

Full Length Research Paper

Inhibitory effect of alpha-momorcharin and its PEGylated conjugates on the migration and invasion of human choriocarcinoma JAR cells via reduced matrix metalloproteinase-2 (MMP-2) activity

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Alpha-momorcharin (α -MMC) is a ribosome-inactivating protein (RIP) with antitumor and antiviral activities. α -MMC has a tumoricidal effect on choriocarcinoma, which is a malignant trophoblast-derived tumor that can arise during any type of gestation. To develop the therapeutic potential of α -MMC, we performed surface site-specific modification by covalent attachment of a 20 kDa amino derivative poly(ethylene glycol), which is (mPEG)₂-Lys-NHS. We evaluated the potency of α -MMC and the PEGylated α -MMC as potential anti-metastatic drugs to combat this disease. We conducted a quantitative 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test, adhesion assay, wound healing assay, matrigel invasion assay and gelatin zymography to test the cell viability and proliferation, cell adhesion, migration and invasiveness, and the activities of matrix metalloproteinases (MMPs) on JAR cells after protease treatment. We found that native and PEGylated α -MMC significantly inhibited the growth of choriocarcinoma JAR cells in a dose- and time- dependent manner. Zymography showed that they both potently attenuated the activity of matrix-metalloproteinase-2 (MMP-2), which is associated with decreased cell movement potential. The *in vitro* migration and invasion assays confirmed these results. The inhibitory effect of α -MMC and its PEGylated conjugates on the metastatic stage of human choriocarcinoma JAR cells suggests a possible novel role for RIPs such as α -MMC in tumor progression.

Key words: Alpha-momorcharin (α -MMC), choriocarcinoma, migration and invasion, matrix-metalloproteinase-2 (MMP-2), Alpha-momorcharin PEGylated anti-tumor activity (PEG- α -MMC).

INTRODUCTION

Alpha-momorcharin (α -MMC) is a type I ribosome-inactivating protein (RIP), which is found in abundance in *Momordica charantia* (bitter melon) seeds. α -MMC has a wide spectrum of biological activities including enzymatic (DNase, RNase and N-glycosidase) activities (Go et al., 1992; Mock et al., 1996; Ren et al., 1994), abortifacient (Tam et al., 1984) and immunosuppressive effects (Leung

et al., 1987). It has also demonstrated anti-tumor growth activity (Ng et al., 1994; Xiaoxiao Bian et al., 2010) and anti-HIV function (Au et al., 2000; Lifson et al., 1988; Zheng et al., 1999). However, it is a foreign protein so it is typically susceptible to degradation by proteases, which leads to a short circulatory half-life *in vivo*, rapid kidney clearance, high immunogenicity and high antigenicity

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(Leung et al., 1987). Surface site-specific modification by the covalent attachment of polyethylene glycol (PEG) is regarded as a valuable technique for overcoming the problems inherent to protein drugs because it allows protein drugs to be tolerated immunologically, makes them resistant to proteolytic digestion (Veronese, 2001), and it also preserves unmodified activity (Werlen and Bernkop-Schnurch, 2006). We modified α -MMC with a branched 20 kDa bis-monomethoxy polyethylene glycol (mPEG)₂-lysine attached to an *N*-hydroxysuccinimidyl ester; (mPEG)₂-Lys-NHS. The carboxyl group of the derivative could be activated by NHS because it reacts directly with amino groups in the protein molecules at a pH ranging from 7.0 to 9.0 (Abuchowski et al., 1984).

Choriocarcinoma is a malignant and aggressive trophoblastic cancer that usually spreads to the lung and brain. Metastatic disease is responsible for death rather than the primary tumor itself (Yang et al., 2004). The metastasizing process involves the detachment of cancer cells from the primary tumor, followed by migration, adhesion and invasion of cancer cells into the blood or lymphatic vessels, which is facilitated by matrix metalloproteinases (MMPs) (Hart and Saini, 1992). MMP-2 and MMP-9 are considered to be crucial for cellular invasion and tissue remodeling. To determine whether RIPs might affect this complex scenario of adhesion, migration and invasion, we treated the choriocarcinoma JAR cells with the ribosome inactivating protein α -MMC in a trial. We found that α -MMC suppressed JAR cell adhesion to a matrigel-coated substrate in a concentration-dependent manner, while PEG- α -MMC virtually eliminated this ability. Native and modified α -MMC inhibited the invasion and wound healing migration of JAR cells. Zymography showed that the activity of MMP-2 was attenuated after the protease treatment. To the best of our knowledge, this is the first report of the involvement of an RIP in tumor progression.

MATERIALS AND METHODS

Preparation of α -MMC and PEG_{20k}- α -MMC

Natural and modified α -MMC were prepared as described previously (Li et al., 2009). The purification of α -MMC was accomplished in the following four steps: The supernatant was extracted from bitter melon seeds with acetate buffer (50 mM, pH 5.0), followed by ammonium sulfate fractionation precipitation (35 to 70%), MacroCap SP chromatography with a 0 - 0.2 M NaCl linear gradient in 20 mM phosphate buffer (PB, pH 7.0) and Superdex 75 chromatography with 20 mM PB containing 0.15 M NaCl, pH 7.0. Next, 40 mg PEG (PEG/ α -MMC molar ratio = 5:1) was added to 5 ml α -MMC (2.5 mg/ml in 0.1 M borate buffer, pH 8.5), which was then applied directly to a Sephacryl S100 column. The purity was assessed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration high performance liquid chromatography (HPLC).

Cell culture

JAR choriocarcinoma cells were purchased from the Cell Bank at

the Shanghai Institute of Cell Biology (Shanghai, China) and were maintained in an incubator (Thermo Forma 3110, USA) with a humidified atmosphere of 5% CO₂ at 37°C where the medium was replaced every 2 to 3 days. The culture medium was Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) containing 20 mM HEPES, which was supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone Laboratories, USA).

Cell survival and proliferation

A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test was performed as previously described (Mosmann, 1983). Number of cells was adjusted to 3×10^4 cells/ml after trypan blue staining and counting using a haemocytometer, before cell suspensions were plated with 100 μ L in each well of the 96-well plates. Six hours after initial cell attachment, we added 20 μ L of medium or an appropriately native or PEG- α -MMC diluted by stock solutions to produce a final concentrations of 0.012, 0.06, 0.3 and 1.5 mg/ml, which were incubated 24, 48 and 72 h (four replicates per concentration). The procedure was based on a previously described method (Li et al., 2009). Cell proliferation was determined as the formazan absorbance 570 nm, which was expressed as the ratio of treated to control values.

Detection of apoptosis assay by Hoechst staining

A Hoechst 33258 staining kit (Applygen, Beijing, China) was used to detect apoptotic morphological changes in the nuclear chromatin of JAR cells. Cells were seeded into 60 mm dishes containing cover slips and they were allowed to grow to 90% confluency before treatment with or without 1.5 mg/ml α -MMC for 48 h or PEG- α -MMC for 72 h. After fixation, cells were stained on the cover slips according to standard methods.

Cell adhesion

We coated 96-well plates with matrigel, which was diluted 1: 4 in media with 40 μ L/well followed by at incubation at 4°C overnight. Plates were blocked with 0.5% BSA/DMEM at 37°C in a CO₂ incubator for 45 to 60 min. Cells were pretreated with 0.3 mg/ml α -MMC or 1.5 mg/ml PEG- α -MMC for 24 h (three replicates per concentration), while untreated cells were used as the control. Subsequently, the cells were harvested and adjusted to 3×10^5 /ml with 100 μ L/well and cultivated in a CO₂ incubator at 37°C for 40 min.

The medium was carefully removed and each well was washed three times with PBS. A colorimetric MTT assay was used to determine the number of adherent cells. Replicates from four wells were averaged. Cell adhesion data was expressed as the absorbance of the treated cells relative to the control.

Cell migration

A wound healing assay was performed to test whether (PEG-) α -MMC had any effects on JAR cell migration. Cells were seeded in 35 mm dishes and allowed to grow to 90% confluency in complete medium. Cell monolayers were then scratched with a pipette tip. Wounded monolayers were washed with PBS to remove any cell debris and they were then incubated at 37°C with serum-free culture in the absence or presence of α -MMC or PEG- α -MMC for 24 h. Scratches were photographed with an inverted fluorescent microscope (Olympus TH4-200, Japan) immediately after wounding and 24 h incubation.

Cell invasion

A matrigel invasion assay was performed to investigate the effects of (PEG-) α -MMC on JAR cell invasiveness. First, we coated 24-well Transwell (BD Biosciences, USA) inserts with 8 μ m porosity polycarbonate filters (Millipore, USA) using 70 μ L matrigel (Vigorous, Beijing, China), which was diluted 1:3 in media, followed by incubation at 37°C for at least 4 h to promote gelling. JAR cells (2×10^5 cells with 0.2 ml DMEM) were placed into the upper compartment and incubated with PEGylated or native α -MMC at a final concentration of 1.5 mg/ml for 24 h at 37°C in a humidified atmosphere with 5% CO₂ (three replicates per concentration). Finally, cells were removed from the upper surface with a cotton swab and the invaded cells on the lower surface of the filter were stained with 0.2% crystal violet. The dye was eluted with 33% acetic acid and the amount of invaded cells was estimated based on the absorbance at 570 nm. The invasion level was calculated based on the filter absorbance relative to the control.

Gelatin zymography

MMP-2 and MMP-9 protease activities were detected in media samples from JAR cells by gelatin substrate zymography. JAR cells were grown to 90% confluency, washed and placed in serum starvation conditions, before adding equal amounts of either serum-free media, α -MMC (0.36, 1.8 and 9 mg/ml), or PEG- α -MMC (9 mg/ml), and incubating the cells for 24 h. The conditioned medium containing gelatinase was incubated at 37°C for 10 min with non-reducing loading buffer and applied to a 10% polyacrylamide gel that was copolymerized with 0.1% gelatin (Bio-Rad). After electrophoresis, the gels were washed with agitation using 50 mM Tris-HCl (pH 7.6) containing 2.5% (v/v) Triton X-100 for 1 h with at least two changes, before washing out the Triton X-100 residue. Gels were subsequently incubated for 38 h at 37°C with 50 mM Tris-HCl (pH 7.6) containing 50 mM CaCl₂ and 0.5 M NaCl. Finally, gels were stained with Coomassie Blue R-250 and destained to reveal zones of gelatinolytic activity. Bands were analyzed by scanning densitometry (Umax Colour Scanner, USA) and the Quantity One Quantitation program (Bio-Rad Laboratories).

Statistical analysis

Values were reported as the mean \pm SEM from two independent samples. We used one-way ANOVA followed by Scheffe's *post hoc* test with an alpha value of 0.05 to compare changes among the different groups. Statistical significance was indicated at * $P < 0.05$ and ** $P < 0.01$.

RESULTS

Alpha-MMC and PEG- α -MMC inhibited the proliferation of JAR cells

Cancer cells have defective regulatory circuits, which govern proliferation and homeostasis in normal cells (Hanahan and Weinberg, 2000). To investigate the effect of α -MMC and its PEGylated conjugates on cell viability and proliferation, we seeded JAR cells on 96-well plates, which were treated with increasing concentrations of α -MMC or PEG- α -MMC for 72 h (Figure 1A), and for different time periods at 1.5 mg/ml (Figure 1B). The growth curves (Figure 1) showed that the proliferation of

treated cells was significantly slower and less abundant than the control cells. Both treatments produced inhibition in a dose-dependent and time-dependent manner. Statistical analyses showed that preparations treated with 1.5, 0.3 and 0.06 mg/ml of α -MMC and PEG- α -MMC began to exhibit significant effects after 48 and 72 h incubation. Figure 1A shows that PEGylated conjugates had approximately 30% lower activity compared with the native α -MMC in the test at the highest concentration. Moreover, Figure 1B shows that the anti-proliferation effect on cultured cells was not evident after 24 h treatment, while continued incubation for 48 or 72 h with the proteins enhanced the cytotoxic effect on cells.

In general, alpha-MMC and PEG- α -MMC induced alterations in the morphology of the treated cells (Figure 2). Untreated JAR cells were extended and flat (Figure 2A), whereas the treated groups produced less cells, which had abnormal shapes such as shrinkage, blebbing and loss of membrane asymmetry. This illustrated the cytotoxic effect of α -MMC and its modified form after treatment for 72 h, particularly the α -MMC groups (Figure 2C and D).

Alpha -MMC and PEG- α -MMC induced the apoptosis of JAR cells

The morphological changes shown in Figure 2 appeared to be related to apoptosis. The results were confirmed by Hoechst 33258 staining (Figure 3). In contrast to untreated normal JAR cells (Figure 3A), the processed cells exhibited typical apoptotic morphological changes in the nuclear chromatin such as nuclei condensation and fragmentation, boundary aggregation or splitting (Figure 3B and C). The PEGylated conjugates maintained the bioactivity and they could also induce cell death by apoptosis.

Alpha-MMC and PEG- α -MMC suppressed the progression of JAR cells *in vitro*

The MTT assay showed that treatment with α -MMC or PEG- α -MMC at a concentration of 1.5 mg/ml had no cytotoxic effect in JAR cells (Figure 1B). A lower time and concentration were applied in the following experiments. Tumor cell adhesion to extracellular matrices and basement membranes are considered to be an initial step in the invasive process of metastatic tumor cells (Saiki et al., 1990). Figure 4 shows that α -MMC caused a delay in JAR cell adhesion in a dose-dependent manner, whereas no statistically significant effect was found with 1.5 mg/ml PEG- α -MMC ($P > 0.05$). The quantitative analysis detected 41 and 29% reductions in trophoblast adhesion with 1.5 and 0.3 mg/ml of α -MMC, respectively, compared with the control ($P < 0.05$).

Cell motility and tissue invasion are considered to be

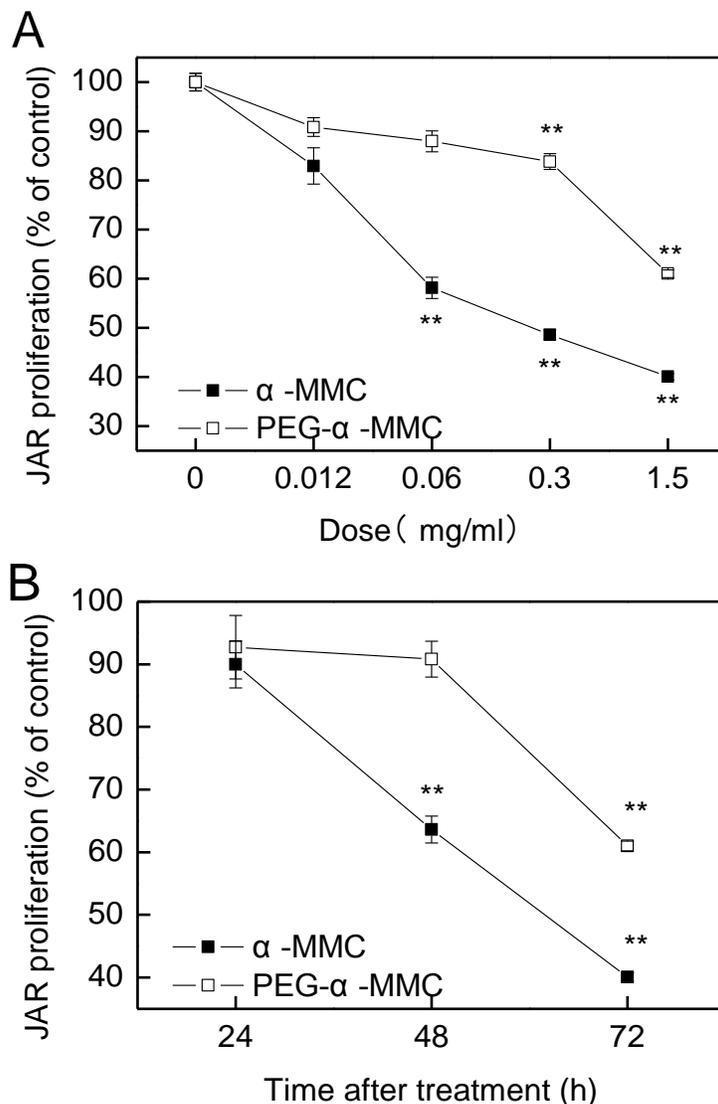


Figure 1. Inhibitory effect of α -MMC and PEG- α -MMC on the proliferation of human choriocarcinoma JAR cells. JAR cells were treated with increasing concentrations of native or PEGylated α -MMC as indicated for 72 h (A) and for various times periods using 1.5 mg/ml of each compound (B). Each data point represents the average of three independent experiments performed in quadruplicate. * $P < 0.05$, ** $P < 0.01$.

essential alterations in the cell physiology that collectively lead to malignant growth (Hanahan and Weinberg, 2000). The capacity for cell migration was evaluated with a wound healing assay. As shown in Figure 5, both native and PEGylated α -MMC significantly inhibited the motility of JAR cells. After 24 h, cells in the control group efficiently spread into the wound area to such an extent that only traces of the wounded region remained (Figure 5A), whereas very few cells spread into the wound in the 0.3 mg/ml α -MMC or 1.5 mg/ml PEG- α -MMC treated groups (Figure 5B and C). Cell mobility was associated with pixel values in the wounded area. In contrast to the

control, cellular migration was inhibited by up to 78 and 82% at 24 h with 0.3 mg/ml α -MMC or 1.5 mg/ml PEG- α -MMC, respectively (Figure 5D, $P < 0.01$).

Matrigel cultures have been reported to be useful for *in vitro* assays when evaluating trophoblast invasiveness. We found that incubation with 1.5 mg/ml α -MMC or PEG- α -MMC significantly decreased JAR cell invasiveness. Figure 6 shows the results of the 30 h *in vitro* Matrigel invasion assay. α -MMC and PEG- α -MMC had a significant effect on cell invasiveness, with reductions of 53 and 28%, respectively, compared with the control (Figure 6D, $P < 0.01$).

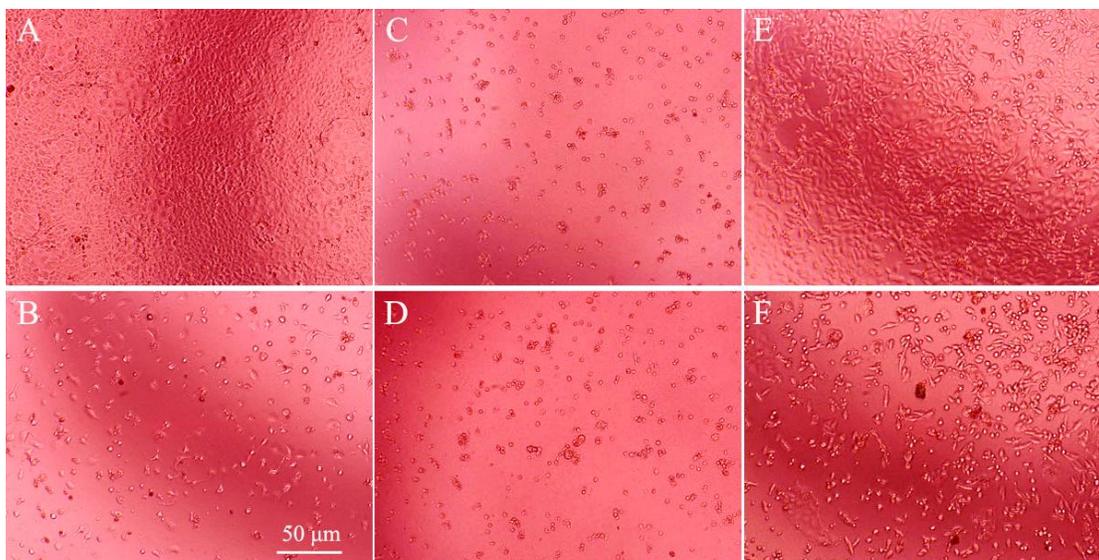


Figure 2. Morphological differences in JAR cells for after 72 h culture as observed by phase contrast microscopy. JAR cells were untreated (A), or treated with 15 µg/ml 5-fluorouracil (B), 0.3 mg/ml α-MMC (C), 1.5 mg/ml α-MMC (D) 0.3 mg/ml PEG-α-MMC (E), and 1.5 mg/ml PEG-α-MMC (F).

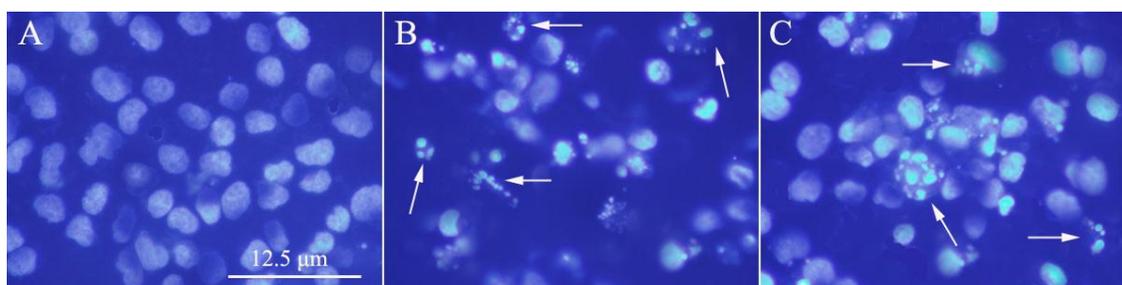


Figure 3. Apoptosis observed in the JAR cells treated with α-MMC or PEG-α-MMC. Hoechst 33258 staining of JAR cells incubated without α-MMC or PEG-α-MMC (A), with 1.5 mg/ml α-MMC for 48 h (B) and with 1.5 mg/ml PEG-α-MMC for 72 h (C). Arrows indicate typical morphological features of apoptotic cells (DNA condensation, fragmentation, and nuclear shrinkage) observed in treated JAR cells.

Alpha -MMC and PEG-α-MMC reduced the activity of MMP-2

MMP-2 has major roles during migration and invasion processes by cancer cells (Sato et al., 1994). Figure 7A shows several lytic bands in the zymograms, which can be seen as clear areas of lysis in the uniformly stained blue gel. The major gelatinolytic bands were pro- and active-MMP-2. As shown in Figure 7B, the MMP-2 enzyme activity was significantly reduced in the conditioned medium treated with 0.06 mg/ml α-MMC or a higher concentration. Similarly, 1.5 mg/ml PEG-α-MMC reduced the MMP-2 activity by about 30% compared with the control ($P < 0.01$). MMP-9 activity was hardly detectable in the control and tested groups, which disagreed with previous reports that detected active MMP-9 in media samples derived from JAR cell culture

wells (Staff et al., 2000; Tseng et al., 2009). The incubation period with serum-free conditioned medium was insufficiently long for the active form of the enzyme to be degraded, which may account for the different results.

DISCUSSION

This study demonstrated that α-MMC and PEG-α-MMC reduced the migration and invasiveness of a chorio-carcinoma cell line, JAR cells incubated with α-MMC at concentrations of 0.06 mg/ml or greater concentrations exhibited had reduced MMP-2 enzyme activity, while 1.5 mg/ml PEG-α-MMC produced a similar effect. Our results suggested that the decreased of proliferation due to apoptosis and the overall survival of JAR cells were a

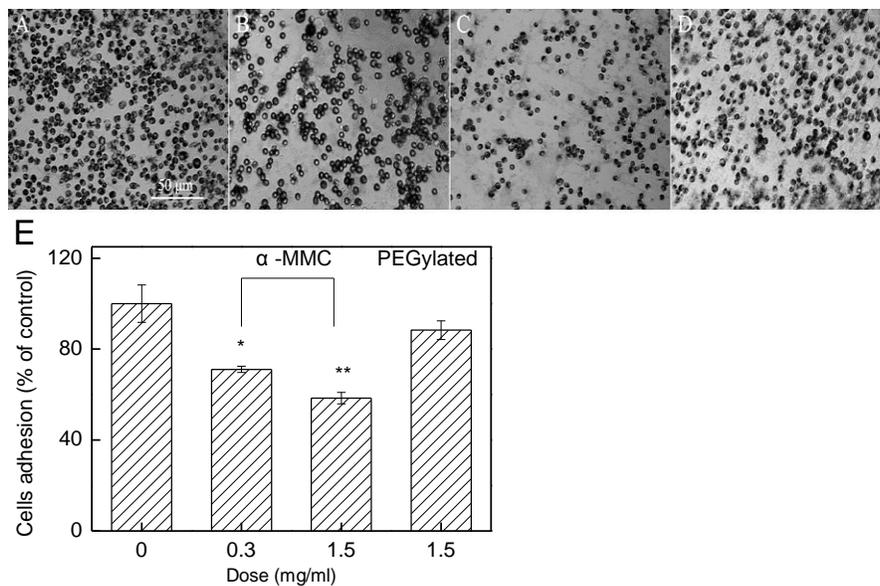


Figure 4. Effects of native and modified α -MMC on JAR cell adhesion to matrigel. Adherent photos of JAR cells without pretreatment (A), or pretreated with 0.3 mg/ml α -MMC (B), 1.5 mg/ml α -MMC (C) and 1.5 mg/ml PEG- α -MMC (D) for 24 h. An MTT assay was used to determine the number of adherent cells. The data are expressed as the relative absorbance of treated sample compared with the control (E). Each value is the mean of triplicate measurements.

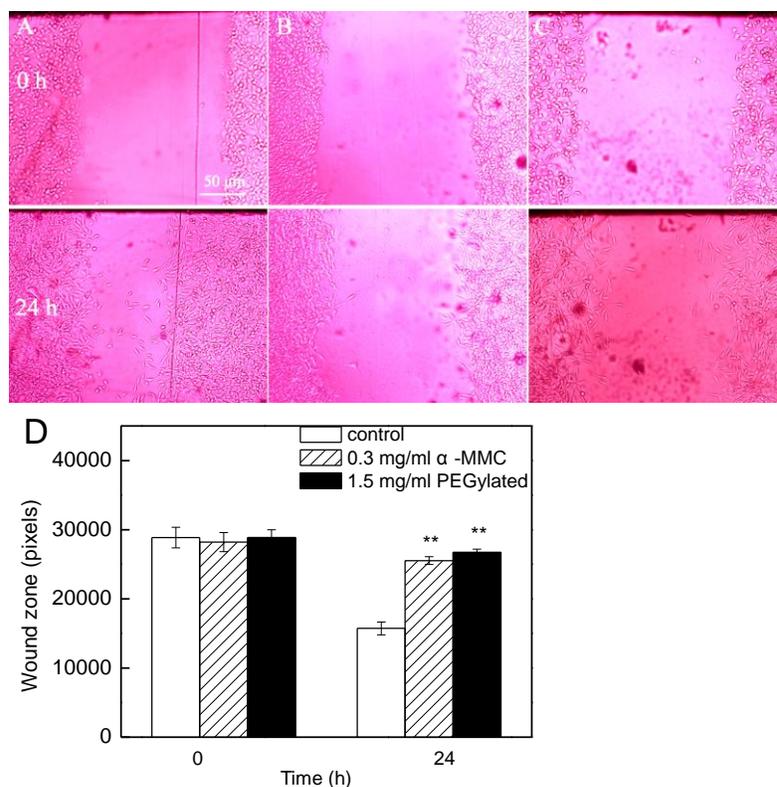


Figure 5. Cell migration observed during a wound closure assay of JAR cells. Microphotographs of untreated control cells (A), cells treated with 0.3 mg/ml α -MMC (B) and 1.5 mg/ml PEG- α -MMC (C) at the start and after 24 h incubation. (D) Quantitative data for the wound area. ** $P < 0.01$.

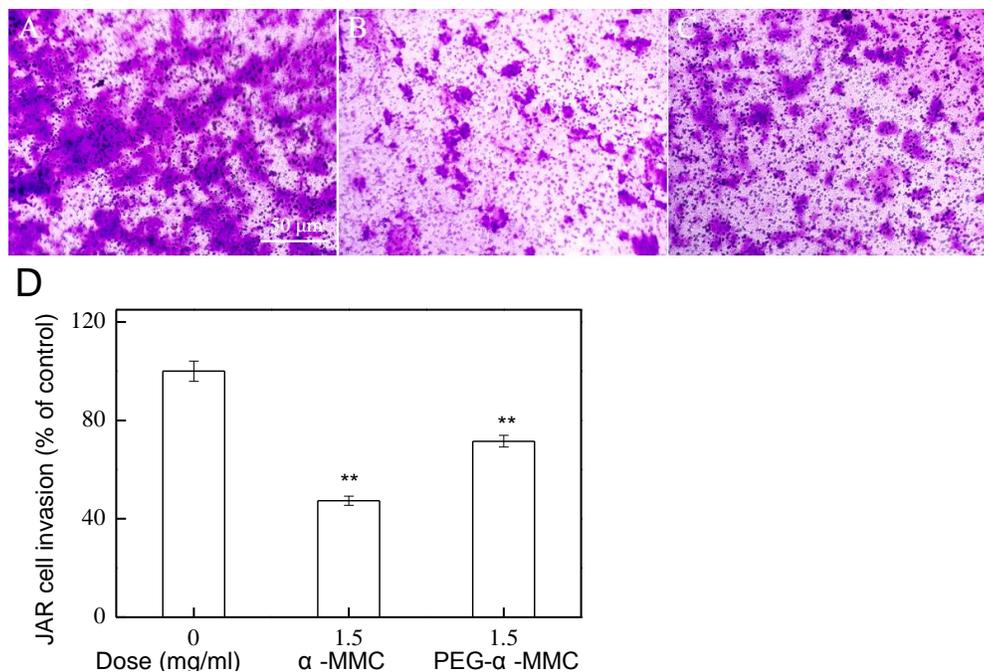


Figure 6. Effects of α -MMC or PEG- α -MMC on JAR cell invasion *in vitro*. JAR cells were photographed after crystal violet staining. JAR cells were treated without α -MMC or PEG- α -MMC (A), with 1.5 mg/ml α -MMC (B) and PEG- α -MMC (C) for 24 h. (D) Inhibition of the invasion by α -MMC and its PEGylated conjugates. Each of the three independent experiments was performed in triplicate. ** $P < 0.01$.

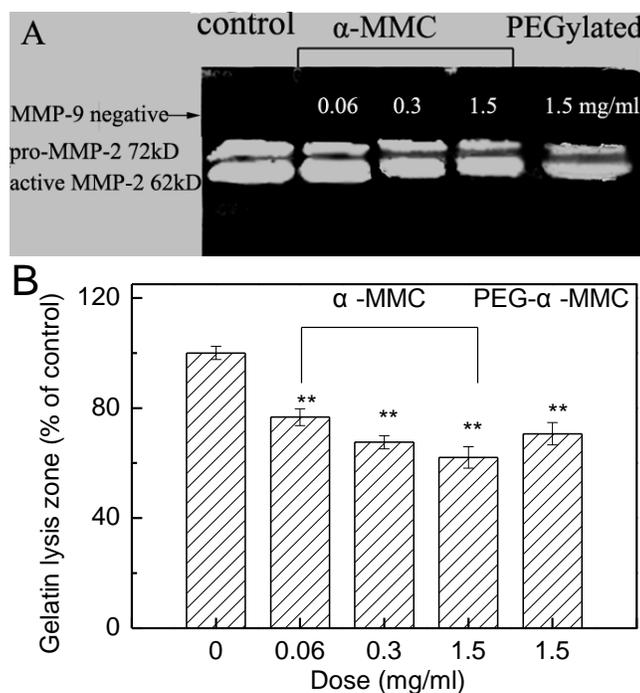


Figure 7. Effects of α -MMC and PEG- α -MMC on the secretion of MMP-2 according to gelatin zymography. (A) Gelatin zymography analysis of serum-free media conditioned by JAR cells treated with α -MMC (0.06, 0.3 and 1.5 mg/ml) or PEG- α -MMC (1.5 mg/ml) for 24 h. (B) Bar graph showing the relative enzyme activity of MMP-2 (pro- and active type). ** $P < 0.01$.

consequence of the proteins treatment. α -MMC is a type I RIP, which are considered to have very low toxicity against cells because they have no receptors at on the cell surface. However, after entering the cytosol, they inhibited protein synthesis at minute concentrations and lead to cell death. Figure 1B demonstrated this well because 24 h treatment with α -MMC did not significantly lower the proliferation of cultured cells. Depending on endocytosis, α -MMC may have required more time to reach a specific level in the cytoplasm and produce a marked effect (Roberts and Lord, 2004). Furthermore, PEG- α -MMC did not significantly inhibit the growth of JAR cells until 48 h of treatment (Figure 1B). Moreover, (mPEG)₂-Lys conjugation masked the protein's surface and increased the molecular size of α -MMC, which made it more difficult to engulf PEG- α -MMC, thereby delaying its effectiveness.

RIPs are known to induce cell death by apoptosis (Sriram et al., 2005). Apoptosis is a well-regulated cellular machinery that removes unnecessary, aged and damaged cells, and its mechanism has been studied with the goal of treating cancer (Ghobrial et al., 2005). Both native and modified α -MMC showed increased the apoptosis of JAR cells (Figure 3), although we did not investigate the mechanism or pathways mediating this effect. However, caspase proteins are recruited during classical apoptosis via the activation of the apoptotic signaling pathway (Vaux et al., 1999). Our previous study indicated that α -MMC and its PEGylated conjugates decreased the proliferation of human epidermal carcinoma A431 cells via caspase 3- dependent apoptosis (Li et al., 2009). The established choriocarcinoma JAR cell line has important advantages for *in vitro* study, such as homogeneity, a low spontaneous rate of apoptosis, and the practicality of its progression procedures (Gaus et al., 1997).

In this study, we utilized the JAR cell line as a model for studying trophoblast proliferation, apoptosis, and progression in response to the biological inducer α -MMC and its PEGylated conjugates. JAR cells invade the extracellular matrix (ECM), because of their ability to secrete MMPs. MMP-2 and MMP-9 are involved in trophoblast invasion into the ECM of the maternal decidua. Thus, MMP inhibitors (MMPi) have been explored widely for the treatment of malignancy (Bjorklund and Koivunen, 2005). Our results showed that α -MMC inhibited MMP-2 activity at the protein level, as did PEG- α -MMC (Figure 7). α -MMC also lowered the *in vitro* progression of JAR cells without cytotoxicity, while PEGylated conjugates behaved in the same way except in terms of their effect on cell adhesion (Figure 4-6).

For the first time, our overall data indicated that α -MMC could inhibit the adhesion, migration, and invasion of choriocarcinoma JAR cell by attenuating the MMP-2 enzyme activity, while PEGylated α -MMC preserved most of its activities.

Thus, α -MMC should be considered as a possible therapeutic agent for decreasing the metastasis and invasion

of choriocarcinoma. Further investigations will be required to exploit the potential of native and modified α -MMC in the treatment of cancer.

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