Maslinic acid potentiates the antitumor activities of gemcitabine 
*in vitro* and *in vivo* by inhibiting NF-κB-mediated survival signaling pathways in human gallbladder cancer cells

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**Abstract.** Gemcitabine (GEM) is one of the first-line drugs in the treatment of gallbladder cancer (GBC), although the therapeutic effect is not sustained due to resistance to the drug over time. Maslinic acid (MA) has been shown to inhibit transcription factor nuclear factor-κB (NF-κB), resulting in the suppression of survival signaling. The authors of the present study investigated whether MA enhanced the antitumor activity of GEM in GBC. Anti-proliferative effects of MA, GEM and MA + GEM were assessed using the MTT assay. Apoptosis was assessed using Annexin V and by western blot analysis of various mediators of apoptosis. Xenograft tumors of EH-GB2 GBC cells were established in athymic nude mice and were monitored following treatment with MA, GEM and MA + GEM. Immunohistochemistry of the tumors was used to examine various survival proteins. MA inhibited the *in vitro* proliferation of various GBC cell lines and potentiated the apoptosis and cell invasion inhibition induced by GEM. Western blot analysis showed that the combination of MA and GEM inhibited constitutive NF-κB activation and NF-κB-regulated gene products, including cyclin D1, Bcl-2, Bax, MMP-2 and MMP-9, to a greater extent. *In vivo*, the group that was treated with MA + GEM showed significant reductions in tumor volume and a decreased expression of NF-κB-regulated gene products. In conclusion, the results suggest that MA potentiates the antitumor effects of GEM in human GBC cell lines by suppressing the activation of NF-κB and its downstream gene products, which are involved in survival signaling.

**Introduction**

Gallbladder cancer (GBC) is the most common cancer of the bile duct system and is the fifth most lethal cancer of the digestive system (1). The incidence of GBC in China, Thailand, Chile and Northern India is higher when compared with the United States and European countries (2). At present, radical surgical resection is the most effective treatment of GBC. However, for the majority of patients, surgery is not curative because of late detection and/or early, regional or distant metastasis. Additionally, few patients experience complete responses to chemotherapy, mainly due to chemoresistance. Thus, identifying novel approaches to enhance the antitumor effects of chemotherapeutic drugs and reduce chemoresistance are imperative.

Maslinic acid (MA), a pentacyclic triterpene acid, is widely present in dietary plants, especially in olive fruit skins and hawthorn berries (3). The compound has attracted much interest due to its proven pharmacological safety and its many biological effects, such as anti-inflammation (2), anti-viral (4,5), anti-oxidation (6), anti-diabetogenic (7), anti-inflammatory (8), anti-cancer (9), anti-oxidative (10) and anti-angiogenic properties (11). Maslinic acid has been shown to potentiate the anticancer activity of TNF-α in pancreatic cancer cells through the inhibition of nuclear factor-κB (NF-κB) survival signaling pathways (11).

NF-κB is a transcriptional activator that has been extensively studied for its role in controlling the expression of genes involved in immune and inflammatory processes. The classical form of NF-κB is a ubiquitous heterodimeric complex composed predominantly of IKKα/β and p65 subunits (12). In non-stimulated cells, NF-κB exists in an inactive form in the cytoplasm bound to an inhibitor, IκBα. Aberrant or constitutive activation of NF-κB has been shown to stimulate cell growth and inhibit apoptosis in many human malignancies (13). The constitutive activation of NF-κB widely exists in many tumor types and may play a role in oncogenesis by stimulating cell growth, inhibiting apoptosis and promoting invasion (14,15). Evidence suggests that NF-κB may also be involved in tumor cell resistance to cancer chemotherapy and radiation (16). NF-κB can be activated in response to treatment with anticancer drugs through a variety of mechanisms. For example, in HeLa cells, the topoisomerase I inhibitor SN38...
and the topoisomerase II inhibitor doxorubicin induce NF-κB nuclear translocation and activation of NF-κB target genes directly through mobilization and stimulation of the IKK complex, leading to increased cell survival (17). The activation of NF-κB also leads to the transcription of genes that regulate cell growth, apoptosis, and invasion. Cyclin D1, Bcl-2, Bax, MMP-2 and MMP-9 are among the genes that are transcriptionally regulated by NF-κB (18).

Gemcitabine (GEM) is one of the few chemotherapeutic drugs used in the treatment of advanced and metastatic biliary duct cancer and GBC (19,20). However, GEM has been shown to induce chemoresistance in many types of cancer, including pancreatic (21), lung (22), ovarian (23), bladder (24) and biliary cancer (25).

It has been reported that MA significantly potentiates the antitumor activities of antitumor agents (11,26). In this study, we investigated the effects of MA alone and in combination with GEM on human gallbladder carcinoma \textit{in vitro} and \textit{in vivo}, and its underlying mechanisms.

Materials and methods

Cell culture and reagents. GBC cell lines were reserved by the Eastern Hepatobiliary Surgery Institute. The gallbladder EH-GB1 (27), EH-GB2 (28), and GBC-SD human cancer cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA, USA). The cells were regularly checked for mycoplasma by the PlasmoTest mycoplasma detection kit (InvivoGen, San Diego, CA, USA) and found to be negative. MA (>98%) was purchased from Cayman (Ann Arbor, MI, USA) and was prepared as 20 mg/ml stock solutions in dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO, USA) and stored at -20°C.

MTT and drug interaction analysis. For measurement of proliferation, the human GBC cell lines, EH-GB1, EH-GB2 and GBC-SD, were placed into 96-well plates at 1x10^4 cells/well. After 24 h, the cells were treated with various concentrations of MA, GEM (Lilly, France) and MA + GEM. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to evaluate cell growth and viability. MTT solution was added to each well and incubated for 4 h at 37°C. An extraction buffer (20% SDS and 50% dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO, USA) and stored at -20°C.

Mouse xenograft model and tumor treatment. Xenograft mouse models were performed as previously described (31). Six-week-old athymic nu/nu male mice were purchased from SIBS (Shanghai, China). The experiments performed on the animals were approved by the Animal Ethics Committee of the Second Military Medical University prior to the study. EH-GB2 tumor cells were subcutaneously injected into the mice (2x10^6 cells/mouse).

After the tumors were established (~100 mm³), the mice were randomized in the following treatment groups (n=5): i) untreated control; ii) subcutaneously injected with 30 mg/kg bodyweight of MA every 2 days for 30 days; iii) GC 50 mg/kg of bodyweight intraperitoneal every 2 for 30 days; and iv) MA and GEM following the schedule for the individual treatments. The control mice were injected with DMSO. The mice body weight and tumor sizes were recorded every other day, and the tumor size was determined by vernier caliper measurements and calculated as maximal diameter x minimal diameter² x 0.5 (32). After 36 days, mice with tumors were...
sacrificed. One section of the tissue was fixed in formalin and another section was frozen in liquid nitrogen.

**Histology and immunohistochemistry (IHC).** After the tumors were removed, they were weighed, fixed with 10% formalin, and embedded with paraffin. H&E and IHC for Bcl-2 and Bax were performed on paraffin-embedded tissue sections. The rabbit antibodies against Bcl-2 and Bax were purchased from Santa Cruz Biotechnology, Inc. and applied for IHC staining (1:1,000 dilution). PBS was used as a negative control. After IHC was performed, images from each group were captured using an Olympus BX60 upright microscope (Olympus, Tokyo, Japan).

**Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labelling (TUNEL) staining.** Apoptotic cells in EH-GB2 tumor xenograft tissue sections were detected by TUNEL using a commercially available kit (EMD Millipore Corporation, Billerica, MA, USA). The tissue sections were processed according to the manufacturer's instructions.

**Statistical analysis.** Data are presented as mean ± SD as indicated in the vertical axis of figures. The statistical significance of differential findings between experiments and controls was determined by using the Student's t-test or analysis of variance (ANOVA). Statistical analyses were computed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was P<0.05 unless otherwise stated.

**Results**

**MA inhibits proliferation, and potentiates the apoptosis induced by GEM.** We first examined the effects of MA (Fig. 1A), GEM and MA + GEM on the proliferation of human GBC cells (EH-GB1, EH-GB2 and GBC-SD) using the MTT assay. The results showed that MA and GEM as single agents both inhibited the proliferation of the GBC cell lines in a dose-dependent manner (Fig. 1B and C). MA and GEM combined significantly inhibited cell proliferation of all three GBC cell lines (Fig. 1D). A combination index (23) was calculated for all combinations of GEM and MA examined in the three cell lines, indicating that the interaction between the two drugs was synergistic (Fig. 2).

Subsequently, we examined whether the inhibition of cell proliferation was due to enhanced apoptosis with the combination of MA and GEM compared to single agents. Accordingly, relative to the single agents, MA treatment (25 µmol/l) followed by GEM treatment elicited significantly (P<0.05) higher apoptosis in the investigated cancer cell lines, suggesting that the loss of viable cells by MA + GEM was due to the induction of cell death pathways (Fig. 3A).

**MA enhances the inhibition of GEM in cancer cell migration and invasion.** Migration assays were performed to determine the effects of MA on EH-GB2 migration. We found that the combination of MA and GEM strongly inhibited the migration of EH-GB2 cells (Fig. 3B). We performed Transwell assays to evaluate the ability of EH-GB2 to filter through the membrane barrier after treatment with MA, GEM and MA + GEM. MA, at low concentrations of 10 µmol/l, significantly potentiated the properties of GEM, inhibiting cancer cell invasion (Fig. 3C).

**NF-κB activation is inhibited by the combination of MA and GEM.** We examined whether the combination of MA and GEM was able to inhibit NF-κB activation. EH-GB1 and EH-GB2 cells were incubated with suboptimal concentrations of MA and GEM alone and in combination. IKKα/β and p65 activation were determined by western blot analysis. MA potentiated
Yu et al.: MA Potentiates the Activities of Gemcitabine

MA potentiates the effect of GEM in downregulating the expression of NF-κB-regulated gene products in vitro. We examined the effect of MA on the expression of NF-κB-regulated gene products involved in cell proliferation (cyclin D1), apoptosis (Bax and Bcl-2), and metastasis (MMP-2 and MMP-9) (Fig. 5). The cells were exposed to MA (25 μmol/l) for 48 h prior to the addition of GEM for 24 h. The results showed that the expression of cyclin D1 and Bcl-2 were significantly downregulated in the combination group compared with the individual treatment groups. By contrast, Bax expression was substantially increased after the combinatorial treatment when compared to single agents. Furthermore, the activities of MMP-2 and MMP-9 were significantly reduced by the treatment with MA + GEM.

MA potentiates the antitumor effects of GEM in vivo. To determine whether MA enhances the antitumor effects of GEM in GBC, we established a human GBC xenograft in nude mice using EH-GB2 cells. Treatment was initiated...
1 week after tumor cell implantation and was continued up to 28 days (Fig. 6A). The tumor diameters were measured at 1-week intervals. Animals were sacrificed 35 days after tumor cell injection and 28 days after the treatment initiation date.

Figure 4. The treatment of maslinic acid (MA) + gemcitabine (GEM) alters the expression of cyclin D1, Bax, Bcl-2, MMP-2 and MMP-9. Various mediators of survival signaling and apoptosis were assessed by western blot analysis. (A) EH-GB1 and (B) EH-GB2 were treated with cell medium containing 25 nmol/l GEM alone or combined with 25 µmol/l MA for 0, 12, 24 and 48 h. β-actin protein was used as the internal control.

Figure 5. NF-κB activities can be inhibited by combined treatment with gemcitabine (GEM) and maslinic acid (MA) western blot analysis for IKKα/β and p65 subunit of (A) EG-GB1 and (B) EH-GB2 cells treated with cell medium, GEM, MA or MA + GEM. TNF-α was used as the irritant, and β-actin protein was used as the internal control.
Yu et al.: MA Potentiates the Activities of Gemcitabine

At this time the tumors were excised and the tumor diameters were measured. We found that the tumor volume increased rapidly in the control group compared with the other treatment groups (Fig. 6B). MA alone and GEM alone moderately decreased the tumor volume. However, the tumor volume in the combination group was significantly lower than that in the GEM and MA alone groups at day 28 after treatment (P<0.05).

**TUNEL staining of xenograft sections.** To investigate whether MA potentiated GEM-induced apoptosis in vivo, we stained the solid tumor sections with an apoptosis staining kit. Significant differences in the percentage of TUNEL-positive cells were noted in tumors derived from the combination group relative to the single treatment groups (P<0.05).

**Immunohistochemical and western blot analyses.** Various mediators of apoptosis and survival signaling were also examined in tumors collected from the xenograft study. The expression level of Bcl-2 was significantly decreased, while Bax was increased by IHC staining (Fig. 6C). The same trend of Bcl-2 and Bax was identified in western blot assay (Fig. 6D). The expression of cyclin D1, MMP-2 and MMP-9, which were involved in tumor progression, was inhibited significantly by combining MA and GEM (Fig. 6D).

**Discussion**

MA, a natural triterpenoid that can be extracted from olive skin, has been assessed for its antitumoral property in colonic cancer (10), melanoma (33,34), and astrocytoma (35) cells. GEM, which is a nucleoside analog of deoxycytidine that inhibits DNA synthesis (36), has been increasingly prescribed for GBC. Findings of recent studies have focused on improving the drug efficacy by combining GEM with other agents (37,38). We designed this study to determine whether MA can sensitize GBC to GEM.

The constitutive activation of NF-κB is associated with the growth and survival of cancer cells (39). In addition, studies have shown that MA suppressed NF-κB activation (40). In the present study, we investigated the mechanism of how...
MA enhanced the apoptotic effects of GEM in cultured GBC cells. In vitro, we have demonstrated that MA enhanced GEM-induced apoptosis and suppressed NF-κB activity. A recent review by Nakanishi and Toi (41) details the activation of NF-κB by chemotherapeutic agents as the major factor contributing to chemoresistance. MA can inhibit IKKα/β degradation, block p65 nuclear translocation and phosphorylation, and downregulate the expression levels of NF-κB-mediated genes/proteins involved in proliferation, apoptosis and invasion (42). As previously reported, proteins including cyclin D1, Bax, Bel-2, MMP-2 and MMP-9, have been associated with tumor growth, apoptosis, invasion, and metastasis. In previous studies, it was shown that combining a NF-κB inhibitor with a front-line anticancer drug enhances the overall antitumor response (43,44). In this study, we have concluded that the downregulation of NF-κB by MA can enhance the sensitivity of GBC cells to GEM.

Our in vitro results were recapitulated in vivo in a subcutaneous xenograft GBC model, wherein MA significantly enhanced the antitumor efficacy of chemotherapeutics. Although none of the mice from the combinatorial treatment group were tumor-free, the therapeutic effect was significant compared with single-drug treatment. These findings are concomitant with increased TUNEL staining and reduced MA and GEM immunoreactivity indicative of apoptosis and reduced cell proliferation within tumors. These features are of significant value in predicting improved therapeutic outcomes and require further investigation.

Given the pharmacologic safety of MA (45), our studies suggest that this compound has great potential as a chemopreventive and a chemotherapeutic agent, especially when used in combination with existing agents. Whether the concentrations of MA used in our studies are achievable in the clinic remains to be determined. MA metabolism in the cells is also unclear at present. Our results also show that patient-derived GBC cells exhibit constitutive NF-κB activation. This NF-κB activation was suppressed significantly by GEM in combination with MA, thus leading to the inhibition of proliferation, apoptosis and invasion of the cells.

In this study, MA decreased the protein expression of cyclin D1, which is a cell cycle-positive regulator, indicating a role for MA in GBC proliferation (46). Cyclin D1 adversely affects clinical outcomes and serves as an independent marker in predicting decreased survival for patients with GBC (47,48).

In the present study, the cells treated with GEM and MA had a downregulated anti-apoptotic Bel-2, but an increased expression of pro-apoptotic Bax. The Bel-2 family is important in the regulation of apoptosis and comprises pro-apoptotic and anti-apoptotic members (49). In EH-BG1 and EH-BG2 cell lines, Bel-2 was significantly downregulated and Bax was upregulated in the combination group when compared with the GEM treatment group and the non-treated control group.

MMPs are central mediators of tumor metastasis due to their ability to degrade basement membrane and extracellular matrix components (50). MMP-2 and MMP-9 expression is positively associated with Nevin stage, distant metastasis, and the degree of histological differentiation in GBC (51). The results showed that MA inhibited the invasion of GBC by reducing MMP-2 and MMP-9 activation through the suppression of NF-κB/p65 activation.

In conclusion, to the best of our knowledge, this is the first study to demonstrate that MA can potentiate GEM activity in vitro and in vivo in GBC models. The results suggest that MA is a potentially valuable agent in the development of a new class of drugs to assist in potentiating the anticancer effects of conventional chemotherapeutics targeting specific pathways for the treatment of human GBC. Future studies should be conducted in the clinical setting to validate the biological relevance of these results.

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References


