND METHODS

The α-MMC and α-MMC-PEG (di-PEGylated α-MMCs) were isolated and characterized in our laboratory (Figure 1). The new α-MMC and α-MMC-PEG of optimal concentration was stored at 4°C for three months. Other experiments listed in the text were done with the new prepared α-MMC. The human MDA-MB-231 cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Grand Island, USA), supplemented with 10% heat-inactivated fetal bovine serum,100 mg/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Plasmid pET28a was a product of Promega Corporation, USA, and the human MDA-MB-231 cells were from ATCC. Other reagents were all of analytical grade.

The female BALB/C mice were received 1 week before the experiments and were acclimatized in the animal room. They were housed on aspen bedding (Scanbur BK) in standard type macrolone cages on a 12:12 light/dark cycle at 21 to 23°C.

Cell inoculation and RIPs treatments

Logarithmically growing cells were harvested by scraping in complete culture medium. $3×10^6$ MDA-MB-231 cells in 0.2 ml of culture medium were inoculated subcutaneous (s.c) on the flank immediately caudal to the axilla, through a 22-gauge needle tunneled 1 to 2 cm to prevent leakage of cell inoculum. For each strain of mouse, 80 animals were randomized into 8 groups and treated with anti-tumor by s.c injection of the drugs {high dose (H) 2.5 mg/kg, middle dose (M) 0.5 mg/kg and low dose (L) 0.1 mg/kg, respectively every other day for 10 days}, α-MMC (1 to 3) and α-MMC-PEG (4 to 6) in physiological saline (0.2 ml). Negative controls received physiological saline alone (Group 7). Positive controls received 0.2 ml epirubicin (0.5 mg) (Group 8). Symptoms and time of death were recorded. All animals were treated according to the Norwegian law on research on animals.

Tumor measurement

Tumor diameters were measured at least once every four days, and tumor volume (cumm) was calculated by the formula (Osborne et al., 1985):

Tumor volume = $(\text{width})^2 \times \text{length}/2$

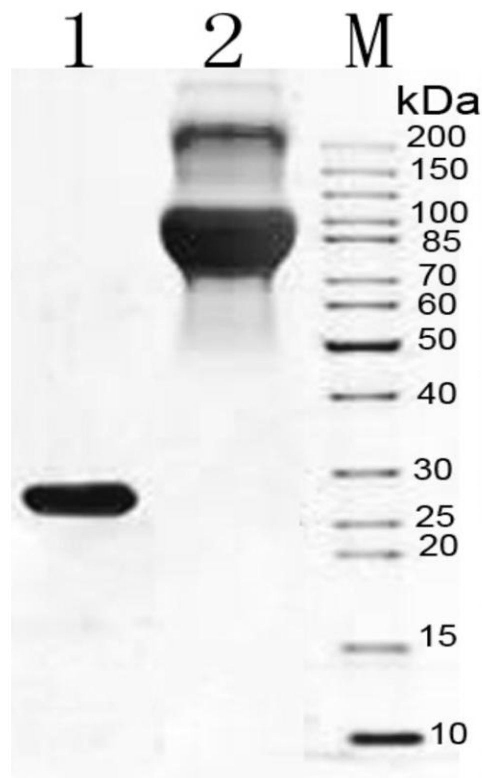

Figure 1. SDS-PAGE assessment of α-MMC and α-MMC-PEG. Lane 1: α-MMC; lane 2: α-MMC-PEG; lane M: HMW markers.

Assays for acute toxicity

For each strain of mouse, 80 animals were randomized into 8 groups and treated with antitumor by s.c. injection of the drug {high dose (H) 2.5 mg/kg, middle dose (M) 0.5 mg/kg and low dose (L) 0.1 mg/kg, respectively}, α-MMC (Groups1 to 3) and α-MMC-PEG (Groups 4 to 6) in physiological saline (0.2 ml). Negative controls received physiological saline alone (Group 7). Positive controls received 0.2 ml epirubicin (0.5 mg) (Group 8). The mice were observed for 2 weeks. Symptoms and time of death were recorded. All animals were treated according to the Norwegian law on research on animals.

Methyl thiazolyl tetrazolium (MTT) assay

Human MDA-MB-231 was inoculated into 96 well plates at a density of 1×10^3 cells/well, 24 h before the treatment. Then, these cells were exposed to the α-MMC and α-MMC-PEG at 1, 10, 100 and 1000 µg for 48 h. For MDA-MB-231 cells, 100 µg α-MMC and α-MMC-PEG was added, followed by 24, 48 and 72 h incubation. After removal of the medium, 20 µl of MTT [3-(4,5-dimethylthiozol-2-yl)-3-(5-dipheryl tetrazolium bromide] (Sigma solution) (5 mg/ml) was added to each well, followed by 4 h incubation. To each well, 100 µl of acidified isobutyl alcohol (40 mM HCl in isopropanol) was added. The OD of each well was read by a microplate spectrophotometer (Model 680, Bio-RAD, Hercules, USA), equipped with a 570 nm filter. Cells without the α-MMC and α-MMC-PEG were used as control. The percentage of inhibition was calculated by the following formula (Li et al., 2009):

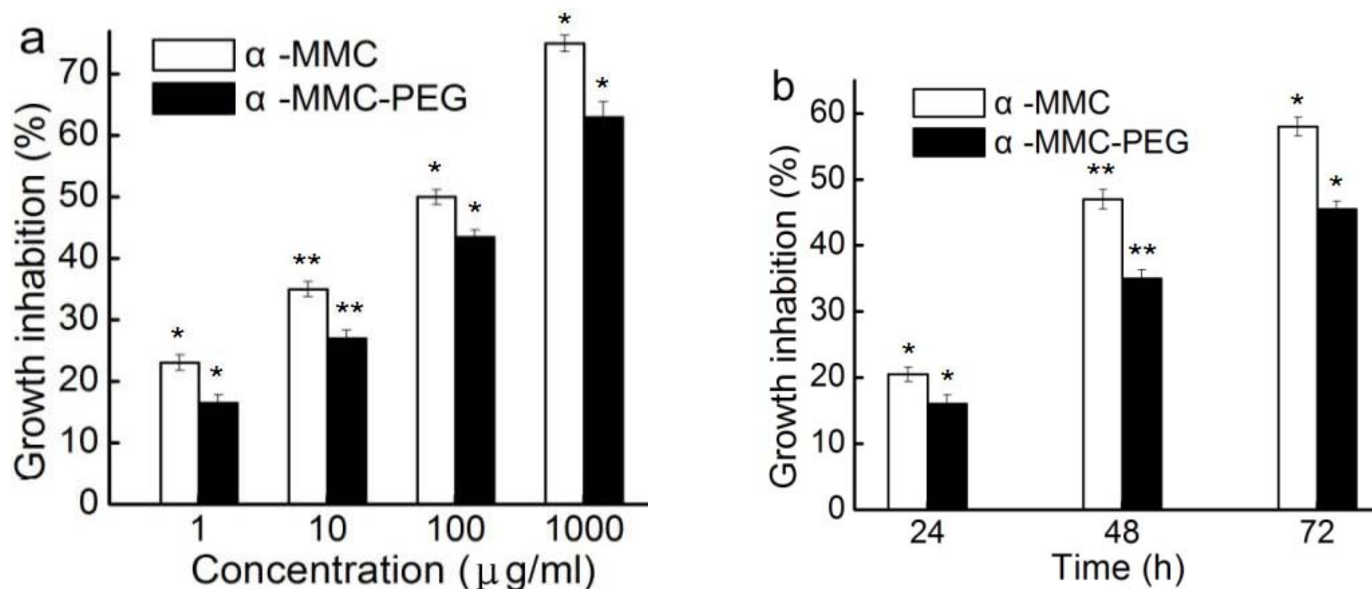

Figure 2. Inhibition effects of α-MMC and α-MMC-PEG on the proliferation of MDA-MB-231 cells. (a) Dose-dependent inhibition of the proliferation of MDA-MB-231 by α-MMC or α-MMC-PEG at different concentrations. (b) Time-dependent effects of the 100 μg α-MMC and α-MMC-PEG on the inhibition of MDA-MB-231. Value represents the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ when compared with the control group.

$$\text{Inhibition (\%)} = \frac{\text{OD570control} - \text{OD570sample}}{\text{OD570control}} \times 100\%$$

N-Glycosidase assay

The human MDA-MB-231 cell line was incubated at 37°C with α-MMC and α-MMC-PEG for 72 h. Total RNA was extracted with phenol/chloroform, recovered by ethanol precipitation, and allowed to react with 1 M aniline/0.8 M acetic acid (pH 4.5) for 5 min at 60°C, prior to electrophoresis in 1.2% agarose gel containing 3.7% formaldehyde, for 20 min at a constant voltage of 150 V. The gel was stained in ethidium bromide (0.5 μg/ml) and destained with distilled water. The RNA bands were visualized on a UV transilluminator and photographed with Polaroid 667 instant film.

DNA glycosidase assay

0.5 μg of supercoiled DNA (pET28a) was incubated with serial amounts of RIPs in a final volume of 10 μl, containing 10 mM Tris/HCl, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.5, at 37°C for 30 min. Electrophoresis was carried out under non-denaturing condition in 0.5 × TAE buffer (40 mM Tris-acetate 1 mM EDTA) in a 1% agarose gel. DNA bands were visualized by staining with ethidium bromide, and the line DNA concentration in agarose gel was measured by using quantity-one software.

Statistical analysis

All assays were repeated at least three times. Statistics were performed by using SPSS Software, and the results were described as: mean value ± standard error (SE) or mean value ± standard deviation (SD).

RESULTS

MTT assay

We examined the effect of α-MMC and α-MMC-PEG on the cell viability of MDA-MB-231 human breast cancer cells, using a conventional tetrazolium-based (MTT) assay. The results shown in Figure 2 reveal a dose and time dependent inhibition of cell viability. After 48 h, α-MMC and α-MMC-PEG inhibited completely the cell viability, with an EC_{50} value of about 100 and 130 μg. This represents a decrease of 30% from α-MMC to α-MMC-PEG. The decreased cell viability could be the result of the cover of PEG to, or near the active site (Bian et al., 2010). Results show that the pegylation of α-MMC retained the high capacity of suppressing tumor cell proliferation *in vitro*.

RNA N-glycosidase activity

RNA N-glycosidase activities of α-MMC and α-MMC-PEG, cleaved from 28S RNA isolated from MDA-MB-231. As shown in Figure 3, aniline treatment of rRNA, extracted from RIPs treated MDA-MB-231cell ribosomes, resulted in generation of a specific RNA fragment (R-fragment), due to its N-glycosidase activity. On the contrary, without RIPs treatment showed no such released fragment. The results show that α-MMC-PEG retained its RNA N-glycosidase activity with reduced potency *in vitro*.

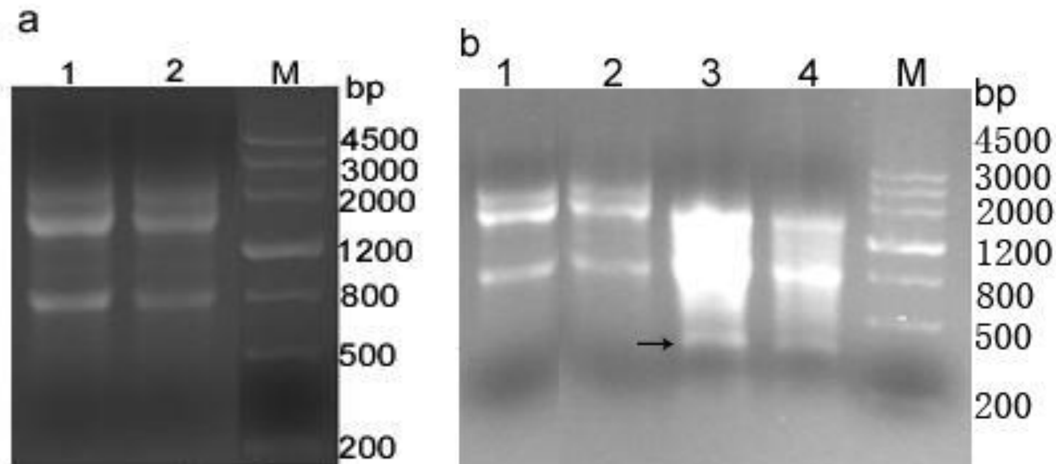

Figure 3. RNA N-glycosidase activity of RIPs assayed by formation of an R-fragment from MDA-MB-231 cells 28S rRNA. (a) Incubation without RIPs, with (lane 1) or without (lane 2) aniline treatment; (b) MDA-MB-231 cells were incubated with 200 µg α-MMC (lanes 1 and 3) and 200 µg α-MMC-PEG (lanes 2 and 4) with (lanes 3 and 4) or without (lanes 1 and 2) aniline treatment. Arrows show the location of the R-fragment.

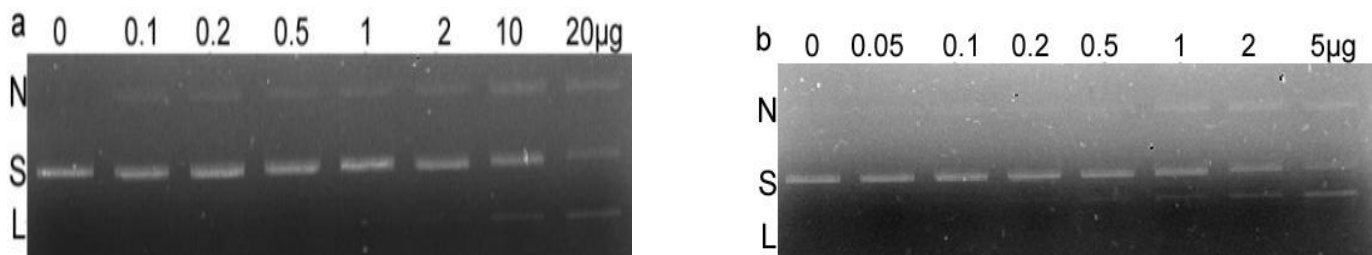

Figure 4. Effect of substrate concentrations (kept at 4°C for three months until used) on the enzymatic activity of the pET28a DNA. pET28a DNA (0.5 µg) were incubated with different concentrations of α-MMC (a) and α-MMC-PEG (b) at 37°C for 30 min (N, nicked DNA; L, linear DNA; S, supercoiled DNA).

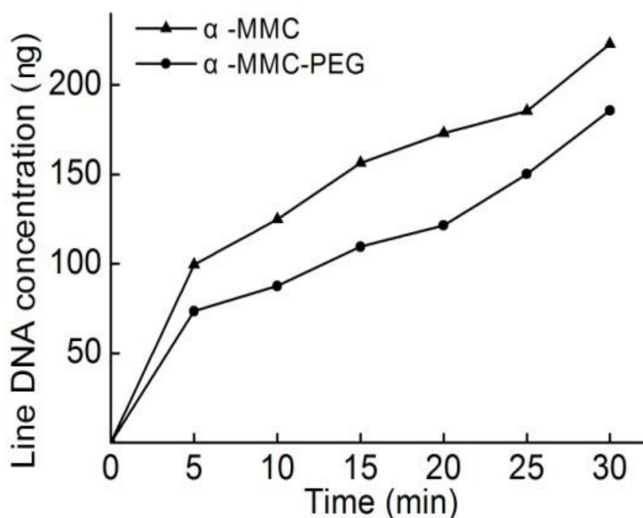

Figure 5. Time-course of line DNA yield with 0.5 µg concentrations of plasmid pET28a DNA. The reaction was performed at 37°C for 30 min in 10 µl of Tris/HCl 10 mM, pH 7.5, 5 mM $MgCl_2$, 50 Mm (NaCl) with α-MMC and α-MMC-PEG of 5 µg.

Effect of substrate concentrations and time on the DNA glycosylase

The optimal concentration of RIPs (kept at 4°C for three months until used) on plasmid pET28a DNA, were 10 and 5 µg α-MMC and α-MMC-PEG, respectively (Figure 4). This represents a decrease of 50% from α-MMC to α-MMC-PEG. Time needed for the line DNA of the cleavage of supercoiled DNA appeared at 15 min, when reaction was performed at 37°C (Figure 5). But in order to get the clear bands of line DNA, we increased the time to 30 min, and the time of later experiments on plasmid pET28a DNA was 30 min. The concentrations of line DNA represent a decrease of 25% from α-MMC to α-MMC-PEG (Figure 4).

Effect of pH and temperature on the DNA glycosylase

The optimal pH and reaction temperature for the DNA glycosylase were 8.0 and 40°C, respectively (Figure 6). A

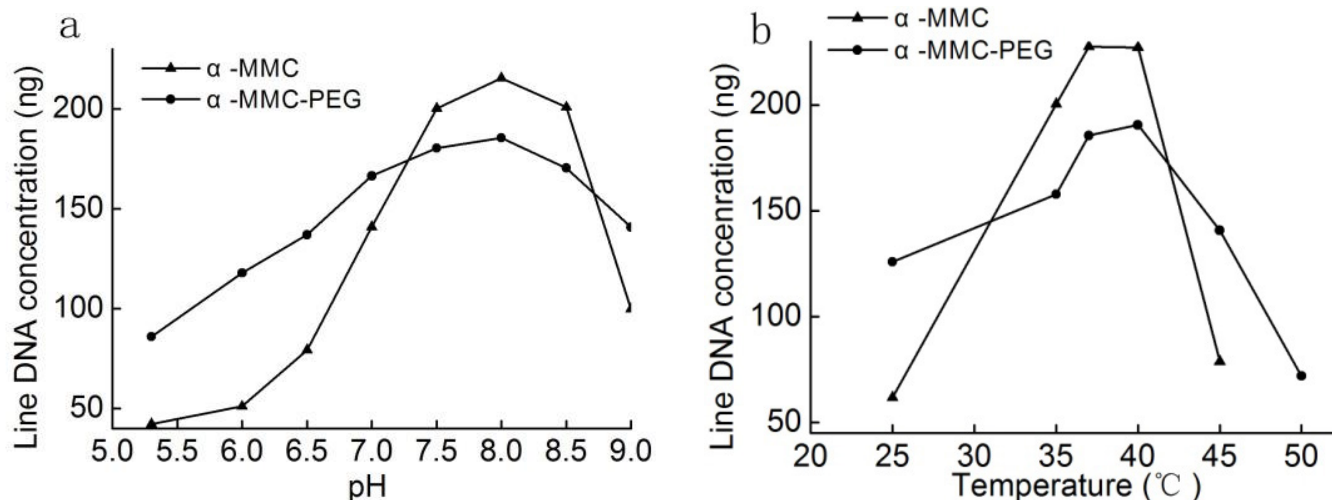

Figure 6. Effect of reaction pH (a) and temperature (b) on the DNA glycosylase activity. Both experiments were carried out with 0.5 µg plasmid pET28a DNA and 5 µg α-MMC and 5 µg α-MMC-PEG. Buffer used in the experiment: 0.1 M sodium citrate buffer (pH 5.3 to 6.0); 0.1 M sodium phosphate buffer (pH 6.0 to 7.5); 0.1 M Tris/HCl buffer (pH 7.0 to 9.0).

working pH of 7.5 was selected for subsequent work for the normal BALB/C mice, and the pH of human was near 7.5 (Figure 6a). The α-MMC enzyme activity was easier to deactivate above 40°C than the α-MMC-PEG (Figure 6b). The effect of the pH value in the test medium on the current measurements of α-MMC and α-MMC-PEG gives a maximum response between pH 7.0 to 8.0 and 6.0 to 9.0, respectively.

Assay for acute toxicity

The amount of weight loss were estimated at 3.32 g for α-MMC (H), 0.8 g for α- MMC-PEG (H) and 3.27 g for epirubicin as the drug treating times increased (Figure 7a). With morphology observations on pathological injuries to liver cells of BALB/C mice in acute toxicity (Figure 7b), we found α-MMC(H) and epirubicin group in BALB/C mice with severe lesion of the liver cells, α-MMC-PEG with minor lesion and negative control group without affect. The results of α-MMC-PEG with lower toxicity and the anticipated results were similar. The α-MMC-PEG had low toxicity in BALB/C mice than α-MMC. As expected, the α-MMC-PEG can reduce toxicity.

In vivo antitumor tests

At the beginning of medication on the 4th day, negative control had a sustainable tumor growth (Figure 8a). The tumor weight was 35.73 (positive control), 36.10, 54.34 and 73.96% (α-MMC), and 25.55, 47.39 and 69.72% (α-MMC-PEG), respectively when compared with the negative controls (Figure 8b). The surface of tumor tissue presented necrosis in the high drug group was different from drug (Figure 8c). The neoplastic volume of the mice significantly reduced. At the same time, the size of the neoplastic volume apparently decreased. Results show that the antitumor activity of α-MMC-PEG was slightly higher than that of α-MMC.

DISCUSSION

α-Momorcharin showed cytotoxicities against choriocarcinoma and melanoma cells (Tsao et al., 1990), and human placental choriocarcinoma and sarcoma (S180) cell lines (Ng et al., 1994). α -Momorcharin enhanced the tumoricidal effect of mouse macrophages on mouse mastocytomal (P815) cells (Ng et al., 1994). Based on these biological activities, researchers are attempting to take advantage of the multi-functions of the RIPs to develop RIP-based drugs for anti-cancers. Although, α-MMC is well known to be effective in inhibiting tumor cell proliferation, its side effect is also very significant at the same time. The major ones include antigenicity and short plasma half-life. All these factors could be addressed by PEGylation which will reduce antigenicity as well as improve plasma half-life. Our results show that the α-MMC or α-MMC-PEG suppressed human MDA-MB-231 cell proliferation in a time and dose dependent manner. Under the same concentration, the inhibition rate of α-MMC-PEG for human MDA-MB-231 is about 70% of α-MMC. The antitumor activity and acute toxicity of the α-MMC-PEG were tested, and there was a substantial drop in tumor activity but smaller decrease in cytotoxicity. Causes of the decrease can be viewed from two areas. First, the conjugate is much larger; this sterically affected the active site, and reduced the ribosome inactivating activity that contributed to the anti-

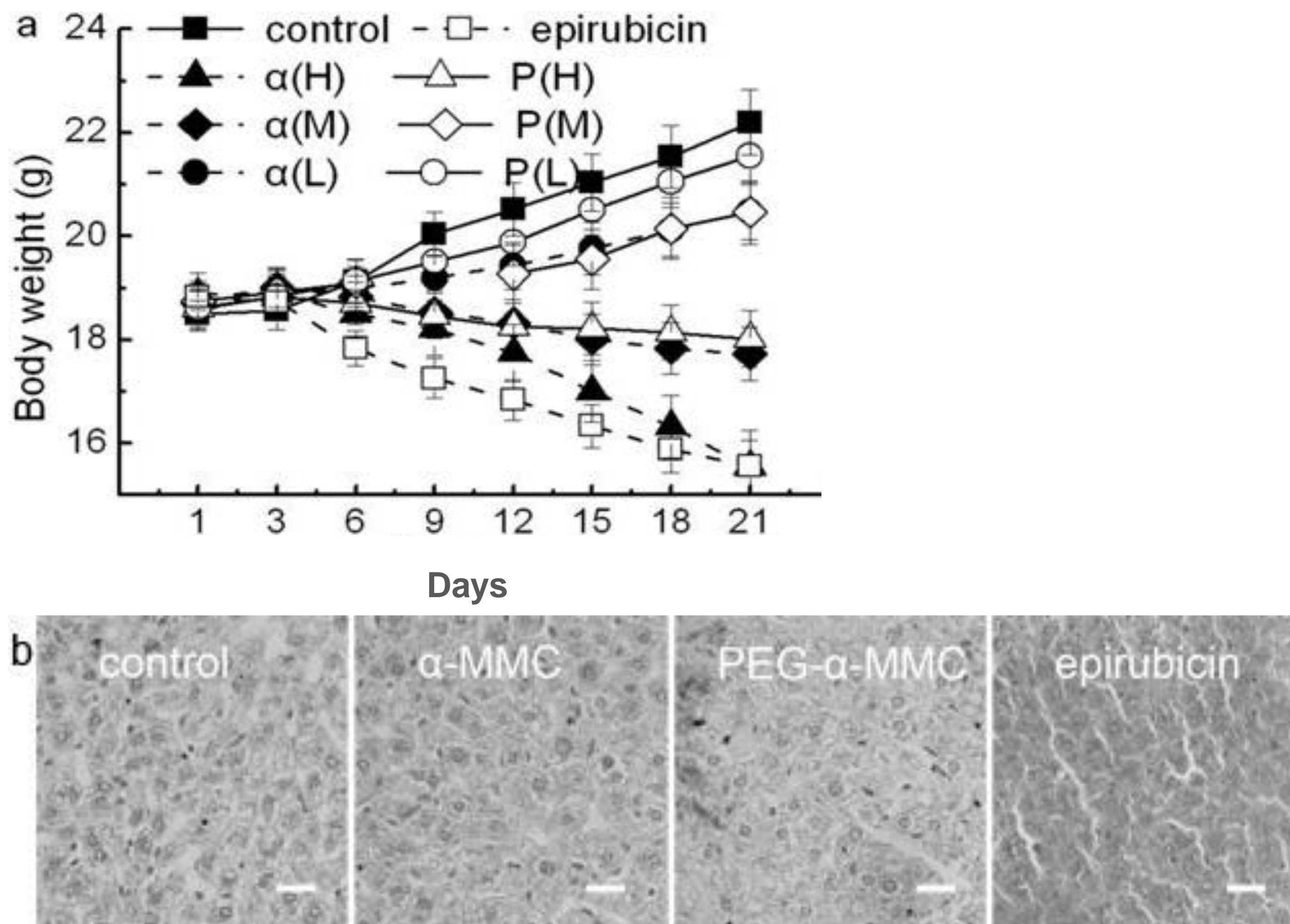

Figure 7. Assay for acute toxicity. (a) Dose and time-dependent effects of the drugs on the inhibition of the body weight of mice on the flank immediately caudal to the axilla. (α(H), α-MMC high dose; α(M), α-MMC middle dose; α(L), α-MMC low dose; P(H), α-MMC-PEG high dose; P(M), middle dose; P(L), α-MMC-PEG low dose; control, negative control; epirubicin, positive control). (High dose, (H) 2.5 mg/kg; middle dose, (M) 0.5 mg/kg; low dose, (L) 0.1 mg/kg). Value represents the mean ± SD of three independent experiments. Each point represents the mean value ± (SD) deviation obtained from 10 animals.

tumor activity. This was supported by the parallel decrease in ribosome activity and anti-HIV activity. Second, the larger α-MMC conjugate may affect its entry into cells.

A longer half-life for α-MMC is desirable because the plasma can thus maintain a higher concentration for clinical effectiveness. It is well known that conjugation of α-MMC with $mPEG_2$ can improve thermostability and stability, due to the beneficial effects from the mPEG chains. It is reasonable to deduce that such beneficial effects may also lead to increment of *in vitro* half-life (Zhang et al., 2004). The DNA glycosylase activity of α-MMC-PEG on plasmid pET28a DNA was increased greatly, as compared to natural α-MMC, and the half-life was increased by about 2-fold after storage at 4°C for three months. Our experimental results revealed that the Thermal and pH-stability of the α-MMC-PEG was also strengthened.

Nonspecific interactions and *in vivo* uptake of α-MMC can be minimized when methoxy polyethylene glycol is anchored to their surfaces. The reduced biological recognition leads to prolonged circulation in blood, and selective localization into many different sites of pathology: tumors, infections and inflammations (Zalipsky et al., 1994). At the same time, increasing the apparent molecular size of the protein, result in increased resistance to protease stabilization to salt and pH (Jensen-Pippo et al., 1996). During *in vivo* antitumor tests, an important observation in our study is that an antitumor activity enhanced *in vivo* effect of α-MMC-PEG. α-MMC-PEG enhanced the activity of antitumor activity as compared to natural α-MMC *in vivo*. The discrepancy between studies *in- vivo* and *vitro* could be useful for the therapy of tumor.

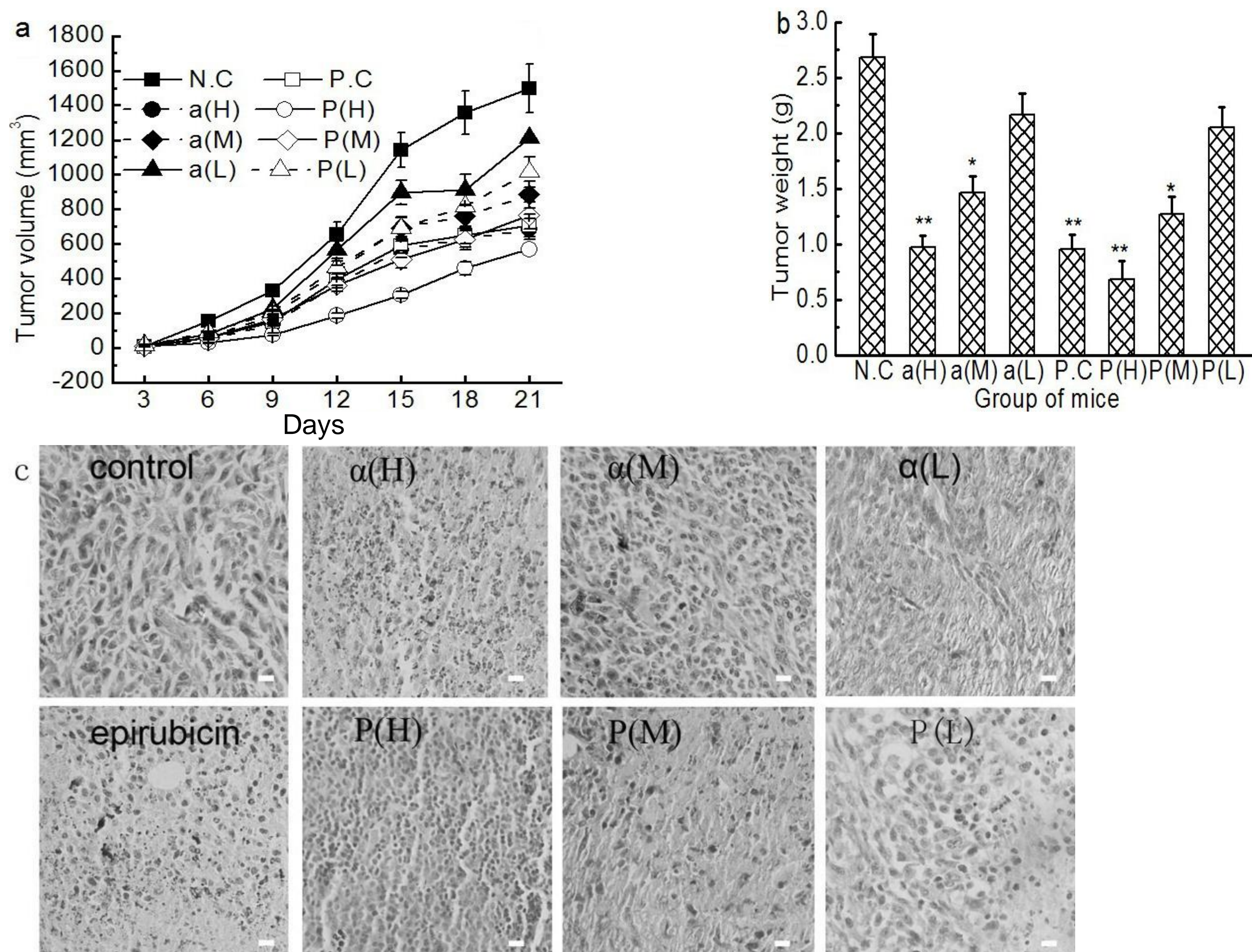

Figure 8. *In vivo* anti-tumor tests. (a) Tumor volume with the change of time (b) tumor weight treated with drug (high dose (H) 2.5 mg/kg; middle dose, (M) 0.5 mg/kg; low dose, (L) 0.1 mg/kg). The value represents the mean ± SD of three independent experiments. Each point represents the mean value ± (SD) deviation obtained from 10 animals. *p<0.05, **p<0.01 when compared with control group, respectively.

Conclusion

The antitumor activity of α-MMC-PEG was retained but decreased in potency only slightly *in vitro*. However, the antitumor activity is expected to increase only slightly *in vivo*, and longer half-life, thermal and acid-base stability can usually compensate for the reduction in activity. Together with the other benefits of PEGylation, like reduction in antigenicity, view of the magnitude of the tumor pandemic, the absence of a protective vaccine and the paucity of nontoxic therapy, the potential clinical application of α-MMC-PEG should be considered.

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