AA-PMe, a novel asiatic acid derivative, induces apoptosis and suppresses proliferation, migration, and invasion of gastric cancer cells

Abstract: Asiatic acid (AA; 2α,3β,23-trihydroxyurs-12-ene-28-oic acid) is widely used for medicinal purposes in many Asian countries due to its various bioactivities. A series of AA derivatives has been synthesized in attempts to improve its therapeutic potencies. Herein we investigated the anti-tumor activities of N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-1-proline methyl ester (AA-PMe), a novel AA derivative. AA-PMe exhibited a stronger anti-cancer activity than its parent compound AA. AA-PMe inhibited the proliferation of SGC7901 and HGC27 human gastric cancer cells in a dose-dependent manner but had no significant toxicity in human gastric mucosa epithelial cells (GES-1). AA-PMe induced cell cycle arrest in G0/G1 phase and blocked G1-S transition, which correlated well with marked decreases in levels of cyclin D1, cyclin-dependent kinase CDK4, and phosphorylated retinoblastoma protein, and increase in cyclin-dependent kinase inhibitor P15. Further, AA-PMe induced apoptosis of human gastric cancer cells by affecting Bcl-2, Bax, c-Myc, and caspase-3. Moreover, AA-PMe suppressed the migration and invasion of human gastric cancer cells (SGC7901 and HGC27) cells by down-regulating the expression of MMP-2 and MMP-9. Overall, this study investigated the potential anti-cancer activities of AA-PMe including inducing apoptosis and suppressing proliferation, migration and invasion of gastric cancer cells, as well as the underlying mechanisms, suggesting that AA-PMe is a promising anti-cancer drug candidate in gastric cancer therapy.

Keywords: Asiatic acid derivatives, gastric cancer cells, anti-tumor effect, cytotoxicity, apoptosis, cell cycle arrest, migration, invasion, mobility

Introduction

Gastric cancer has a high mortality rate. Among all the malignant tumors, gastric cancer is the second leading cause of global cancer-related death, especially in East Asian countries such as the People’s Republic of China, Japan, and Korea. The development of gastric cancer is a multi-step process, starting from normal mucosa and developing to chronic gastritis, then to precancerous lesions including gastric atrophy, intestinal metaplasia and dysplasia, and eventually to invasive cancer. Gastric cancer is also frequently associated with lymph node metastasis, peritoneal dissemination, and hematogenous metastasis. It is believed that alterations of various regulators (eg, Bcl-2 and c-Myc), tumor suppressor genes (eg, Bax), DNA repair genes, cell cycle regulators (eg, cyclin D1, CDK4, CDK6, and P15), cell adhesion molecules (eg, Matrix metalloproteinase [MMP]-2 and MMP-9), and growth factor/receptor system are collectively involved in the onset and progression of gastric cancer. Rapid proliferation, migration, invasion, and multidrug resistance of tumor cells are responsible for poor prognosis of gastric cancer. At present, surgery and chemotherapy are the two main
ways to treat gastric cancer. However, the existing chemo-
therapeutic drugs for gastric cancer lack robust efficacy and
come with many side effects. As a result, current research has
begun to focus on drugs that are more effective at preventing
and treating gastric cancer as well as other malignant tumors
with much lower side effect.

Asiatic acid (AA; 2α,3β,23-trihydroxyurs-12-ene-28-
oic acid) is a pentacyclic triterpenoid widely distributed in
plant kingdom. AA has been reported to have a variety of
pharmacological effects, including anti-inflammatory,12,13
antioxidative,14 anti-tumor,15,16 neuroprotective,17,18 and
wound healing19,20 activities. In addition, AA and its
derivatives have been shown to have anti-tumor activities
in various cancer cell lines.21,22 They are also inhibitors of
glycogen phosphorylases23,24 and have hepatoprotective
effect.25–27

We have synthesized a series of novel AA derivatives with
amino acid conjugates and compared their bioactivities. Among
them, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-L-proline methyl
ester (AA-PMe) exhibited the strongest anti-tumor activities
in most of the cancer cell lines used. In the present study, we
sought to elucidate the effects of AA-PMe on the malignant
phenotype of SGC7901 and HGC27 gastric cancer cells.
Our result indicated that AA-PMe could efficiently inhibit
proliferation and induce apoptosis of the gastric cancer cells via
arresting cell cycle in G1/S phase. Moreover, AA-PMe could
suppress migration and invasion of gastric cancer cells.

Materials and methods
Materials and reagents
AA was extracted from Centella asiatica (L.) and AA-PMe
was synthesized in our laboratory.28 The structures of AA and
AA-PMe are shown in Figure 1. Proline was purchased from
Aladdin (Shanghai, China). DMEM, RPMI 1640, and fetal
bovine serum (FBS) were purchased from Gibco (Thermo
Fisher Scientific, Grand Island, USA). Rabbit anti-caspase
3, anti-c-Myc, anti-phosphorylated Rb (Ser807/811), anti-
p15, anti-MMP-2 and anti-MMP-9 polyclonal antibodies
and mouse anti-cyclin D1, anti-CDK4, and anti-CDK6
monoclonal antibodies were obtained from Cell Signaling
Technology (Beverly, MA, USA). Rabbit anti-Bax and anti-
Bcl-2 polyclonal antibodies and the horseradish peroxidase
(HRP)-conjugated anti-rabbit IgG secondary antibody were
purchased from ABCam (Cambridge, MA, USA). The
HRP-conjugated anti-mouse IgG secondary antibody was
purchased from Boster (Pleasanton, CA, USA). Annexin
V-FITC/propidium iodide (PI) double staining apoptosis
detection kit, cell cycle kit, and cell counting kit-8 (CCK8)
were purchased from Vazyme Co. Ltd (Nanjing, Jiangsu,
People’s Republic of China). All other reagents and chemi-
cals were purchased from Sigma (St Louis, MO, USA). All
animal experiments in this investigation were conducted in
compliance with the Guide outlining the Care and Use of
Laboratory Animals, published by the US National Institutes
of Health (NIH publication No. 85-23, revised 1996) and was
approved by the Animal Care and Use Committee of Nanjing
Normal University, China (Permit Number: 2090658).

Cell culture
The human gastric mucosa epithelial GES-1, gastric
cancer cell lines SGC7901 and HGC27, were purchased from
the Cell Bank of the Shanghai Institute of Biochemistry and
Cell Biology, Chinese Academy of Science, and cultured in
DMEM and RPMI 1640 media, respectively, supplemented

Figure 1 Chemical structures of AA and its derivative.
Notes: Chemical structures of AA (A) and AA-PMe (B).
Abbreviations: AA, Asaiatic acid; AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-L-proline methyl ester.
with 10% FBS in a humidified atmosphere containing 5% CO₂ in air at 37°C. This study was approved by the Ethics Committee of Nanjing Normal University, and conformed with the provisions of the Declaration of Helsinki.

**Measurement of cell viability with CCK8 assay**

Cell counting kit-8 was used to examine cell viability. Briefly, gastric cancer cells (5×10⁴/well) were seeded in 96-well plates. Then AA and AA-PMe were added at a series of concentrations (1–100 µM) and incubated for 24, 48, or 72 hours, followed by addition of 10 µL CCK8 and incubated for another hour. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Grand Island, USA).

**Cell count using trypan blue dye exclusion**

SGC7901 cells were seeded in a six-well plate (10⁵ cells/well) and cultured overnight. Then AA and AA-PMe were added at a series of concentrations and incubated for 24, 48, or 72 hours. After that, cells were harvested and mixed with Trypan blue solution (0.14% in Hank’s balanced salt solution; Gibco BRL Co. Ltd., USA). The colored (dead) and dye-excluding (live) cells were counted using a hemocytometer.

**Cell cycle analysis by flow cytometry**

Cells were seeded in a six-well plate (1.5×10⁶ cells/well) and treated with AA-PMe or AA (1–50 µM) for 24 hours. Then the cells were harvested, fixed with 75% ethanol for 2 hours at 4°C, washed with PBS, and stained with PI (5 mg/L containing RNase A (1 g/L), sodium citrate (1 g/L), and 0.5% Triton X-100 for 30 minutes in the dark. Then the cells were analyzed by a FACS Vantage cell sorter (BD Bioscience, Franklin Lakes, NJ, USA) equipped with a 488 nm argon laser. Cell distribution in different phases of the cell cycle was estimated using ModFit LT™ software.

**Annexin V-FITC/PI assay**

SGC7901 and HGC27 cells were seeded in six-well plates and cultured overnight to reach 80% confluence. Apoptosis was induced by 24 hours of treatment with AA or AA-PMe at a series of concentrations (1, 5, 10, 25, and 50 µM). Apoptosis was analyzed using an Annexin V-FITC and PI double-staining apoptosis detection kit by following the manufacturer’s instruction. Briefly, after treatment, cells were harvested, washed with PBS, centrifuged, and resuspended in 195 µL Annexin V-FITC binding buffer. The cells were incubated with 5 µL Annexin V-FITC solution for 10 minutes at room temperature in the dark, then the solution was removed and 190 µL Annexin V-FITC binding buffer and 10 µL PI were added and incubated for another 5 minutes. Cell suspension was analyzed by FACS with CellQuest version 3.3 software.

**Wound-healing assay**

Cells were seeded into six-well plates and grew into monolayer, and a plastic pipette tip was used to scratch across the center of wells to generate a 1 mm-wide wound area and washed twice with PBS to remove detached cells. The photomicrographs of initial wounds were taken using Canon Power Shot A640 digital camera. Then, cells were treated with AA or AA-PMe (1, 5, 10, 30, and 50 µM) in FBS-free medium for 6, 12, or 24 hours, and the photomicrographs of wounds at different time points were taken under a phase-contrast microscope and analyzed.

**Invasion assay**

A 24-well plate with transwells (Costar, Corning, NY, USA) precoated with matrigel (BD Biosciences) was used to analyze cell invasion. Briefly, 600 µL of culture medium containing 10% FBS with or without AA or AA-PMe was added to the lower chamber as chemoattractant. Cells were placed in the upper chamber in 200 µL medium containing 1% FBS at a density of 1×10⁵ cells/well; 24 hours later, the nonmigrated cells were removed with cotton swabs, and the cells migrated to the lower surface of the filters were fixed with 90% alcohol for 30 minutes at room temperature and stained with 0.1% crystal violet for visualization. Images were captured under a phase-contrast microscope and the invasive cells were quantified by manual counting. A total of 33% acetic acid was then used to decolor invasive cells for 10 minutes. The destaining solution was collected and the absorbance was measured at 570 nm using a microplate reader (Thermo FC, USA). The inhibition of invasion was calculated as follows:

\[
\text{Inhibition percentage of invasion (\%)} = \frac{\text{OD treated} - \text{OD blank}}{\text{OD control} - \text{OD blank}} \times 100\%
\]

**Protein extraction and Western blot analysis**

Cells were lysed in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.1% SDS, 0.2% EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.01% PMSF) supplemented with protease and phosphatase inhibitors cocktail (Roche, Basel, Switzerland) by 30 minutes of incubation on ice and centrifuged at 12,000 rpm for 15 minutes at 4°C to remove nuclei and cell debris (Eppendorf centrifuge 5804R, Eppendorf, Germany). The protein concentration of the extracts
Table 1 The primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5′-TGTTGAGAAGCCGCACTGGA-3′</td>
</tr>
<tr>
<td>Forward</td>
<td>5′-GACCCGCTAAGGGCTGAGAACAC-3′</td>
</tr>
<tr>
<td>Box</td>
<td>5′-CAACTGTTGCTCAAGGCC-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-GACCTGGCCCAACAGAT-3′</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5′-ATGTGTTGGAAGCACAGTGACC-3′</td>
</tr>
<tr>
<td>Forward</td>
<td>5′-TGGAGAAGCTTCTAGACAGACC-3′</td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-TCGAGGCTCATATGCTTGTT-3′</td>
</tr>
<tr>
<td>c-Myc</td>
<td>5′-GCTGCTTAGAAGCTGATTTTT-3′</td>
</tr>
<tr>
<td>Forward</td>
<td>5′-CACCAAGTGGCGGCACT-3′</td>
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<tr>
<td>Reverse</td>
<td>5′-GCTGACGGTAAGGAGACCT-3′</td>
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<tr>
<td>Cyclin D1</td>
<td>5′-GGGCAGTTTTCTAATGGAGATTG-3′</td>
</tr>
<tr>
<td>Forward</td>
<td>5′-GCCACAGTGCGCCTAC-3′</td>
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<tr>
<td>MMP-2</td>
<td>5′-AGGCGGACAGAAGGTTTTG-3′</td>
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<td>Forward</td>
<td>5′-CATGACGTAAGGGAGAGAAGTTG-3′</td>
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<tr>
<td>MMP-9</td>
<td>5′-TTTGGACCGCAGAACAGATGG-3′</td>
</tr>
<tr>
<td>Forward</td>
<td>5′-AGGCGGACAGAAGGAGAGTACTGAGG-3′</td>
</tr>
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</table>

Real-time reverse transcription PCR

Total RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and quantified using a spectrophotometer (Nano Drop ND-1000, Thermo Fisher Scientific, Grand Island, USA). Reverse transcription was performed using a cDNA synthesis kit (Invitrogen). Quantitative RT-PCR was performed by following the manufacturer’s protocol (Takara, Takara, Tokyo, Japan). The sequences of primers used are listed in Table 1. The target mRNA was quantified in comparison with GAPDH mRNA. The data were calculated with ΔΔCt method.

Statistical analysis

All the data were presented as mean ± standard deviation (SD) of at least triplicate measurements. The data shown are representatives of at least two independent experiments with similar results. Statistical analysis was performed using SPSS software (version 13.0). The significant differences between groups were evaluated by one-way analysis of variance (ANOVA). Statistical significance was defined as *P* < 0.05.

Results

AA-PMe reduces the viability of gastric cancer cells

The anti-cancer effect of AA-PMe and its parent compound AA was compared by exposing tumor cells at a series of concentrations and time points. The CCK-8 analysis results showed that both AA-PMe and AA reduced the viability of SGC7901 and HGC27 cells in a concentration- and time-dependent manner (Figure 2). The half maximal inhibitory concentration (IC$_{50}$) of AA-PMe for SGC7901 cells was 4.48 µM, which was much lower than that of AA (36.9 µM) (Table 2 and Figure 2A). We also evaluated cell viability using a trypan blue exclusion assay. It was shown that AA-PMe could inhibit cell survival at a low concentration of 5 µM (Figure 2B), whereas AA inhibited cell survival only at a high concentration of 50 µM (Figure 2C). Similar results were also obtained in HGC27 cells. AA-PMe exerted a stronger inhibition than AA in HGC27 cells with an IC$_{50}$ of 7.92 µM and 61.23 µM for AA-PMe and AA, respectively (Figure 2D–F).

We also examined the toxicity of AA-PMe and AA on human gastric mucosa epithelial GES-1 cells. Both AA-PMe and AA had no significant effect on viability of GES-1 cells, except for a slight inhibition at high concentration of 100 µM (Table 2 and Figure 3).

AA-PMe arrests cell cycle of gastric cancer cells in G$_{0}$/G$_{1}$ phase

We examined the cell cycle after treatment with AA-PMe and AA. We found that both AA-PMe and AA concentration-dependently induced cell cycle arrest in G$_{0}$/G$_{1}$ phase (Figure 4). Again, AA-PMe exhibited a stronger activity than AA. After a 24-hours exposure, the proportion of cells in G$_{0}$/G$_{1}$ phase treated by 25 µM AA-PMe increased from 51.31% to 77.7% in SGC7901 cells (Figure 4A and C), and from 52.12% to 77.26% in HGC27 cells (Figure 4B and D). AA increased the proportion of cells in G$_{0}$/G$_{1}$ phase from 50.81% to 65.08% in SGC7901 cells (Figure 4A and C) and from 54.66% to 65.63% in HGC27 cells (Figure 4B and D).

We next observed the influence of AA-PMe and AA on cell cycle regulators including cyclin D1, CDK4, CDK6, p15, and phosphorylated Rb. We found that AA-PMe remarkably decreased the expression of cyclin D1, CDK4, and...
Figure 2 Inhibitory effect of AA and AA-PMe on the viability of SGC7901 and HGC27 cells. 
Notes: (A) Viability of SGC7901 cells determined by CCK8 assay. Cells were exposed to AA or AA-PMe for 72 hours. The relative survival rate was determined in relation to that of untreated control cells, which was set as 100%. Data are mean ± SD of three replicate experiments. (B and C) Growth curve of SGC7901 cells. Cells were incubated with different concentrations of AA or AA-PMe for 24, 48, and 72 hours, then harvested, stained with trypan blue and counted. Figure 2 (D–F) results of the same experiments performed in HGC27 cells as (A–C). *P<0.05, **P<0.01 vs control.
Abbreviations: AA, asiatic acid; AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester; CCK8, cell counting kit-8; SD, standard deviation.

Table 2 Comparison of anti-tumor activities of AA and AA-PMe in SGC7901 and HGC27 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA-PMe</td>
</tr>
<tr>
<td>SGC7901</td>
<td>4.48</td>
</tr>
<tr>
<td>HGC27</td>
<td>7.92</td>
</tr>
<tr>
<td>HEK293</td>
<td>109.7</td>
</tr>
</tbody>
</table>

Notes: Cells were treated with AA and AA-PMe at different concentrations for 72 hours and IC$_{50}$ was determined by CCK-8 assay.
Abbreviations: AA, asiatic acid; AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester; CCK8, cell counting kit-8; IC$_{50}$, half maximal inhibitory concentration.

phosphorylated Rb, and increased the amount of p15, but failed to affect the CDK6 level or only slightly decreased it. In contrast, AA only had effects on these cell cycle regulators at high concentration of 50 µM (Figure 5).

AA-PMe induces apoptosis of gastric cancer cells

We used Annexin V-FITC/PI double staining to analyze AA-PMe-induced apoptosis. SGC7901 and HGC27 cells were treated with AA-PMe and AA (0–50 µM) for 24 hours. As
showed in Figure 6, the fluorescent microscopic images show that both AA-PMe and AA induced significant cell apoptosis in SGC7901 and HGC27 cells in a concentration-dependent manner. The green light shows the cells at early stage of apoptosis, while the red light shows those at late stage of apoptosis. Consistently, AA-PMe exhibited a stronger activity than AA. The apoptotic cells induced by 10 μM AA-PMe were much more than those induced by 10 μM AA in both SGC7901 and HGC27 cells.

To further quantify the apoptosis of SGC7901 and HGC27 cells induced by AA-PMe and AA, we performed Annexin V-FITC/PI double-staining assay by flow cytometric analysis, which detects different stages of apoptosis. The results indicated that AA-PMe had a stronger capability to induce apoptosis than AA. A total of 50 μM AA-PMe induced an apoptosis rate of 65.4% in SGC7901 cells and 59.02% in HGC27 cells after 24 hours of treatment. In contrast, 50 μM AA induced an apoptosis rate of 42.68% in SGC7901 cells and 54.6% in HGC27 cells (Figure 7).

We next analyzed the effects of AA-PMe and AA on apoptosis-related regulators including Bcl-2, caspase 3, c-Myc, and Bax in SGC7901 and HGC27 cells. Results showed that AA-PMe treatment downregulated the expression of Bcl-2, caspase 3, and c-Myc, and increased the accumulation of proapoptotic protein Bax in a concentration-dependent manner in both SGC7901 and HGC27 cells (Figure 8). AA exhibited weaker activities in affecting these signaling events. Overall, these results clearly showed that AA-PMe could decrease viability and induce apoptosis in SGC7901 and HGC27 cells through altering the expression of pro-apoptotic and anti-apoptotic molecules.

**AA-PMe inhibits migration and invasion of gastric cancer cells**

Cancer cell migration and invasion are critical events for tumor growth and metastasis. So we next studied the effect of AA-PMe and AA on the migration and invasion of SGC7901 and HGC27 cells using wound-healing and transwell assays. As shown in Figure 9, AA-PMe dramatically inhibited the migration of both SGC7901 and HGC27 cells in a concentration- and time-dependent manner in wound-healing assay. Similarly, AA-PMe also significantly suppressed the invasion of both SGC7901 and HGC27 cells through matrigel in a concentration-dependent manner. Again, AA-PMe showed a much stronger activity than AA in these experiments (Figure 10). It is well known that MMP-2 and MMP-9 could degrade the intercellular mesenchyme and promote tumor migration. Expressions of MMP-2 and MMP-9 were detected following treatment with AA-PMe for 24 hours. Western blot results showed that AA-PMe downregulated the expression of both MMP-2 and MMP-9 (Figure 11).

**AA-PMe modulates gene expression**

To determine whether AA-PMe affects the transcription in SGC7901 and HGC27 cells, the mRNA expression of Bax, Bcl-2, cyclin D1, c-Myc, MMP-2, and MMP-9 was examined. As shown in Figure 12, AA-PMe treatment increased the expression of proapoptotic Bax gene and suppressed the expression of the rest – Bcl-2, cyclin D1, c-Myc, MMP-2, and MMP-9 genes at high concentration used, suggesting that AA-PMe modulates the expression of these genes at transcriptional level.

**Discussion**

In this paper, we have studied the anti-tumor activities of a novel AA derivative, AA-PMe, in human gastric cancer cells, which has been proved to have the highest anti-cancer efficacy among all the AA derivatives in numerous cancer cell lines, suggesting that the increased anti-cancer activity of AA derivatives may result not only from increased water solubility but also from the optimization of hydrophilic–lipophilic balance to a great extent. Moreover, it suggests that proline methyl ester and acetoxy groups could lead to an increased activity jointly when put together.

Our studies showed that AA-PMe had stronger anticancer activities than its parent compound AA in gastric cancer cells. AA-PMe remarkably inhibited the proliferation of gastric cancer cells by arresting cell cycle in G0/G1 phase.
Figure 4 (Continued)
Figure 4 Effect of AA-PMe and AA on cell cycle distribution in SGC7901 and HGC27 cells. 

Notes: Gastric cancer cells (2×10^6/mL) were synchronized by incubation overnight in the absence of serum and then treated with different concentration of AA and AA-PMe for 24 hours, after which the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Figure 4 (A, C) analyzed for SGC7901 cells and figure 4 (B, D) was analyzed for HGC27 cells.
Figure 5: Changes in the expression levels of cell-cycle regulatory proteins induced by AA-PMe and AA.

Notes: Lysates were prepared from the cells after treatment with 0, 1, 5, 10, 25, and 50 µM AA-PMe or AA for 24 hours. *P<0.05 and **P<0.01 represent the difference of AA and AA-PMe at same concentration. Gastric cancer cells were then subjected to Western blot analysis using the indicated antibodies. Equal protein loading was confirmed by probing the Western blots with an anti-GAPDH antibody. The density of each band was measured and normalized to that of GAPDH. (A) Western blots of cyclin D1, CDK4, CDK6, p15, and pRb in SGC7901 cells. (C) Densitometric analysis of the relative level of cyclin D1, CDK4, CDK6, p15, and pRb in SGC7901 cells. (B, D) The same methods as A and C in HGC27 cells. Error bars represent standard errors of the mean (SEM).

Abbreviations: AA, asiatic acid; AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester.
Figure 6 The morphological feature changes and apoptosis induced by SGC7901 and HGC27 cells.

Notes: Gastric cancer cells were treated with 0.1% DMSO (control); 5 µM AA or AA-PMe; 10 µM AA or AA-PMe; 25 µM AA or AA-PMe and then double stained with Annexin V-FITC/PI kit. The green light shows early-stage apoptotic cells while the red light shows the late apoptotic cells (×100).

Abbreviations: AA, asiatic acid; AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-L-proline methyl ester; PI, propidium iodide.

Figure 7 (Continued)
via affecting cell cycle regulators including cyclin D1, CDK4, p15, and phosphorylated Rb, and induced cell apoptosis by regulating apoptosis-related signaling molecules including Bcl-2, caspase 3, c-Myc, and Bax. Moreover, AA-PMe also suppressed the migration and invasion of gastric cancer cells by downregulating the expression of MMP-2 and MMP-9, two critical proteins in cell migration and invasion. Furthermore, we have found that AA-PMe regulated the expression of these related genes including Bax, Bcl-2, cyclin D1, c-Myc, MMP-2, and MMP-9 at transcriptional level. In contrast, AA-PMe exhibited very weak toxicity in normal human embryonic kidney 293 cells, suggesting that AA-PMe may have some selectivity for tumor cells over normal cells, which is very important for an anti-cancer drug candidate.

Cell cycle arrest induced by AA-PMe might be the mechanism underlying the anti-proliferation effect of AA-PMe. AA-PMe increased the percentage of tumor cells in G₀/G₁ phase but decreased the proportion of cells in S phase, suggesting that AA-PMe might induce cell cycle arrest into G₀/G₁ phase and block the entry of cells into DNA synthesis phase. The cell cycle progression is driven by CDK family proteins, which are positively regulated by cyclin proteins and negatively regulated by CDKI. During the G₁ phase, cyclin D engages with CDK4 and/or CDK6 to form a complex, resulting in the activation of CDKs. The activated CDKs can phosphorylate Rb protein, which subsequently disrupts the binding of Rb to E2F, leading to E2F activation and the gene transcriptions necessary for G₁→S transition and cell cycle progression. In our study, AA-PMe treatment decreased the levels of CDK4, cyclin D, and phosphorylated Rb proteins but increased the levels of p15, a specific inhibitor of CDK4 and CDK6, thereby regulating cell cycle progression.

Then, we elucidated the mechanism underlying AA-PMe-induced apoptosis in gastric cancer cells. Apoptosis is essential for the normal development and maintenance of tissue homeostasis. On the other hand, apoptosis is also involved in pathologic conditions especially cancers. Bcl-2 family proteins cytochrome c and caspas are well-known apoptosis-inducing signaling pathways. Induction of cancer cell apoptosis has become a promising direction for...
Figure 8 Compared to AA, AA-PMe showed a stronger ability in enhancing the expression of proapoptotic protein Bax and suppressing the antiapoptotic protein Bcl-2, c-Myc, and caspase 3.

Notes: SGC7901 and HGC27 cells (2×10⁶/mL) were treated with different concentrations of AA-PMe or AA for 24 hours, and then the whole-cell extracts were prepared and analyzed using Western blot using the indicated antibodies. Equal protein loading was confirmed by probing the Western blots with an anti-GAPDH antibody. The density of each band was measured and normalized to that of GAPDH. (A) Western blots of Bcl-2, Bax, c-Myc and caspase 3 in SGC7901 cells. (B) Western blots of Bcl-2, Bax, c-Myc and caspase 3 in HGC27 cells. (A and B) The same methods as A and C in HGC27 cells. Significant increases are denoted by *P<0.05, **P<0.01, compared with their respective controls.

Abbreviations: AA, asiatic acid; AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester.
Figure 9 AA-PMe inhibits the migration of SGC7901 and HGC27 cells.

Notes: Effect of AA-PMe on the migration of SGC7901 (A, C) and HGC27 (B, D) cells was analyzed using wound healing assay. Representative photomicrographs of initial and final wounds are shown at 100× magnification and the migration distance was measured using the formula: (initial wound size – final wound size)/2. Cell migration data shown are mean ± SD of three samples for each treatment. Significant increases are denoted by *P<0.05, **P<0.01, compared with their respective controls.

Abbreviations: AA-PMe, N-(2β,3β,23-acetoxyurs-12-en-28-oyl)-L-proline methyl ester; SD, standard deviation.
Figure 10 AA-PMe inhibits motility in SGC7901 and HGC27 cells.

Notes: Effects of AA-PMe on the invasion potential of SGC7901 (A, C) and HGC27 (B, D) cells were examined by manual counting and measuring absorbence at 570 nm, respectively. Cell invasion inhibition percentage data shown are mean ± SD of three samples from each treatment. Significant increases are denoted by *P < 0.05, **P < 0.01, compared with their respective controls.

Abbreviations: AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester; SD, standard deviation.
Figure 11 AA-PMe reduced MMP-2 and MMP-9 protein levels in SGC7901 and HGC27 cells analyzed by Western blot.

Notes: Whole-cell extracts were prepared and analyzed using Western blot with anti-MMP-2 and MMP-9 antibodies. Equal protein loading was confirmed by probing the Western blots with an anti-GaPDH antibody. The density of each band was measured and normalized to that of GaPDH. (A) Western blots of MMP-2, MMP-9 in SGC7901 cells. (C) Densitometric analysis of the relative level of MMP-2 and MMP-9 in SGC7901 cells. (B, D) The same methods as (A) and (C) in HGC27 cells. Significant reductions are denoted by *P<0.05, **P<0.01, compared with their respective controls.

Abbreviations: AA-PMe, N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester; SD, standard deviation.

Figure 12 (Continued)
anti-cancer drug development. Apoptosis is directly mediated by caspase-3, a family member of cysteine proteases, which is activated by the formation of apoptosisome, relying on the release of cytochrome c from mitochondria to cytosol regulated by Bax and Bcl-2. The proapoptotic protein Bax favors cytochrome c release by forming pores in the outer mitochondrial membrane, while the antiapoptotic protein Bcl-2 prevents the opening of mitochondrial transition pore by binding to Bax. In our study, we found that AA-PMe could induce apoptosis of gastric cancer cells by decreasing the levels of Bcl-2, caspase 3, and c-Myc proteins and by increasing the levels of Bax.

We also investigated the antimigration/invasion activities of AA-PMe as well as the underlying mechanisms. Cell migration and invasion results demonstrated that AA-PMe efficiently decrease the migration and invasion of SGC7901 and HGC27 cells. MMPs are critical molecules favoring tumor invasion and metastasis by degrading extracellular matrix surrounding the tumor, especially the basement membrane. Higher expression levels of MMP-2 and MMP-9 have been correlated with increased tumor metastasis and poorer prognosis. Our study indicated that AA-PMe downregulated MMP-2 and MMP-9 levels, which may at least partially explain the antiinvasion activity of AA-PMe.

Conclusion
Our studies demonstrated that AA-PMe, a novel derivative of AA, has stronger anti-cancer activities in human gastric cancer cells than its parent compound AA. However, AA-PMe almost has no toxicity in normal human gastric mucosal epithelial cells. AA-PMe inhibited the proliferation of gastric cancer cells by blocking G₁-S phase transition and arresting cell cycle in G₁/S phase via affecting cell cycle regulators. AA-PMe induced cell apoptosis by enhancing the expression of proapoptotic protein and suppressing the expression of antiapoptotic proteins. Moreover, AA-PMe also suppressed the migration and invasion of gastric cancer cells by downregulating the expression of MMP-2 and MMP-9. Our study suggests that AA-PMe is a promising anti-cancer drug candidate in gastric cancer therapy.

Disclosure
The authors report no conflicts of interest in this work.

References


