

ORIGINAL ARTICLE

PEGylation is effective in reducing immunogenicity, immunotoxicity, and hepatotoxicity of α -momorcharin *in vivo*

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Abstract

Background and aim: α -momorcharin (α -MMC), a type I ribosome-inactivating protein (RIP) from *Momordica charantia*, is well known for its antitumor and antiviral activities. However, the immunotoxicity and hepatotoxicity hampers its potential therapeutic usage. In order to reduce its toxicity, we had modified the α -MMC with polyethylene glycol (PEG), and detected the toxicity of the PEGylated α -MMC conjugates (α -MMC-PEG) *in vivo*.

Materials and methods: After α -MMC purified from bitter melon seeds, α -MMC-PEG was constructed with a branched 20 kDa (mPEG) 2-Lys-NHS, the tests of immunogenicity, immunotoxicity, and general toxicity of α -MMC-PEG were conducted in guinea pig and rat.

Results: The titer of specific IgG in rats, immunized by α -MMC-PEG, were approximately one-third of those that by α -MMC, all the guinea pigs treated with α -MMC died of anaphylaxis shock within 5 min, while no animals treated with α -MMC-PEG died in the active systemic anaphylaxis (ASA) test. The passive cutaneous anaphylaxis (PCA) reaction of α -MMC-PEG challenge in rats was significantly smaller than that of the α -MMC. The liver damage was greatly released, such as the change of globulin (GLB), aspartate aminotransferase (AST), total bilirubin (TBIL) cholesterol (CHOL), albumin (ALB), and the degree of hepatocyte necrosis in repeated toxicity study.

Conclusions: PEGylation is effective in reducing the immunogenicity, immunotoxicity, and hepatotoxicity of α -MMC *in vivo*.

Keywords: α -MMC, α -MMC-PEG, immunogenicity, immunotoxicity, hepatotoxicity

Introduction

α -momorcharin (α -MMC) is a type I ribosome-inactivating protein (RIP) found in abundance in *Momordica charantia* seeds. By deadenylation of ribosomal RNAs, α -MMC can inactivate ribosomes, inhibit cell protein synthesis, and even induce cell death

in its target cells. A wide spectrum of biological activities of α -MMC, especially the antitumor and antiviral activities, has been addressed either *in vitro* or *in vivo*.^(1–4) However, as a botanic protein, α -MMC can induce body to generate high titers of neutralizing antibody, which subsequently inhibits the pharmacological activities and accelerates

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the blood clearance. Furthermore, hepatotoxicity and severe systemic anaphylaxis have been observed in our previous guinea pig and rat studies of α -MMC. These toxicities strongly limit the potential therapeutic usage of α -MMC and hence promote us to develop the structural modification of it with polyethylene glycol (PEG), which is one of the most valuable strategies to reduce the immunological tolerance and extend the half-life of biotechnological drugs *in vivo*.⁽⁵⁻⁸⁾

Our research team had previously published the synthesis and characteristic of α -MMC-PEG.^(9,10) In those studies, α -MMC-PEG showed moderate reduction in antitumor activity *in vitro*, but its antigenicity was significantly reduced in BALB/c mice compared with the un-PEGylated α -MMC. In the present manuscript, we further evaluate the *in vivo* immunogenicity, immunotoxicity, and general toxicity of α -MMC-PEG in guinea pig and rat. Our data show that PEGylation lowers the titers of anti- α -MMC antibody, reduces the active and passive systemic anaphylaxis, and significantly alleviates the hepatotoxicity of α -MMC. These results suggest that PEGylation of α -MMC is may be beneficial to its clinical use.

Materials and methods

Chemicals and reagents

Bitter melon seeds were obtained from the Institute of Agricultural Science and Technique of Sichuan Province, China. MacroCap™ SP, Superdex 75, Sephacryl S100 were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Twenty kilodalton of (mPEG) 2-Lys-NHS was obtained from Shearwater Polymers (Huntsville, AL, USA). Biotin-labeled Goat Anti-Rat IgG was purchased from ZSGB-BIO (Beijing, China). Ovalbumin (OVA, grade V) and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation and analysis of α -MMC and α -MMC-PEG

All steps in the process of purification were carried out at 4–6°C unless specifically stated. Briefly, the powder of bitter melon seeds was extracted with phosphate buffer containing 0.15 mM NaCl the supernatant was precipitated with ammonium sulfate (30–60%). The part from 60% ammonium sulfate was dialyzed in sodium phosphate buffer (20 mM, pH 6.5), and loaded onto SP-Sephacryl FF, Superdex 75 and Macro-Cap-SP columns, respectively. The purified α -MMC was assessed by SDS-PAGE, acidic PAGE, HPLC, IEF, the N-terminal amino acid sequencing and electrospray ionization quadrupole mass spectrometry (ESI-QUAD-MS). The protein concentration was determined by the method of Lowry.

PEG (PEG/ α -MMC mass ratio = 5:1) was added to α -MMC solution (5 mg/mL in 0.1 M borate buffer, pH 8.5). The reaction mixture was gently shaken for 30 min at 5–6°C, then directly subjected to a Sephacryl S100 column (2 cm × 100 cm). The yield and purity of α -MMC-PEG were monitored by SDS-PAGE and RP-HPLC. The

molecular weight of α -MMC-PEG was determined by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). The α -MMC-PEG was stored at 4°C for use. The exhaustive procedure have been shown in our previous study.^(9,10)

Experimental animals

Sprague-Dawley (SD) rats (specific pathogen free-quality, 180–220 g) and guinea pigs (conventional animal-quality, 300–350 g) were purchased from Sichuan laboratory Animal Center (Chengdu, China) and used equal gender. Animals were housed in rooms maintained at 22–25°C with 12 h light–dark cycle and relative humidity of 50–70%, provided with fresh water and standardized food *ad libitum*, unless otherwise noted. Animals were acclimatized for at least 6 days prior to entering the studies and all the animals studies were conducted in accordance with the Guideline for Animal Experimentation of Chengdu Medical College, as well as the Good laboratory Practice Regulations for Nonclinical Laboratory Studies issued by Chinese Food and Drug Administration (SFDA, 2007).

Acute toxicity study in rats

SD rats were randomized into eight groups, with six rats in each, male and female in half. α -MMC was single intravenously administrated to rats at the dose of 4.00, 5.00, 6.25, 7.81, 9.77, 12.21, and 15.26 mg/kg (0.2 mL/animal), respectively. The safety dose acquired by a preliminary study was 4.00 mg/kg. After dose, clinical observation, body weight, and food consumption were recorded daily for 14 days as well as the animals in sodium control group.

Immunogenicity study in rats

A total of 30 SD rats were divided into three groups, 10 rats per group. Either α -MMC-PEG or α -MMC of 2.08 mg/kg in 0.2 mL in 2 of the 3 groups, 0.2 mL of normal saline (NS) in NS control group were intravenously administered via the caudal vein every other day for 2 weeks. The first blood collection via retro-orbital vein from the rats anesthetized by ether was made on day 14 after initial administration, with the blood volume of 0.5 mL. Following which, blood samples were raised every week for 5 weeks. The serum was centrifuged and stored approximately at –20°C until analyses.

The reaction wells were covered with α -MMC or α -MMC-PEG respectively and blocked with 3% bovine serum albumin. The following procedures included additions of serum in multiple dilutions, HRP-labeled Goat Anti-Rat IgG and substrate (tetramethyl benzidine, TMB). Titers of specific antibody to α -MMC (IgG) in the response panel were detected by ELISA microplate reader (BIO-RAD Model 680).⁽¹¹⁾

Active systemic anaphylaxis study in guinea pigs

Guinea pigs were randomized into four groups, with six rats in each, male and female in half. Guinea pigs were

sensitized by subcutaneous administration of either α -MMC-PEG or α -MMC at 1.81 mg/kg (0.2 mL/animal), every other days, for five times. Negative and positive control animals received saline or BSA (10 mg/kg), respectively. The dose level was based on the maximum tolerated dose obtained from the rats. Twenty days after the first dose, 0.6 mL of the same test articles at the same concentration were intravenously injected for challenge. Sign of the systemic anaphylaxis were observed for 30 min after dose. Classification criteria for active systemic anaphylaxis (ASA):⁽¹²⁾ nonreactive (–), slight symptoms of scratching of the nose, shivering, or piloerection (+), symptoms of catching a few coughs, with scratching of the nose, shivering, or piloerection (++), symptoms of catching frequent coughs, including convulsive spasms, dyspnoea, and so on. (+++), symptoms of convulsive spasms, dyspnoea, involuntary urination, shock, or death (++++).

Passive cutaneous anaphylaxis in rats

Eight SD rats were divided into four groups, with two rats in each. α -MMC of 2.08 mg/kg, α -MMC-PEG of 2.08 mg/kg, egg albumin solution of 15 mg/kg (positive control), or normal saline (negative control) in 0.2 mL was subcutaneously injected every other day for five times, respectively. On day 10, after the last sensitizing injection, blood samples collected from the orbital sinus under etherization condition were centrifuged, and the sera were stored at -20°C until analyses.

A total of 24 rats were randomized into four groups, with six rats in each. The rats were depilated on the back with a predetermined area of 4 cm $\times$ 5 cm. The above serum samples in each group were 1:16 and 1:64 diluted with normal saline to sensitize animals.

Diluted serum of 0.1 mL was intradermally injected into both sides of dorsal skin of each rat, left with 1:16 diluted serum, right with 1:64 diluted serum. After 48 h, antigen challenge was carried out by injection of 1 mL mixture of the homologous antigen and Evans blue dye via caudal vein (mixture of 0.5 mL sensitizing antigen and 0.5 mL 1% Evans blue dye). After 30 min, the animals were euthanized, with the dissection of the dorsal skin. Then, diameters of the ceruleus around the injection sites were detected and each ceruleus was smashed and soaked into 5 mL of acetone-saline solution (7/3, v/v). After 24 h, the leachate was centrifuged at 3000g for 15 min, the absorbance of the supernatant was measured at 610 nm by a spectrophotometer.

Repeated toxicity study in rats

SD rats were randomized into 3 groups, with 24 rats in each, male and female in half. Based on the MTD gained from acute toxicity study, α -MMC-PEG or α -MMC was respectively intravenous dosed to rats every 2 days for 28 days at 6.25 mg/kg (0.2 mL/animal). Control animals received sodium solutions. The characteristics of the each animal including appearance, behavioral activity, gland secretion, respiration, stool, genital, death,

and other toxic reactions were gotten each time before administration. In each group, blood was collected from the 14 rats anesthetized with pentobarbital 24 h after the last administration via abdominal aorta for serum chemistry test and cytology test. And then, the animals were sacrificed for gross necropsy, bone marrow biopsy, and other tests. At the end of the withdrawal (14 days after the last dose), specimens were collected from the remaining animals for above tests.^(13,14)

Statistical analyses

Data were declared as mean $\pm$ SEM. Significance was estimated by an ANOVA unpaired test (comparison between the groups) or paired *t*-test (comparison with the initial value within one group). *p* < 0.05 was used as a level of statistical significance.

Results

Identification of both α -MMC and α -MMC-PEG

The α -MMC was obtained through extraction and chromatographies with SP-Sepharose FF and Superdex 75, and Macro-Cap-SP. This protein with 30 kDa, pI of 9.0, N-Asp-Val-Ser-Phe-Arg was assessed by SDS-PAGE, PAGE, gradient PAGE and N-amino acid sequencing.

α -MMC-PEG was separated on Superdex 200 molecular sieve column and analyzed by SDS-PAGE. Then one-mer, two-mer, three-mer PEG-RIPs were identified by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS). The exhaustive identification have been shown in our previous study.^(9,10)

MTD in rats

After single intravenous administration of α -MMC at the dose levels from 7.81 mg/kg to 15.26 mg/kg, the rats showed the signs of lethargy, piloerection, hunched posture, calm behavior, labored respiration, hemafecia, and convulsion even succumb. The body weight of 4.00 mg/kg, 5.00 mg/kg, 6.25 mg/kg, and control animals kept increasing steady over the whole study, however, that of the animals in 7.81 mg/kg groups were observed mildly decreased from day 3 then recovered by day 7. While for animals in higher dose level at 9.77 mg/kg, 12.21 mg/kg, and 15.26 mg/kg, the body weight dropped dramatically from day 3 then began to grow slightly after day 7. Till day 14, 1, 3, 2 dead rats were observed in groups with dose of 9.77, 12.21, and 15.26 mg/kg, respectively, no overt adverse effects were observed in groups with dose of 4.00, 5.00, and 6.25 mg/kg groups. The MTD was therefore addressed as 6.25 mg/kg.

Rats immunogenicity study

Rats received intravenous injections of either α -MMC-PEG or α -MMC every 2 days for 2 weeks, blood samples were serially collected to evaluate the IgG anti- α -MMC-PEG or anti- α -MMC by an enzyme-linked immunoassay and the data are illustrated in Figure 1. The mean antibody titers of α -MMC group were firstly detected on day 14, reached the peak level (1:46.4) on day 28 then decreased

and maintained till day 49 (1:12.0). However, α -MMC-PEG-treated group showed significantly ($p < 0.05$) lower titers, which were only approximately one-fourth at the peak level and one-fifth on day 49, compared to that of the α -MMC-treated group.

Guinea pig ASA

Guinea pigs were sensitized with either α -MMC-PEG or α -MMC and then challenged on day 20 after sensitization. After the challenge, α -MMC induced strong ASA reactions in guinea pig 1 min right after challenge, and all six animals were died of anaphylaxis shock within 5 min, while only three of the BSA-treated animals died. However, none of PEGylated α -MMC-treated animals died and only piloerection, restlessness, and cough were observed after challenge (Table 1), the most severe clinical observation was labored respiration which fully recovered 5 min later.

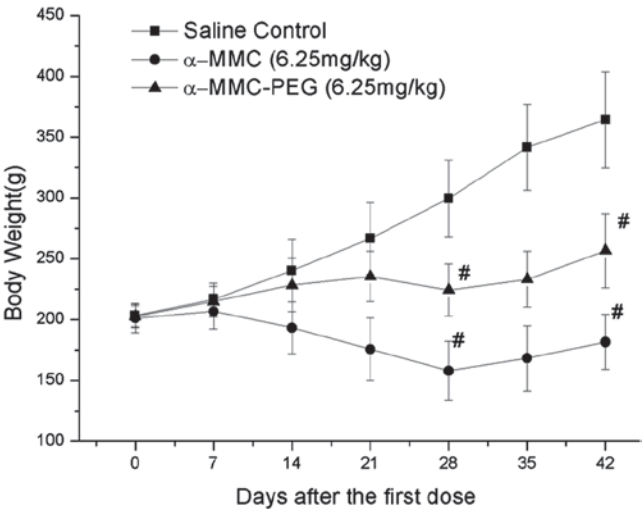


Figure 1. Titter changes of anti- α -MMC or anti- α -MMC-PEG IgG in rats sera. ELISA was used to detect the titter of IgG in serum, and the results were expressed as means $\pm$ SEM. Mean titers of anti- α -MMC IgG in group of 2.08 mg/kg α -MMC were 1:3.0, 1:15.2, 1:46.4, 1:28.8, 1:24.0, 1:12.0, respectively from week 2 to 7, and mean titers in α -MMC-PEG group were 1:1.6, 1:4.8, 1:10.8, 1:8.8, 1:2.4. * $p < 0.05$ compared to α -MMC-PEG group. 53 $\times$ 38 mm (300 $\times$ 300 DPI).

Rats PCA

Rats were sensitized passively by subcutaneous injection of the diluted sera from the either of α -MMC-PEG or α -MMC-treated rats, the challenged with the intravenous administration of the same antigen (α -MMC-PEG or α -MMC, respectively) and Evans blue, then the diameters of the ceruleus were measured 30 min later, the ceruleus leachate was evaluated as the optical absorptions at 610 nm (Figure 2, Table 2). When dosed by 1:16 diluted sera, the mean PCA reaction of α -MMC-PEG sensitized/challenged animals was significantly smaller than that of α -MMC-treated (ceruleus diameter 0.60 ± 0.12 cm vs 2.2 ± 0.46 cm, leachate optical density 0.090 ± 0.013 vs 0.304 ± 0.104 , $n = 6$, $p < 0.05$). When sensitized by 1:64 diluted sera,

Table 1. Results of active systemic anaphylaxis in guinea pigs (n = 6).

Group	No. of rats with following severity of reaction				
	–	+	++	+++	++++
NS control	6	0	0	0	0
10 mg/kg egg albumin control	0	0	0	3	3
1.81 mg/kg α -MMC	0	0	0	0	6
1.81 mg/kg α -MMC-PEG	0	2	4	0	0

α -MMC, α -momorcharin; α -MMC-PEG, α -MMC with polyethylene glycol.
Severity of anaphylaxis: “–”no obviously reaction; “+” restlessness, gently seize nose, shudder, or piloerection; “++” a few cough, seize nose, shudder, or piloerection; “+++” multiple or continuously cough, or having anhelation, spasms; “++++” anhelation, spasms, gatism, shock, or death.

Table 2. Ceruleus leachate absorption at 610 nm.

Group	$A_{610} (\bar{x} \pm s, n = 6)$	
	1:16	1:64
NS control	0.004 ± 0.002	0.003 ± 0.001
15 mg/kg egg albumin control	0.060 ± 0.014^a	0.015 ± 0.005^b
2.08 mg/kg α -MMC group	0.304 ± 0.104^a	0.096 ± 0.012^b
2.08 mg/kg α -MMC-PEG group	0.090 ± 0.013^a	0.002 ± 0.002

^a $p < 0.05$, compared to the NS control (sensitized with 16-fold diluted serum).

^b $p < 0.05$, compared to the NS control (sensitized with 64-fold diluted serum).

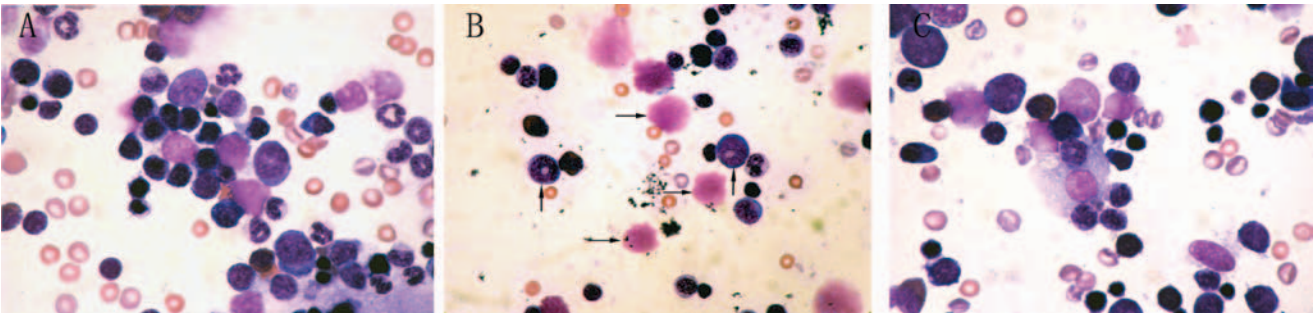


Figure 2. Ceruleus in rats induced by α -MMC or α -MMC-PEG in passive cutaneous anaphylaxis. (A) Negative control group, no ceruleus was found. (B) Positive control group, ceruleus was mostly seen in the injection sites with 1:16 dilution antiserum. (C) Group with 2.08 mg/kg α -MMC, deep blue ceruleus with an average diameter of about 2.2 cm was seen in the injection site with 1:16 dilution antiserum, light blue ceruleus with an average diameter of about 0.7 cm was observed in the 1:64 dilution antiserum injection site. (D) Group with 2.08 mg/kg α -MMC-PEG, only light blue ceruleus was seen in the injection site with 1:16 dilution antiserum. 941 $\times$ 462 mm (120 $\times$ 120 DPI).

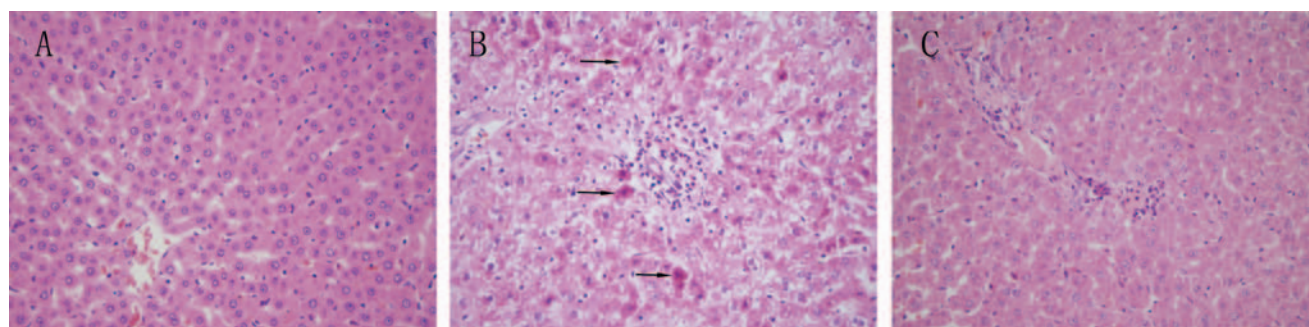


Figure 3. Body weight changes (g) in the NS control, 6.25 mg/kg α -MMC group and 6.25 mg/kg α -MMC-PEG group. Body weights were showed as means $\pm$ SEM. $^{\#}p < 0.05$ compared to that in NS control group on day 28, $^{*}p < 0.05$ compared to that in NS control group on day 42. 915 $\times$ 462 mm (120 $\times$ 120 DPI).

Table 3. WBC and WBC differential count of samples collected on day 28 after first administration ($\bar{x} \pm s$).

Group	WBC ($\times 10^9/L$)	Leukocyte differentiation (%)				
		NEU	LYM	MONO	EOS	BASO
NS control ($n = 14$)	8.57 ± 2.74	11.10 ± 2.80	81.83 ± 6.40	4.81 ± 2.08	1.41 ± 0.69	0.85 ± 0.64
6.25 mg/kg α -MMC ($n = 14$)	7.10 ± 2.03^a	19.95 ± 7.18^a	60.65 ± 12.14^a	11.03 ± 1.39^a	2.91 ± 0.76^a	5.46 ± 1.26^a
6.25 mg/kg α -MMC-PEG ($n = 14$)	6.90 ± 1.95^a	$22.08 \pm 4.70^{a,b}$	$62.70 \pm 10.03^{a,b}$	$9.66 \pm 1.25^{a,b}$	2.69 ± 0.40^a	$2.87 \pm 0.08^{a,b}$

α -MMC, α -momorcharin; α -MMC-PEG, α -MMC with polyethylene glycol; BASO, basophile; EOS, eosinophil; LYM, lymphocyte; MONO, monocyte; NEU, neutrophils count; WBC, white blood cell.

$^a p < 0.05$ compared to the control group.

$^b p < 0.05$ compared to the α -MMC group.

the ceruleus diameter was 0.71 ± 0.19 cm for the α -MMC-treated animals but almost no ceruleus was found by the gross observation.

Repeated toxicity study in rats

For rats treated with α -MMC at 6.25 mg/kg every 2 days for 28 days, the mean body weight gain was found significantly slower than that of the control or α -MMC-PEG treated animals, even began to lose the body weight from day 7 (Figure 3), calm behavior and decreased food consumption were observed from day 12 while piloerection and lethargy were found from day 21. Two deaths were found in group with α -MMC on day 20 and on day 24. For animals treated with α -MMC-PEG, no clinical signs of toxicity were observed during the treatment period, except that the mean body weight gain was slightly decrease at day 21 and the mild depression were observed after that as well.

While compared with the control animals, the rats treated with α -MMC or α -MMC-PEG had the significantly lower ($p < 0.05$) white blood cell count (WBC) at the end of treatment period (day 28), mostly due to the decreased lymphocyte (LYM) percentage. However, the neutrophils count (NEU), monocyte (MONO) percentage, eosinophil (EOS), and basophile (BASO) percentage were all increased ($p < 0.05$) at the same time, and the differential counts was also significant different between the α -MMC and α -MMC-PEG treatment groups ($p < 0.05$) (Table 3). Beside these, mild decrease of red blood cell (RBC) and hemoglobin (HGB) were observed in the α -MMC treated rats ($p < 0.05$ compare to that of the control) while these inhibition seemed slightly reduced in the α -MMC-PEG treated group ($p > 0.05$ while compared with that of the control) (data not shown). No significant difference was

found in hematology results of rats treated with α -MMC or α -MMC-PEG between day 28 after administration and day 14 after withdrawal (data not shown).

No treatment related effects were found in the coagulation and serum chemistry parameters of electrolyte and kidney function (data not shown) at the end of 28-days dose. Abnormal changes occurred mainly in some relevant indicators of liver function. Compared to the control animals, α -MMC treatment rats had significantly increased in globulin (GLB), aspartate aminotransferase (AST), total bilirubin (TBIL), and cholesterol (CHOL) but decreased in albumin (ALB). These differences were also observed between the α -MMC-PEG and control groups but not so obvious. Biochemical results showed mainly a slight decrease in AST, GLB and a slight increase in ALB at the end of recovery period (Table 4).

No abnormal morphology was observed in the Wright-Giemsa stained bone marrow smear collected from the control animals (Figure 4A). However, the bone marrow of animal treated with α -MMC showed lower myeloid/erythroid (M:E) ratio than that of the control group (2.36 ± 0.48 vs 3.72 ± 0.60), and the slides illustrated vacuolar degeneration and karyolysis of lymphocyte, megaloblastoid of the basophilic normoblast and matured but abnormal morphological erythroid series, including target erythrocyte, stomatocyte, elliptocyte, and so on, the olistherozone of the RBC was enlarged, and the numbers of EOS and MONO with normocellularity were increased (Figure 4B). For the smear of animals treated with α -MMC-PEG, the M:E ratio was 3.43 ± 0.55 , vacuolar degeneration, karyolysis lymphocyte, and abnormal morphological erythrocyte were scarcely observed (Figure 4C). No significant differences were

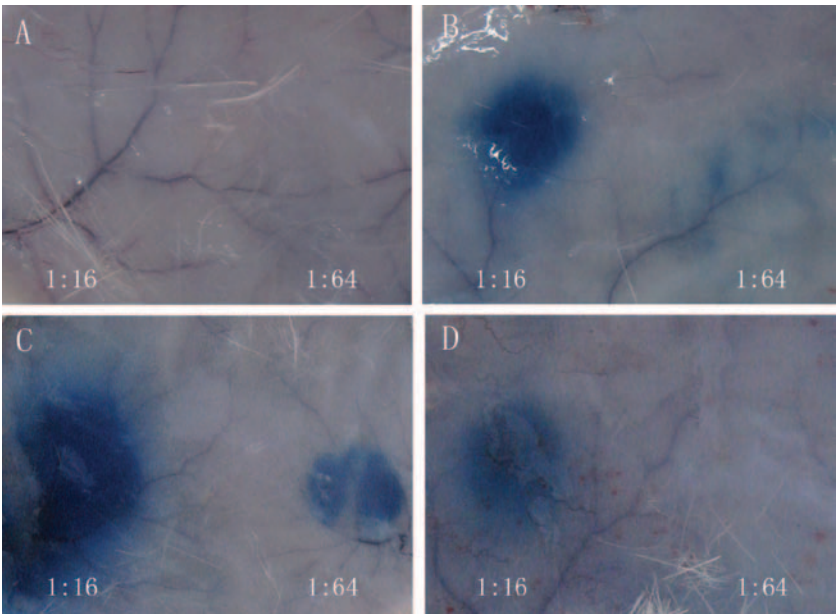


Figure 4. Observations of the bone marrow by optical microscope (Wright-Giemsa, 40 × 10). (A) NS control group, morphology was normal. (B) Group with 6.25 mg/kg α-MMC, vacuolar degeneration (↑) and karyolysis (→) of lymphocytes were observed. (C) Group with 6.25 mg/kg α-MMC-PEG, no obvious morphologic changes were observed. 368 × 462 mm (120 × 120 DPI).

Table 4. Serum chemistry data of samples collected on administration day 28 and recovery day 14 ().

	Day 28			Recovery day 14		
	NS control (n = 14)	α-MMC (n = 14)	α-MMC-PEG (n = 14)	NS control (n = 10)	α-MMC (n = 8)	α-MMC-PEG (n = 10)
TP (g/L)	66.90 ± 7.10	69.00 ± 13.82	65.71 ± 11.25	67.63 ± 6.14	69.37 ± 11.47	68.23 ± 12.42
ALB (g/L)	38.50 ± 3.94	27.85 ± 8.66 ^a	31.68 ± 9.08	38.14 ± 3.21	29.63 ± 8.35 ^b	35.55 ± 7.34
GLB (g/L)	28.40 ± 3.27	41.15 ± 4.16 ^a	34.03 ± 3.68	29.49 ± 3.36	39.74 ± 4.67 ^b	32.68 ± 3.82
A/G	1.36 ± 0.28	0.68 ± 0.11 ^a	0.93 ± 0.12	1.29 ± 0.15	0.75 ± 0.14 ^b	1.09 ± 0.18
AST (U/L)	89.43 ± 8.71	175.00 ± 25.25 ^a	83.62 ± 13.48	85.43 ± 6.26	186.24 ± 21.57 ^b	84.57 ± 10.61
TBIL (μmol/L)	1.85 ± 0.37	2.65 ± 0.59 ^a	1.78 ± 0.37	1.85 ± 0.31	2.42 ± 0.21 ^b	1.96 ± 0.27
CHOL (mmol/L)	1.06 ± 0.21	1.29 ± 0.04 ^a	0.89 ± 0.12	1.09 ± 0.24	1.45 ± 0.42 ^b	1.02 ± 0.31

α-MMC, α-momorcharin; α-MMC-PEG, α-MMC with polyethylene glycol; ALB, albumin; A/G, albumin/ globulin; AST, aspartate aminotransferase; CHOL, cholesterol; GLB, globulin; TBIL, total bilirubin; TP, total protein.

^a*p* < 0.05 compared to the day 28 data of control group.

^b*p* < 0.05 compared to the recovery day 14 data of control group.

found between the slide for day 28 and the end of recovery period.

No dose related effects on organ weights were seen in rats at the end of the 28-days administration and the 14 days recovery period, and no significant changes either in absolute or relative weights of any organs except liver were found at the same timepoint. For rats treated with α-MMC which sacrificed at the end of treatment period, gross pathological observation of the liver showed enlargement, gray and little increased weight. Mild enlargement and congestion were also found in the spleen and lymphocyte node; however, no such finding occurred in the animals treated with α-MMC-PEG. Microscopic observation of the liver tissue of the animals treated with α-MMC indicated massive hepatocyte swelling degeneration, inflammatory cell infiltration, congestion, and diffusive necrosis (Figure 5B), but only pathological changes such as mild swelling and inflammatory cell infiltration were seen in the liver tissue of rats treated with α-MMC-PEG

(Figure 5C). Also, regularly arrayed liver cells with normal structure were shown in NS control group (Figure 5A).

Discussion

Polyethylene glycol (PEG) is a polyether compound and has been approved by the US FDA for internal use. PEGylation is the act of covalently coupling a PEG compound to another large molecule, for example, a biomedical molecule for drug use. PEGylation changes the physical and chemical properties of the biomedical molecule, such as its electrostatic binding, and hydrophobicity and results in an improvement in the pharmacokinetic behavior of the drug.⁽¹⁵⁻²³⁾ Since the first PEGylation was developed in the 1970s, a variety of therapeutic proteins, peptides, and antibody fragments, such as PEG-asparaginase (Oncaspar®), PEG-interferonα-2a (Pegasys®), and PEG-epoetin β (Mircera®), have been PEGylated and approved by FDA.⁽²⁴⁾

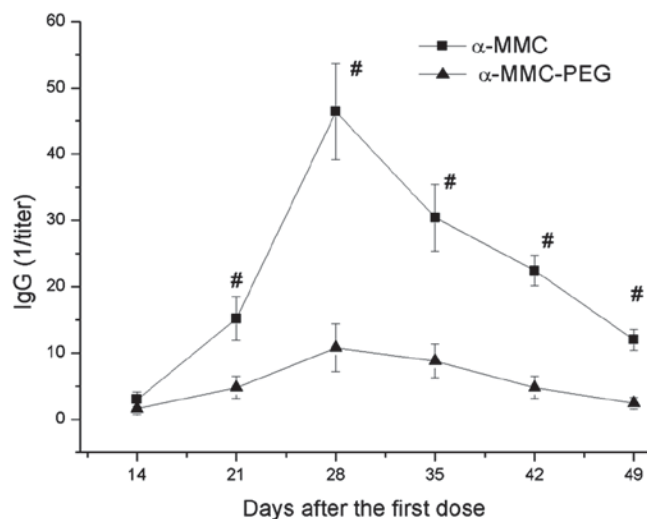


Figure 5. Pathologic characteristics of liver observed by optical microscope (HE, 40×10). (A) NS control group, no significant pathological changes in liver. (B) Group with 6.25 mg/kg α -MMC, massive hepatocyte swelling degeneration, inflammatory cell infiltration, congestion and diffusive necrosis ($\rightarrow$) were observed. (C) Group with 6.25 mg/kg α -MMC-PEG, mild swelling and inflammatory cell infiltration were seen. 53×38 mm (300×300 DPI). HE, hematoxylin-eosin staining.

We constructed mono-, di-, and tri-PEGylated α -MMCs by the lysine-directed PEGylation with a branched 20 kDa (mPEG) 2-Lys-NHS, and then conducted the tests of immunogenicity, immunotoxicity, and general toxicity of α -MMC-PEG in guinea pig and rat. Compared with α -MMC, we found that (i) the serum titers of anti- α -MMC-PEG IgG were approximately one-third–one-fifth of that of anti- α -MMC (Figure 1); (ii) only mild symptoms of ASA were observed in α -MMC-PEG group; (3) the PCA reaction of α -MMC-PEG challenged in rats was significantly smaller.

Why PEGylation leads to notably reduced immunological reactions? The main explanation is that PEGylation masks many antigenic epitopes on α -MMC. In fact, PEG is highly soluble in both aqueous and organic milieu, and α -MMC-PEG is surrounded by a water cloud after injected into body. This water cloud shields most of the antigenic determinants on α -MMC, which in turn greatly reduces the generation of antibodies, such as IgG and IgE. As a result, undesired immune response against α -MMC is weakened, and the reaction of hypersensitivity is inhibited.

α -MMC belongs to type I RIP, which is known to have both antitumor activity and antiviral activity. Furthermore, we found that it had cytotoxicity, such as hepatotoxicity which we had reported in previous study (published in *Journal of Ethnopharmacology* online, doi:10.1016/j.jep.2011.11.057). That study showed that rats treated with α -MMC had serious hepatocellular punctiform necrosis and abnormal liver function. In this study, we also observed that rats treated with α -MMC had low mood, loss of body weight, and abnormal changes in blood biochemistry and liver pathology. Interestingly, these symptoms were

significantly alleviated in the animals treated with α -MMC-PEG. This suggests that PEGylation may have some additional benefits: the liver damage was greatly released, such as the change of GLB, AST, TBIL, CHOL, ALB, and the degree of hepatocyte necrosis.

How α -MMC enters cells and cause cytotoxicity is still largely unknown, but studies on trichosanthin, another type I RIP, suggest that type I RIP penetrates into cells by selectively binding to the lipoprotein receptor-related protein (LRP), expressed on cellular membrane of the target cells.^(25,26) After entering a cell, type I RIP exerts its N-glycosidase activity and eventually leads to cellular necrosis. The expression of LRP is most abundant in hepatocytes, followed by cerebrum and placenta.⁽²⁷⁾ Thus, the liver is apt to be damaged. We presumed that PEGylation may have shielded some binding sites of α -MMC to LRP, which results in dramatically reduced the hepatocyte necrosis. Conversely, PEGylation may have shielded some active sites of α -MMC for its N-glycosidase, then decrease the cytotoxicity.

An intriguing phenomenon we have observed is that although PEGylation could decrease the efficacy of α -MMC *in vitro*,⁽⁹⁾ and decrease its toxicity *in vivo* accompanied, the pharmacodynamic activities *in vivo* were not decreased. The result of our antitumor study *in vivo* having been finished recently showed that the tumor growth inhibition rate of α -MMC-PEG of 2.5 mg/kg was up to 59.58%, but animals administered with the same dose of α -MMC were all died though the general conditions of animals dosed with α -MMC-PEG were bad including appearance, behavior, food intake, body weight, and stool. However, the tumor growth inhibition rates of α -MMC-PEG in doses of 1.25 mg/kg and 0.63 mg/kg were similar with those of α -MMC in the same doses and the rates were 47.24%, 50.78%, and 29.33%, 28.57%, respectively. The activity maintenance may attribute to the drug delayed release property of PEGylation. PEGylation could prolong the half-life of PEGylated drug; therefore, the time of pharmacodynamic action would be increased, thus, the efficacies would be accumulated gradually, and the total activity was not decreased *in vivo* compared with un-PEGylated drug. Our previous study also confirmed that the half-life of α -MMC was 7.25 h long and that of α -MMC-PEG increased to 21.37 h.

Conclusion

PEGylation is effective in reducing the immunogenicity, immunotoxicity, and hepatotoxicity of α -MMC *in vivo*. PEGylation shows the desired effect of reducing the toxicity of α -MMC while preserving the bioactivity of α -MMC. Our results demonstrate that α -MMC-PEG is a highly promising drug candidate for the treatment of cancer.

Declaration of interest

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