Triptolide suppresses growth of breast cancer by targeting HMGB1 in vitro and in vivo

Wei Jiang\textsuperscript{a,1}, Maojian Chen\textsuperscript{b,1}, Chanchan Xiao\textsuperscript{c}, Weiping Yang\textsuperscript{b}, Qinghong Qin\textsuperscript{b}, Qixing Tan\textsuperscript{b}, Zhijie Liang\textsuperscript{d}, Xiaoli Liao\textsuperscript{a}, Anyun Mao\textsuperscript{b}, Changyuan Wei\textsuperscript{b,*}

\textsuperscript{1}These authors contributed equally to this work.

\textsuperscript{a}Department of Medical Oncology, \textsuperscript{b}Department of Breast Surgery, \textsuperscript{c}Department of Experimental Research, The Affiliated Tumor Hospital of Guangxi Medical University, Nanning, Guangxi, 530021, China

\textsuperscript{d}Department of Breast and Thyroid Surgery, The Fifth Affiliated Hospital of Guangxi Medical University & The First People’s Hospital of Nanning, Nanning, Guangxi, 530021, China

\textsuperscript{*}All correspondence should be addressed to Dr. Changyuan Wei, Department of Breast Surgery, The Affiliated Tumor Hospital of Guangxi Medical University, Nanning, Guangxi, 530021, China. (Fax: +86 0771 5308593; email: weicy63@aliyun.com)
Abstract

Triptolide has been indicated potent anti-cancer effect involving multiple molecular targets and signaling pathways. High-mobility group box 1 (HMGB1) is a highly conserved DNA-binding protein taking part in breast cancer development. The therapeutic effect of triptolide on HMGB1 has not been reported. Thus, our study aims to clarify the role of HMGB1 in triptolide-induced anti-growth effect on breast cancer in vitro and in vivo. We demonstrated that triptolide significantly suppressed growth of breast cancer cells by inhibition of cell viability, clonogenic ability. Further studies evidenced that triptolide treatment not only inhibited HMGB1 mRNA expression, but also decreased supernatant level of HMGB1 in vitro. In line with these observations, exogenous recombinant HMGB1 promoted cell proliferation of breast cancer, and triptolide reversed the rHMGB1-promoted proliferative effect. As well, triptolide enhanced the anti-proliferative activity of EP (HMGB1 inhibitor). Furthermore, downstream correlation factors (TLR4 and phosphorylated-NF-κB p65) of HMGB1 were significantly decreased in vitro after triptolide treatment. Consistantly, we confirmed that tumor growth was significantly inhibited after triptolide treatment In vivo. Meanwhile, immunohistochemical analyses showed that triptolide treatment significantly decreased the level of cytoplasmic HMGB1 and TLR4 expression, whereas the expression of NF-κB p65 was relatively higher in cytoplasm, and conversely lower in nucleus as compared to the control group. Collectively, these results demonstrate that triptolide suppresses the growth of breast cancer cells via reduction of HMGB1 expression in vitro and in vivo, which may provide new insights into the treatment of breast cancer.
**KEYWORDS:** triptolide; breast cancer; growth; high-mobility group box 1 (HMGB1)
1. Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-related death in women \(^1\). The 5-year survival rate is merely 20% once the recurrence occurs \(^2\). As standard therapeutic modalities for breast cancer, chemotherapy and radiation significantly decrease tumor recurrence and prolong patient survival. However, the administration of chemotherapy and radiation is restricted for their toxicity in clinical practice. Therefore, there is an urgent need for safer therapeutic approach to improve the survival of patients with breast cancer.

Triptolide, a natural and biologically active compound as a diterpenoid triepoxide, was originally extracted and isolated from the root of traditional Chinese herb Tripterygium wilfordii Hook F \(^3\). Currently, several researches have demonstrated versatile biological activities of triptolide such as anti-inflammatory, anti-oxidant, anti-tumor and anti-angiogenesis \(^4-6\). Anti-tumor effect of triptolide has been investigated for many different types of cancer including hepatocellular carcinoma, prostate cancer, bladder cancer, pancreatic cancer and breast cancer \(^6-9\). All these findings shed some light upon molecular mechanisms of anti-cancer effect of triptolide. However, the exact targets and molecular mechanism remain poorly understood.

High-mobility group box 1 (HMGB1) is a highly conserved DNA-binding protein present in the most cell types. As a nuclear protein, HMGB1 modulates chromatin structure, promotes interaction of proteins, and plays a role as a transcription factor in gene expression regulation \(^10\). In addition to its nuclear expression, HMGB1 can be passively released by necrotic cells \(^11\). As well, it can be actively secreted by inflammatory cells \(^12\) and some cancer
cells \(^{13, 14}\) into the extracellular matrix, where it acts as a damage-associated molecular pattern (DAMP) mediating inflammation or plays a role as chemoattractant factor \(^{15}\). Binding to different cell surface receptors such as Toll-like receptors (TLRs) and advanced glycation end products (RAGE) in direct or indirect way \(^{12}\), HMGB1 can promote the biologic development of cancer including tumor proliferation, invasion and metastasis \(^{16}\). Ni et al. showed that the induction of HMGB1 contributed to breast cancer tumorigenesis, while HMGB1 silence suppressed the progression of breast cancer \(^{17}\). Tang et al. found that exogenous HMGB1 promoted differentiation syndrome in acute promyelocytic leukemia cells \(^{18}\). As well, excessive secretion of HBGB1 was associated with promoted proliferation and invasion of malignant mesothelioma \(^{14}\) and hepatocellular carcinoma \(^{19}\). Considering its role as mediator to the progression of tumorigenesis, HMGB1 targeted therapy may be of meaningful value in anti-cancer treatment \(^{20, 21}\).

Given the importance of HMGB1 in tumor initiation and progression, the aim of this study is to investigate whether triptolide could suppress growth of breast cancer by inhibiting HMGB1 expression.
2. Materials and methods

2.1. Materials

Triptolide was purchased from MedChemexpress (Cat. No.: HY-32735; Purity: 99.83%; Manmouth Junction, NJ, USA). Ethyl pyruvate (EP) was purchased from Sigma Chemical (St. Louis, MO, USA). Recombinant HMGB1 (rHMGB1) was purchased from Sino Biological Inc (Beijing, China). HMGB1 ELISA kit was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). The antibody against TLR4 was purchased from Proteintech (Chicago, IL, USA), and the antibodies against HMGB1, NF-κB p65, phosphor-NF-κB p65 and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). A Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.2. Cell culture

MCF-7 and MDA-MB-231 (human breast cancer cell lines) were obtained from the Cell Culture Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin in 5% CO₂ at 37 °C.

2.3. Cell viability assay

Cells were seeded in 96 well plates at a density of 2×10³ cells/100μl/well. After 24 h incubation to attachment, triptolide (0, 5, 10, 20, 40, 80 and 160 nmol/L) was administered to the cells for 24 h, 48h and 72 h, respectively. The other settings were treated with rHMGB1 (10 ng/mL) or EP (10 mmol/L) alone or in combination with triptolide (20 nmol/L) for 24 h, 48h and 72h. Then each well was added with 10 μl CCK-8 and cultivated for 2 h. Absorbance of each well was detected at 450 nm by enzyme immunoassay instrument.
2.4. Colony formation assay

Cells were inoculated in 6 well plates with 500 cells per well. After 24 h incubation to attachment, they were given various concentrations (0, 10, 20, 40 nmol/L) of triptolide for 12 h and then cultured in a normal condition for two weeks. The colonies were fixed with 4% paraformaldehyde solution and stained with 0.1% crystal violet. Colonies with >50 cells were scored.

2.5. Quantitative real-time PCR

Total RNA was isolated using Trizol (Invitrogen, California, USA). Then cDNA was synthesized using Reverse Transcriptase kit (Takara, Kusatsu, Japan). mRNA levels were detected by real-time PCR using SYBR Premix Ex Taq™ II real-time PCR kit (Takara, Kusatsu, Japan) as standard methods. The specific primers as follows: HMGB1 (F) 5’-TGTGCAAACTTGTCGGGAG-3’, (R) 5’-TCTTTTCATAACGGGCCTTGTC-3’; GAPDH (F) 5’-AGAAGGCTGGGGCTCATTTG-3’; (R) 5’-AGGGGCCATCCACAGTCTTC-3’. Each sample was assessed in triplicate, and GAPDH was employed to normalize the results.

2.6. ELISA analysis

Supernatants were collected and undergone ELISA analysis of HMGB1 level using commercial kits as standard method.

2.7. Western blot analysis

The total protein was extracted from the harvested cells using cold RIPA lysis buffer (Solarbio, Beijing, China) containing a protease inhibitor cocktail (Solarbio, Beijing, China) and then quantified via the bicinchoninic acid method. An aliquot of protein lysate was separated by SDS-PAGE and transferred onto PVDF membranes (Solarbio, Beijing, China).
Blocked by 5% non-fat milk for 1 hour at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies against TLR4 (1:1000), NF-κB p65 (1:1000), phosphor-NF-κB p65 (1:500) and β-actin (1:1000), followed by horseradish peroxidase-conjugated secondary antibodies. The protein expression was evaluated using enhanced chemiluminescence system ChemiDoc MP (BioRad, Hercules, CA, USA).

2.8. Immunohistochemical staining

Immunohistochemical staining was performed as previously described. The primary antibody dilution concentration was including anti-HMGB1 (1:400), anti-TLR4 (1:200) and anti-NF-κB p65 (1:800). The immunohistochemical character results were evaluated under a light microscope.

2.9. In vivo xenograft growth study

Female BALB/c athymic mice (4-5 weeks, 14-16 g) were purchased from Guangxi Medical University Experimental Animal Center and maintained under specific pathogen free condition. All procedures were approved by the Guangxi Medical University Experimental Animal Committee. After harvested and resuspended in PBS, suspension (200 μL) containing \(1\times10^7\) MDA-MB-231 cells were injected subcutaneously into the back of each mouse (n=3 mice per group). Mice were administered intraperitoneally with triptolide (0.15 mg/kg or 0.30 mg/kg) for 14 days once the visible tumor reached around about 100 mm\(^3\). Vehicle-treated animals with equal volume of PBS. The size of the shaped tumors was measured using gauged caliper every three days, and the tumor volume was calculated using the formula: \[\text{volume}(V) = (\text{length} \times \text{width}^2)/2.\] At the end of the experiment, the mice were sacrificed and the tumors were dissected, photographed, weighed and harvested.
3.0. Statistical analysis

All data are presented as means ± standard deviation. Statistical differences were assessed by Student’s t test or one-way ANOVA. All statistical analyses were performed using the SPSS v17.0 (SPSS Inc, Chicago, USA). P value less than 0.05 was considered of statistical significance.
3. Results

3.1. Triptolide suppresses the cell viability and clonogenic ability of breast cancer cells

We first investigated the biological function of triptolide on cell proliferation. CCK-8 results showed that triptolide elicited a significant suppression on cell viability of breast cancer cells (MCF-7, MDA-MB-231) in a dose/time-dependent manner (Fig. 1 A), confirmed by results of colony formation assay (Fig. 1 B).

3.2 Triptolide inhibits HMGB1 expression in breast cancer cells

Next, we explored the effect of triptolide on the HMGB1 expression in breast cancer cells (MCF-7, MDA-MB-231). As shown in Fig. 2 A, B, C and D, triptolide suppressed the expression of HMGB1 mRNA in a dose/time-dependent manner, with a significant down-regulation noted as early as 3 h. Then we conducted ELISA analysis of supernatants to examined HMGB1 extracellular release after triptolide treatment. Consistently, supernatant level of HMGB1 was distinctly decreased in a dose/time-dependent manner (Fig. 2 E, F, G and H).

3.3. Triptolide reverses the effect of rHMGB1-promoted proliferation in breast cancer cells

Above results prompted us to explore the relationship between anti-proliferative activity of triptolide and the effect of extracellular HMGB1 in breast cancer cells (MCF-7, MDA-MB-231). CCK-8 results showed that rHMGB1 (10 ng/mL) significantly promoted proliferation of breast cancer cells, whereas treatment with triptolide (20 nmol/L) perceptibly reversed the proliferative effect promoted by rHMGB1 (Fig. 3 A and B), suggesting triptolide antagonized HMGB1 during the process of its cell proliferation inhibition.
3.4. Triptolide enhances the anti-proliferative activity of EP in breast cancer cells

To further obtain evidence to confirm our perception, we observed whether triptolide could enhance the anti-proliferative activity of EP (a potent HMGB1 inhibitor) in breast cancer cells (MCF-7, MDA-MB-231). As expected, CCK-8 results showed that the blockage of HMGB1 by EP (10 mmol/L) effectively inhibited the proliferation of breast cancer cells (Fig. 3 C and D). More importantly, we observed that combined treatment of EP and triptolide (20 nmol/L) significantly suppressed cell proliferation than that of cells treated with EP or triptolide alone (Fig. 3 C and D). And the ELISA results showed that combined treatment of EP and triptolide significantly decreased the supernatant level of HMGB1 compared with EP or triptolide alone (Fig. 3 E and F), implying the synergic effect of EP and triptolide in blockade of HMGB1 during the process of cell proliferation inhibition.

3.5. Triptolide inhibits TLR4 expression and NF-κB phosphorylation in breast cancer cells

Previous researches have indicated that TLR4 and NF-κB played a crucial role as downstream correlation factors on the signaling pathway of HMGB1. Based on the above observations, we next assessed the impact of triptolide on the expression of TLR4 and NF-κB. Consistent with our expectation, the western blot analysis revealed that triptolide inhibits TLR4 expression and phosphorylate-NF-κB p65 but not NF-κB p65 in breast cancer cells (MCF-7, MDA-MB-231) (Fig. 4).

3.6. Triptolide suppresses the tumor growth of breast cancer in vivo

Finally, to assess the biological relevance of the obtained results in vitro, we investigated whether triptolide also exhibits anti-tumor growth effect on breast cancer in vivo.
tumorigenicity of MDA-MB-231 cells was detected by tumor transplantation in athymic mouse with or without triptolide treatment. As shown in Fig. 5 A, B and C, tumor growth indices (tumor size, tumor weight) were significantly reduced after triptolide treatment. Furthermore, the immunohistochemical analyses of shaped tumor specimens showed that triptolide treatment also significantly attenuated the level of cytoplasmic HMGB1 and the expression TLR4 compared to the control group (Fig. 5 D), which was consistent with the in vitro results as aforementioned (Fig. 4). Of noted, the expression of NF-κB p65 was relatively higher in cytoplasm, and conversely lower in nucleus after triptolide treatment as compared to the control group (Fig. 5 D).

4. Discussion

Given the potential sanative efficacy and fewer toxicity and side effects, Chinese herbs attract more and more attention of researchers recently. In 1970s, triptolide was first isolated from traditional Chinese herb Tripterygium wilfordii Hook F and purified\(^{25}\). As the progress in pharmacological research of triptolide, it has been reported to exhibit a clinical significance in anti-cancer treatment\(^{26,27}\). Previous studies have showed that more than one signaling pathway attributing to the anti-tumor effect of triptolide. Gao et al. found that triptolide induced apoptosis of breast cancer in vitro through p38/Erk/mTOR phosphorylation\(^{28}\), whereas ERα-mediated signaling pathway was presented to involve in the inhibitive effect of triptolide on breast cancer MCF-7 cells growth\(^{29}\). In addition, triptolide has been shown to induce FAK cleavage\(^{30}\). More recently, triptolide has been identified a role of anti-angiogenesis via the inhibition of VEGF in breast cancer cells\(^{31}\). Wang and colleagues reported that extensive transcriptional inhibitive activity of triptolide was associated with the induction of
phosphorylation and subsequent proteasome-dependent degradation of RNA polymerase II in breast cancer cells\textsuperscript{32}). In view of the anti-cancer activity of triptolide with exact underlining mechanisms largely unknown, in the current study, we aimed to explore the association of anti-growth effect of triptolide and HMGB1 that is located in another vital molecular pathway taking part in the tumorigenicity of breast cancer. Consistent with the results from Li et al. that triptolide dose-dependently inhibited the viability of both MCF-7 and MDA-MB-231 cells \textsuperscript{8)}, our studies confirmed the treatment efficacy of triptolide in breast cancer cells based on the inhibition of the cell viability and clonogenic ability in a dose/time dependent manner. Moreover, we succeeded in establishing the breast cancer xenograft model in mice and further validated the anti-growth effect of triptolide \textit{in vivo}.

Various factors involved in different signal pathway have been found to take part in the development of cancer. HMGB1 is a conserved nuclear protein and plays a critical role in nucleosome stabilization and gene transcription \textsuperscript{16}). Moreover, HMGB1 up-regulation was recently proved to act as the link between tumor-associated inflammation and tumorigenesis \textsuperscript{33}). Previously, HMGB1 was identified to stably express in nucleus of the quiescent cells. Then, HMGB1 secretion was confirmed as its translocation from the nucleus to the cytoplasm \textsuperscript{10}). Recently, many reports have showed that overexpression of extracellular HMGB1 contributed to cancer carcinogenesis and metastasis by promoting apoptotic evasion, mediating tumor-associated inflammation, and increasing tumor cell proliferation, migration and angiogenesis \textsuperscript{16, 20, 34}). Enhanced HMGB1 expression were demonstrated in patients with cancers such as breast cancer \textsuperscript{35)}, cervical carcinoma \textsuperscript{36)}, metastatic prostate cancer \textsuperscript{37)}, and hepatocellular carcinoma \textsuperscript{38}). Therefore, antagonizing HMGB1 may be a new target for cancer
therapy. In the present study, for the first time, our findings showed that triptolide treatment significantly suppressed the expression and release of HMGB1 of breast cancer in vitro and in vivo. However, in vitro, triptolide potently inhibited the cell viability at more than 20 nmol/L of triptolide, while the inhibitory effect of triptolide (> 20 nmol/L) on the expression of the HMGB1 gene and extracellular release of HMGB1 protein was a little stronger than the viability. Given the extensive transcriptional inhibition activity of triptolide in multiple signaling pathway, we need to take not only HMGB1 but also other factors involved in other pathways into account with respect to the effect of triptolide on cell death. Besides, in accordance with the report that HMGB1 promote MCF-7 breast cancer cells proliferation and silencing HMGB1 by siRNA can inhibit its proliferation 17), our further studies showed that rHMGB1 promoted a perceptible proliferation and it was dramatically attenuated by triptolide treatment. EP is a stable lipophilic pyruvate with an action of inhibiting the release of HMBG1 by interfering with signal transduction through the p38 MAPK and NF-κB signal pathways39). We also demonstrated that triptolide synergistically enhanced the anti-proliferative activity of EP. Taken together, the present research provides reasonable evidence that triptolide-induced down-regulation of HMGB1 is an important mechanism for suppressing growth of breast cancer. However, the mechanisms through which triptolide modulate the expression and release of HMGB1 are yet to be fully illustrated, and future studies with focus to address this issue would be necessary. Nevertheless, results from Lv et al. showed that reactive oxygen species led to ERK phosphorylation, which facilitated release of HMGB123) and triptolide was revealed to reduced reactive oxygen species generation40). It would be interesting to examine whether triptolide down-regulates HMGB1 via reducing reactive oxygen species generation in
breast cancer.

It has been well known that Toll-like receptor 4 (TLR4) has been identified as a main receptor for extracellular HMGB1 \(^{23}\). TLR4 is an important member of the Toll-like receptors (TLRs) family. Except immune cells, TLR4 is also detected in cancer cells, which implicates the biologic function in tumor development \(^{41}\). Activation of TLR4 by HMGB1 contributes to tumor-associated inflammation, tumorigenesis and progression \(^{42, 43}\). Several researches have shown that HMGB1/TLR4 binding could promote the progression of breast cancer through promoting tumor cells proliferation, inhibiting tumor cells apoptosis, tumor immune evasion, etc. \(^{41, 44}\). As a transcription factor, NF-κB is also of crucial importance in the synthesis of mediators involved in tumor development and progression \(^{44}\). Binding to TLR4, HMGB1 activates the NF-κB signaling pathway, which promotes the expression of various genes taking part in tumor cell proliferation, migration and invasion \(^{43, 45}\). Previous studies have confirmed that inhibition of HMGB1 by shRNA or EP significantly reduced TLR4 expression and NF-κB activation\(^ {46, 47}\). In our study, the western blot analysis showed that triptolide treatment significantly inhibited TLR4 expression and phosphorylate-NF-κB p65 but not NF-κB p65 in breast cancer cells. Notably, the immunohistochemical results in vivo showed that a relatively higher expression of NF-κB p65 in cytoplasm, but a conversely lower expression in nucleus after triptolide treatment as compared to the control group. As is well-known, NF-κB exists as an inactive form in the cytoplasm and it is not until IκB is phosphorylated that NF-κB can enter the nucleus, bind to DNA and activate transcription of its target genes\(^ {48}\). For these reasons, depression of NF-κB by triptolide treatment have to represent by a decrease in translocation ratio from cytoplasmic to nuclear in vivo compared
with non-treated control group, which was consistent with our findings. Recent research also confirmed that macrophage migration inhibitory factor (MIF) was able to promote breast cancer metastasis by activating HMGB1/TLR4/NF-κB signaling pathway \(^{23}\). Collectively, these results above suggested that anti-growth effect elicited by triptolide may attribute to the attenuation of HMGB1/TLR4/NF-κB signaling pathway.

In summary, we demonstrated that triptolide significantly suppresses growth of breast cancer \textit{in vitro} and \textit{in vivo}, and the therapeutic effect owned to the down-regulation of HMGB1 expression and release. Our findings propose an interesting mechanism underlying the development of breast cancer and a hopeful therapeutic drug from natural resource for breast cancer treatment.

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**Conflict of interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.
References


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Fig. 1 Triptolide inhibits cell viability and clonogenic ability of breast cancer cells. (A) Cells were treated with the indicated concentration of triptolide for 24 h, 48 h and 72 h. The cell viability was measured using CCK-8 assay. (B) Cells were pre-treated with the indicated concentration of triptolide for 12 h, and then cultured at the normal condition for two weeks. The clonogenic ability was measured using plate colony formation assay. Data are expressed as mean±SD (n=3 per group), *p<0.05, **p<0.01 vs. Control (0 nmol/L).
Fig. 2 Triptolide inhibits the expression and release of HMGB1 in breast cancer cells. (A and C) Cells were treated with the indicated concentration of triptolide for 6 h, and the mRNA expression of HMGB1 was subsequently determined by qRT-PCR. (B and D) Cells were treated with 20 nmol/L of triptolide, and then the mRNA expression of HMGB1 for the indicated times was analyzed by qRT-PCR. (E and G) Cells were treated with the indicated concentration of triptolide for 6 h, and the supernatant of HMGB1 release was subsequently measured by ELISA. (F and H) Cells were treated with 20 nmol/L of triptolide, and then the supernatant of HMGB1 release for the indicated times was measured by ELISA. Data are expressed as mean±SD (n=3 per group), *p<0.05, **p<0.01 vs. Control (0 nmol/L or 0 h).
Fig. 3 Triptolide reverses the effect of rHMGB1-promoted proliferation and enhances the anti-proliferative activity of EP in breast cancer cells. (A, B, C and D) Cells were treated with a dose of rHMGB1 (10 ng/mL) or EP (10 mmol/L) alone or along with triptolide (20 nmol/L) for 24 h, 48 h and 72 h, and the cell viability was measured using CCK-8 assay. (E and F) Cells were treated with a dose of triptolide (20 nmol/L) or EP (10 mmol/L) alone or in combination with each other for 6 h, and then the supernatant of HMGB1 release was measured by ELISA. Data are expressed as mean±SD (n=3 per group), *p<0.05, **p<0.01 vs. Control; &p<0.05, &&p<0.01 vs. Triptolide; ##p<0.01 vs. rHMGB1 or EP.
Fig. 4 Triptolide inhibits TLR4 expression and NF-κB phosphorylation in breast cancer cells.

Cells were treated with triptolide (20 nmol/L) for the indicated times, and then the level of TLR4 expression and NF-κB p65 phosphorylation was determined by western blot. Data are expressed as mean±SD (n=3 per group), **p<0.01 vs. Control (0 h).
Triptolide suppresses the tumor growth of breast cancer in vivo. Athymic mice bearing MDA-MB-231 cells were administrated intraperitoneally with triptolide for 14 days. (A) shaped tumors were obtained and photographed. (B) The development of tumor size during the treatment period was recorded. (C) The tumor weight after the treatment was measured. (D) The expression of HMGB1, TLR4 and NF-κB p65 in shaped tumors was determined by immunohistochemical staining (original magnification ×400). Data are expressed as mean±SD (n=3 per group), *p<0.05, **p<0.01 vs. Control (0 mg/kg).