Original Research Article

Effect of α-momordicine on proliferation and apoptosis of liver cancer, and its associated mechanisms of action

Mei Yang[1], Wen Jin[1], Wei Shi[1], Bo Wang[1], Qing Li[2], Chunfeng Guan[2]*
[1]*Department of Medicine, Tongling Vocational and Technical College, 678 Furong Road, Hefei Economic Development Zone, Anhui Province,* [2]*Department of Obstetrics and Gynecology, Second Affiliated Hospital of Anhui Medical University, Hefei, Anhui Province, China*

For correspondence: **Email: xvk88f@163.com*

Abstract

Purpose: To investigate the effect of α-momordicine (α-MMC) on liver cancer cell proliferation and apoptosis, and to elucidate the mechanisms of action involved.
Methods: In in vitro experiments, hepatoma cell lines were used, while nude mice with hepatocellular carcinoma were used for in vivo studies. Cancer cell proliferation was determined using MTT assay while apoptosis was assayed by flow cytometry and TUNNEL staining. Gene expression was determined with real-time polymerase chain reaction (RT-PCR), while protein expression levels were assayed by Western blot, immunohistochemistry and immunofluorescence.
Results: Alpha-MMC decreased HCC cell viability dose-dependently (p < 0.05). In HepG2 cells, G2/M cell cycle was halted after 48 h intervention with 1.24 mg/mL α-MMC. However, at G0/G1 phase, α-MMC at doses of 1.06 and 0.92 mg/mL caused cell cycle arrest of HCC-LM3 and SMMC-7721 cells. In vivo studies showed that after establishment of the nude mice liver cancer model, exposure to α-MMC at a dose of 0.70 mg/kg or 2.08mg/kg for 4 weeks reduced the size of liver cancer in the treatment group, relative to control group; mean diameter of liver cancer decreased from 2.16 to 0.51 cm, while mean volume decreased from 1.185 to 0.085 cm^3. Moreover, α-MMC increased apoptosis level in liver cancer tissues in nude mice, and down-regulated the expressions of P-AKT, RAGE, MMP-9 and HMGB1, but upregulated Bax/Bcl2 ratio (p < 0.05).
Conclusion: α-MMC inhibits cancer cell growth and proliferation, and facilitates their apoptosis by positively regulating the ratio of Bax/Bcl2. The anti-liver cancer effect of α-MMC is mediated via HMGB1-RAGE and AKT signaling pathways.

Keywords: α-Momordicine, Liver cancer, Proliferation, Apoptosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is a prevalent cancer all over the world. The pathogenesis of liver cancer is not yet clearly understood, but it may be related to gene mutation, infection, immune damage and other factors [1]. Studies have shown that the 5-year survival for advanced liver cancer is extremely low. Radical resection of early liver cancer increases the 5-year survival to more than 60 %, but 60 -70 % of patients still have metastasis and recurrence [2]. Hence, new

treatments are needed to extend patients' survival.

α-Momorcharin (*α*-MMC) is a ribosomal inactivation protein extracted from bitter melon seeds. It hydrolyzes the N-C glycoside bond between adenine base and ribosome at position A4324 of ribosomal 28SrRNA in eukaryotic cells, and prevents the binding of elongator EF-2 to ribosome, thereby inhibiting protein synthesis [3]. Studies have shown that *α*-MMC exerts immune-regulatory effects on breast carcinoma, colorectal carcinoma, hepatoma, non-small cell lung cancer, epithelial cell cancer and glioma [4-6]. The effect of *α*-MMC on apoptosis and proliferation of hepatoma cells has been rarely reported. It has been reported that *α*-MMC may induce hepatocyte apoptosis through low density lipoprotein receptor related protein 1 (LRP1)-mediated Jun N-terminal kinase (JNK) signaling pathway [7].

Receptor of advanced glycosylation end products (RAGE) and high-mobility group Box1 (HMGB1) participate in the proliferation, invasion, migration and apoptosis of liver cancer cells, and are the potential therapeutic targets of HCC [8]. Protein kinase B (AKT) is also important for the apoptosis and proliferation of HCC cells [9].This study investigated the influence of *α*-MMC on HCC proliferation and apoptosis, and its effect on HMGB1, AKT and RAGE expression levels was determined so as to unravel the mechanism involved in the anti-HCC effect of *α*-MMC.

EXPERIMENTAL

Cells and culture

The liver cancer cells (SMMC-7721, HepG2, and HCC-LM3; Shanghai Yaji Biotechnology Co., Ltd.) were cultured in DMEM-1640 medium (Zhengzhou Chuangsheng Bioengineering Co. Ltd.) containing 10 % fetal bovine serum at 5 % CO_2 and 37 °C. Passage was carried out with 0.25 % trypsin. The cells used in this experiment were passage 3 cells.

Evaluation of effect of α-MMC on proliferative potential

The hepatoma cell lines were incubated in at a concentration of 2×10^4 cells per well, and treated with different doses of *α*-MMC (0.45, 0.60, 0.75, 0.90, 1.05, 1.20, 1.35 and 1.50 mg/mL) [6]. The *α*-MMC was obtained from Shanghai Beizhuo Biotechnology Co. Ltd. Untreated cells served as control. Cell proliferation was measured using MTT kit (Shanghai Gefan Biotechnology Co. Ltd.) with absorbance readings obtained at 450 nm in a Rebec microplate reader.

Flow cytometry

Detection of apoptosis was done using flow cytometry (BD, USA), and Annexin V-FITC/PI apoptosis assay kit (Aimei Technology Co. Ltd). Apoptosis in the hepatoma cells was analyzed. Cell cycle analysis was performed flow cytometrically, and the proportions of early and late apoptotic cells were calculated using Modfit LT software.

TUNNEL staining for determination of apoptosis in tumor tissues

Liver cancer tissue was placed in paraformaldehyde for 24 h, and after dehydration and transparency, it was embedded in paraffin. The thickness of unbroken section was 4 to 6 mm. Sodium citrate (0.1 mol/L, 200 mL) was heated in a microwave for 5 min, and the slices were taken out and immersed in distilled water. The TUNNEL test was performed using an *in situ* apoptosis kit. Positive staining was depicted as brown color. The number of apoptotic cells was counted.

RT-PCR

The levels of expression of Bcl2, Bax, matrix metalloprotein-9 (MMP9), HMGB1, and RAGE mRNA were assayed with PT-PCR. The primers sequences used are shown in Table 1.

Table 1: Primers sequences

	Upstream	Downstream
Bcl-2	5'-CATGTGTGTGGAGAGCGTCAA-3'	5'-GCCGGTTCAGGTACTCAGTCA-3'
MMP9	5'-CGTCGTGATCCCCACTTACTATGGAAACTC-3'	5'-GCAGAAGCCATACAGTTTATCCTGGTCATA-3'
Bax	5'-GATCCAGGATCGAGCAGA-3'	5'-AAGTAGAAGAGGGCAACCAC-3'
HMGB1	5'-CGGATGCTTCTGTCAACTTCT-3'	5'-AGTTTCTTCGCAACATCACCA-3'
RAGE	5'-CGGCTGGTGTTCCCAATAA-3'	5'-TGTTCCTTCACAGATACTCCCT-3'
β-Actin	5'-CTGGAACGGTGAAGGTGACA-3'	5'-AAGGGACTTCCTGTAACAATGCA-3'

The test kits were purchased from Shenggong Bioengineering (Shanghai) Co. Ltd. Extraction of total RNA was done using Trizol reagent, and RT-PCR was carried out after reverse transcription. The primer sequences were designed by Shanghai Bioengineering Co. Ltd. Each sample was assayed at least 3 times in a set of 3 duplicate wells. The RT-PCR reaction conditions were: 30 sec pre-denaturation at 95 °C, 5 sec PCR reaction at 95 °C, 60 °C for 30 sec, and a total of 40 cycles.

Western blot assay

The expression levels of BCL2, Bax, HMGB, p-AKT, AKT and MMP9 were assayed with Western blotting. The antibodies were purchased from Santa Cruz Biotechnology, Inc., USA. The proteins were denatured by boiling with SDS buffer for 5 min. Then, SDS-polyacrylamide gel electrophoresis was followed by transfer of the proteins to PVDF membranes, sealing with 5 % skim milk powder, and overnight incubation with the primary antibodies at 4 °C. Thereafter, the membrane was washed 3 times, each for 5 min, and incubated with secondary antibody for 2 h at 37 ℃. The membrane was then washed 3 times with TBST. The blots were visualized with enhanced chemiluminescence (ECL), with β-actin as reference, and the ratio of the target protein band gray scale to that of β-actin was calculated.

Establishment and treatment of liver cancer models

Four-week-old nude mice were purchased from Shanghai Yusen Biotechnology Co. Ltd from 2006 to 2009. The feeding environment temperature was 22±2°C, humidity was 50 ± 10 %, with 12-h dark/12-h light photoperiod. The mice were permitted *ad libitum* access to feed and clean water. The HCC-LM3 cells were maintained in 0.1 L of DMEM: F12 (1:1, v: v) serum-free medium. The nude mice were anesthetized with 2 % pentobarbital (45 mg/kg). After laparotomy, the liver of 30 mice were subjected to inoculation with the cancer cells. The mice were subsequently checked at 3-day intervals (total monitoring period was 4 weeks and 2 days.

Thereafter, the animals were assigned to 3 groups. One group was intraperitoneally injected α-MMC at a dose of 2.08 mg/kg. Another group was intraperitoneally injected α-MMC at a dose of 0.70 mg/kg, while a third group was intraperitoneally injected an equivalent volume of normal saline; all treatments lasted for 4 weeks [10]. The nude mice were sacrificed via cervical dislocation and the tumor size was measured with a Vernier caliper. Ethical approval for the animal experiment was obtained from the Second Affiliated Hospital of Anhui Medical University (approval no. = DE2018008). Animal handling was in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines [11].

Immunohistochemical assessment

Liver cancer tissues were placed in paraformaldehyde and fixed for 24 h. After dehydration and transparency, paraffin embedding was carried out. The thickness of continuous section was 4 to 6 mm. During the test, xylene was used for dewaxing, ethanol was used for dehydration, while 3 % hydrogen peroxide solution was used to block endogenous peroxidase activity. The slices were heated in citrate buffer, pH 6 for 30 min, and incubated with 5 % BSA for 45 min, followed by incubation with antibodies against p-AKT, HMGB1 and MMP9 (Santa Cruz Biotechnology, USA) overnight at 4 ℃. Then, secondary antibody was added and incubation was continued at 37 ℃ for 45 min, after which the sections were stained with H & E.

Immunofluorescence assay

The hepatocarcinoma cells were seeded in 12-well plates for EP treatment [half inhibitory concentration (IC_{50})] or physiological saline for 2 days, and divided into sham group and α-MMC group. The cells were plated on gelatin-coated coverslips, fixed with 4 % paraformaldehyde, and rinsed 3 times with PBS, each rinse for 5 min. Then, the cells were blocked with 1 % BSA for 1 h at 37 ℃ and incubated overnight with primary antibodies. They were then washed 3 times with PBS, and incubated with secondary antibody at 37 ℃ for 1 h.

The cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 15 min, and rinsed for 5 min. After mounting on glycerol, the cells were observed under a fluorescence microscope and photographed. The expressions of HMGB1 and MMP9 were observed under a fluorescence microscope.

Statistics

Data are expressed as mean ± SD, and were statistically analyzed using SPSS version 19.0. Independent sample *t*-test and LSD-t method were used for 2-group comparisons, while one-way ANOVA was used for multiple-group comparisons. Statistical significance was assumed at $p < 0.05$.

RESULTS

α-MMC inhibited proliferation of liver cancer cells

As shown Figure 1A, *α*-MMC caused decreases in the survival of liver cancer cells (SMMC-7721, HepG2, HCC-LM3) in a dose-dependent manner. From MTT results, SMMC-7721 (0.92 mg/mL), HepG (21.24 mg/mL) and HCC-LM (31.06 mg/mL) were selected for subsequent experiments. After establishing a nude mouse model of liver cancer, *α*-MMC was administered at a dose of 0.70 or 2.08 mg/kg, and the mice were sacrificed 4 weeks later. The liver cancer of the treatment group was smaller than that of the control group (Figure 1D). After treatment, the diameter and volume of liver cancer cells were significantly decreased ($p < 0.05$, Figures 1B and 1C). These results indicated that *α*-MMC exerted anti-liver cancer effects.
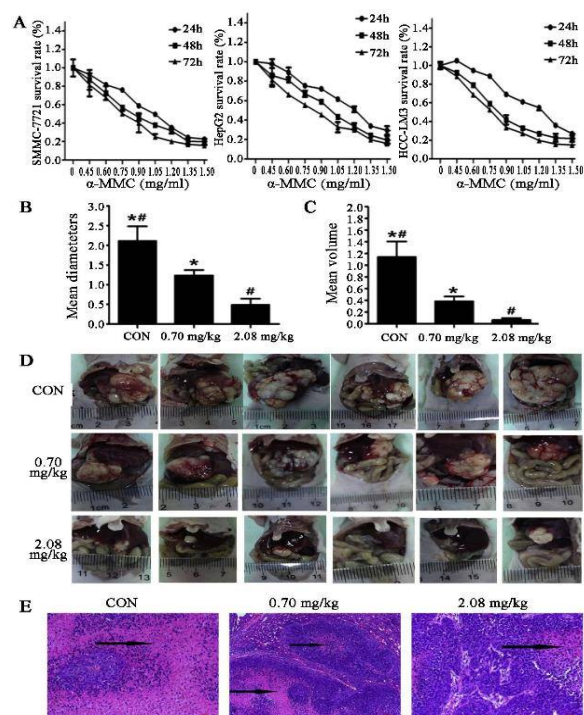

Figure 1: Results of *in vivo* and *in vitro* studies on the effect of *α*-MMC on hepatoma cell proliferation. (A) MTT assay for cell proliferation; (B) tumor diameter changes after injection of *α*-MMC in nude mice; (C) tumor volume changes after injection of *α*-MMC in nude mice; (D) H & E staining showing liver cancer tissue structure (×200)

α-MMC arrested liver cancer cell cycle and induced apoptosis

As shown in Figure 2A, in HepG2 cells, *α*-MMC at a dose of 1.24 mg/mL for 48 h interfered with the cell cycle at G2/M phase; whereas at doses of 0.92 and 1.06 mg/mL, *α*-MMC arrested the cell cycle of the other two cell lines at G0/G1 phase. As shown in Figure 2B, the proportion of early apoptosis and the proportion of late apoptosis increased after *α*-MMC treatment ($p < 0.05$). There was an increase in the ratio of BAX/BCL2 in the hepatoma cell line after *α*-MMC treatment ($p < 0.05$, Figure 2C and Figure 2D). In *in vivo* studies, TUNNEL staining, RT-PCT, and western-blotting assays showed increased apoptosis in hepatoma cells after *α*-MMC exposure (Figure 3).

α-MMC inhibited HMGB1-RACE and AKT signal routes

Results showed that *α*-MMC significantly reduced the expression levels of HMGB1, RAGE and MMP9 genes ($p < 0.05$, Figure 4A). The results of Western-blotting assay showed that *α*-MMC significantly downregulated protein expressions of RAGE, HMGB1, MMP9 and P-AKT ($p < 0.05$, Figure 4B). In addition, immunofluorescence assay showed that *α*-MMC downregulated the expressions of HMGB1 and MMP9 (Figure 4C). The results were similar *in vivo* (Figures 3C-3E).
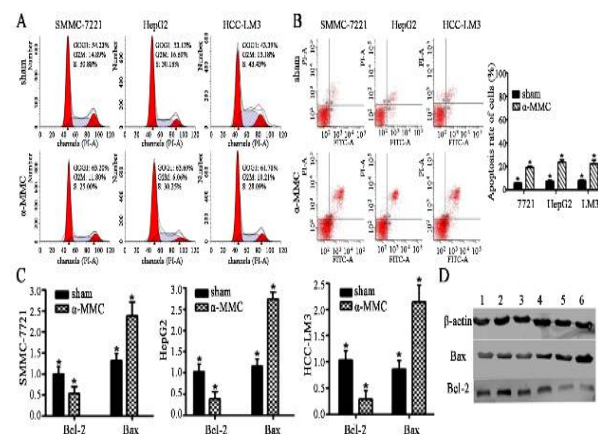

Figure 2: *In vitro* effect of *α*-MMC on hepatoma cell cycle and apoptosis. (A) α-MMC caused cell cycle arrest in different stages of cell cycle in different liver cancer cells; (B) *α*-MMC induced apoptosis of hepatoma cells, and flow cytometric analysis of the hepatoma cell lines with Annexin-V/ PI staining showed that the proportion of cells in early apoptosis and late apoptosis increased after *α*-MMC intervention; (C) results of RT-PCR on the effect of *α*-MMC on Bcl2 and Bax gene expression levels *in vitro*; (D) Bcl2 and Bax expression levels as determined with Western blotting. 1: SMMC-7721 dealt with normal saline; 2: HepG2 dealt with normal saline; 3: HCC-LM3 dealt with normal saline; 4: SMMC-7721 treated with 0.92 mg/mL *α*-MMC; 5: HepG2 treated with 1.24 mg/mL *α*-MMC; 6: HCC-LM3 treated with 1.06 mg/mL *α*-MMC
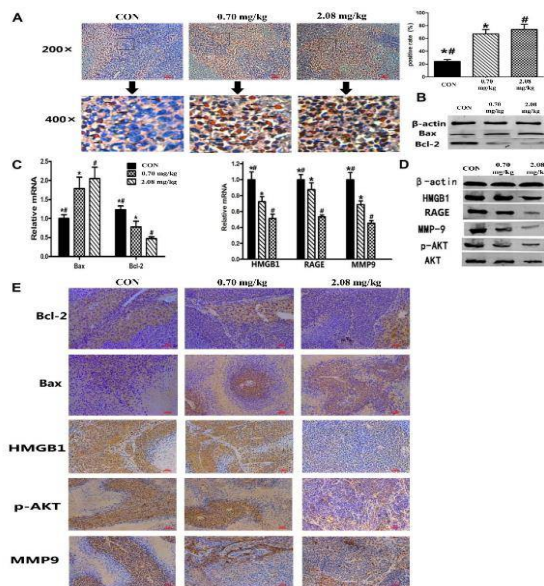
Figure 3: Results of *in vivo* studies on the effect of *α*-MMC. (A) TUNNEL staining showed that *α*-MMC-induced apoptosis of hepatocarcinoma cells in three groups of nude mice; (B) Western blotting results on the effect of *α*-MMC on the expressions of Bcl-2 and Bax in liver cancer tissues; (C) RT-PCR results on the effect of *α*-MMC on expressions of the various genes; (D) levels of expression of RAGE, HMGB1, AKT, MMP9, and p-AKT; (E) immunohistochemical results of the levels of Bax, Bcl-2, p-AKT, HMGB1, and MMP9 in the cancer tissues (×200)
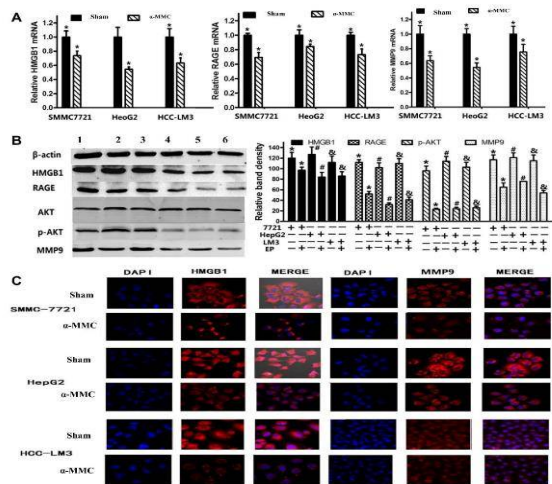

Figure 4: Effect of *α*-MMC on the HMGB1-RACE and AKT signaling pathways. (A) HMGB1, RAGE and MMP9 gene expressions in hepatoma cells; (B) Western-blotting was used to determine the expression levels of HMGB1, RAGE, P-AKT and MMP9 in hepatoma cells *in vitro*. 1: SMMC-7721 treated with normal saline; 2: HepG2 treated with normal saline; 3: HCC-LM3 treated with normal saline; 4: SMMC-7721 treated with 0.92 mg/mL *α*-MMC; 5: HepG2 treated with 1.24 mg/mL *α*-MMC; HCC-LM3 treated with 1.06 mg/mL *α*-MMC; (C) results of immunofluorescence for the expression levels of HMGB1 and MMP9 in hepatoma cells (×200)

DISCUSSION

Liver cancer is a common malignant tumor in the digestive system. *α*-Momordicine **(**α*-MMC) is a type I ribosome-inactivating protein which is extracted from bitter gourd seeds. It modifies the ribosome subunit of ribosomal RNA, thereby inhibiting protein synthesis. Studies have shown that *α*-MMC has anti-AIDS, anti-tumor and anti-fertility effects, but large doses of *α*-MMC impair the immune system when used in animal therapy [12]. Therefore, the present study used medium and low doses of *α*-MMC on liver cancer cells and nude mice in accordance with previous studies [6,10].

It has been reported in *in vivo* and *in vitro* studies on breast cancer that *α*-MMC has anticancer effects which result in inhibition of the proliferation, and induction of apoptosis of cancer cells [4]. This study found that *α*-MMC showed anti-liver cancer effects by inhibiting proliferation and inducing apoptosis. Flow cytometry and MTT results revealed that HCC proliferation was inhibited after 48 h treatment with *α*-MMC, which also induced apoptosis and cell cycle arrest. In *in vivo* studies, H & E staining and TUNNEL staining also confirmed the anti-tumor property of *α*-MMC.

The molecular mechanism involved in the growth-inhibiting effect of liver cancer cells by of *α*-MMC is not yet clear. HMGB1-RACE and AKT signaling pathways are closely related to the occurrence of multifarious cancers such NSCLC, hepatoma and skin squamous cell carcinoma [13-15]. Studies have found that HMGB1 expression level is related to the clinical staging of liver cancer, suggesting that HMGB1 may be a marker of liver cancer. Targeted treatment of HMGB1 gene may contribute to the treatment of liver cancer [16]. The over-expression of RAGE, a membrane receptor which is expressed in many cell membranes, has been reported in several cancers and tumor-related tissues [17].

Previous studies have confirmed that blocking the HMGB1-RAGE pathway inhibits the occurrence of gastric cancer [18]. In addition, abnormal expression of AKT is associated with tumor growth and proliferation. The levels of AKT and p-AKT are elevated in HCC tissues and can help predict clinical outcomes in HCC patients [19]. In the present study, *α*-MMC regulated the HMGB1-RAGE and AKT signaling pathways in liver cancer cells. There is an association between AKT-induced MMP9 and HMGB1 expression [20]. Down-regulation of MMP9 inhibits tumor cell proliferation and induces apoptosis [21]. The results of this study showed

that *α*-MMC downregulated the expression of MMP9 in liver cancer cells. When combined with RAGE, HMGB1 activated MMP9 and promoted tumor proliferation. Therefore, *α*-MMC may play an important role in the treatment of liver cancer by down-regulating HMGB1 and RAGE. During apoptosis, Bax forms oligomers on the outer membrane of mitochondria.

In contrast, Bcl-2 inhibits mitochondrial apoptosis by blocking the Bax-induced oligomer formation. The results of this study showed disturbance in the Bax: Bcl2 ratio in HCC cells, with increased expression of Bax and decreased expression of Bcl2 due to treatment with *α*-MMC. Flow cytometry results showed that the proportions of early apoptosis (quadrant 2) and late apoptosis (quadrant 3) increased after treatment with *α*-MMC. Thus, it can be reasonably speculated that *α*-MMC exerts anti-liver cancer effect by regulating Bax/Bcl2 ratio.

CONCLUSION

This study found that *α*-MMC, an HMGB1 inhibitor, restrains the proliferation of liver cancer cells, and promotes their apoptosis by positively regulating the ratio of Bax/Bcl2. Moreover, this study has shown that the anti-liver cancer effect of *α*-MMC is mediated through the HMGB1-RAGE and AKT signaling pathway. Thus, *α*-MMC may be a potential drug for treating liver cancer but further investigations are required to confirm this.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Guan Chunfeng conceived and designed the study, Yang Mei, Jin Wen, Shi Wei, Wang Bo, Li Qing, Guan Chunfeng collected and analyzed the data. Yang Mei wrote the manuscript.

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