J Ginseng Res 43 (2019) 488–495

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research Article

Synergistic anticancer effects of timosaponin AIII and ginsenosides in MG63 human osteosarcoma cells

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ARTICLE INFO

Article history:
Received 18 September 2018
Accepted 9 November 2018
Available online 16 November 2018

Keywords:
Apoptosis
Ginsenoside
Metastasis
Osteosarcoma
Timosaponin AIII

ABSTRACT

Background: Timosaponin AIII (TA3) is a steroidal saponin extracted from *Anemarrhena asphodeloides*. Here, we investigated the anticancer effects of TA3 in MG63 human osteosarcoma cells. TA3 attenuates migration and invasion of MG63 cells via regulations of two matrix metalloproteinases (MMPs), MMP-2 and MMP-9, which are involved with cancer metastasis in various cancer cells. TA3 reduced enzymatic activities and transcriptional expressions of MMP-2 and MMP-9 in MG63 cells. TA3 also inhibited Src, focal adhesion kinase, extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), p38, β-catenin, and cAMP response element binding signaling, which regulate migration and invasion of cells. TA3 induced apoptosis of MG63 cells via regulations of caspase-3, caspase-7, and poly(ADP-ribose) polymerase (PARP). Then, we tested several ginsenosides to be used in combination with TA3 for the synergistic anticancer effects. We found that ginsenosides Rb1 and Rc have synergistic effects on TA3-induced apoptosis in MG63 cells.

Methods: We investigated the anticancer effects of TA3 and synergistic effects of various ginseng saponins on TA3-induced apoptosis in MG63 cells. To test antimetastatic effects, we performed wound healing migration assay, Boyden chamber invasion assays, gelatin zymography assay, and Western blot analysis. Annexin V/PI staining apoptosis assay was performed to determine the apoptotic effect of TA3 and ginsenosides.

Results: TA3 attenuated migration and invasion of MG63 cells and induced apoptosis of MG63 cells. Ginsenosides Rb1 and Rc showed the synergistic effects on TA3-induced apoptosis in MG63 cells.

Conclusions: The results strongly suggest that the combination of TA3 and the two ginsenosides Rb1 and Rc may be a strong candidate for the effective antiosteosarcoma agent.

1. Introduction

Osteosarcoma is one of the most aggressive malignant neoplasms that occur in adolescents and young adults. It develops in bone. The survival rate for osteosarcoma in 5 years is around 60 % [1]. Although there are many anticancer drugs developed for osteosarcoma, the survival rate of osteosarcoma patients has not been significantly increased [2]. Therefore, there is considerable interest in developing new anticancer drugs for the treatment of osteosarcoma.

Anemarrhena asphodeloides has been used in Asian countries as the traditional treatments for the diseases such as diabetes and hemoptysis. Recent studies have reported that *A. asphodeloides* inhibits tumor growth and induces apoptosis in gastric cancer [3]. Until now, various compounds such as flavonoids and steroidal saponins were separated from *A. asphodeloides*. Steroidal saponins are the major compounds of *A. asphodeloides*, and they are classified as spirostanol saponin and furostanol. Timosaponin AIII (TA3) belongs to spirostanol saponins which has a sugar chain at the C3 position (Fig. 1A) [4]. Spirostanol saponins are reported to have antiinflammatory, antitumor, and antiplatelet effects [5].

TA3 has been studied and reported to regulate proliferations of human breast and prostate cancer cells via mammalian target of rapamycin (mTOR) downregulation, and endoplasmic reticulum (ER) stresses induction [6]. In addition, it triggers autophagy prior to apoptosis, which is mediated by mitochondria, in Hela cells [7]. TA3 also shows antimetastatic effects via controls of matrix metalloproteinase (MMP)-2 and MMP-9 through the suppressions of Src/focal adhesion kinase (FAK), ERK, and β-catenin signalings in A549 non–small cell lung cancer cells [8]. However, the anticancer effect of TA3 on human osteosarcoma is still unclear.

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https://doi.org/10.1016/j.jgr.2018.11.002
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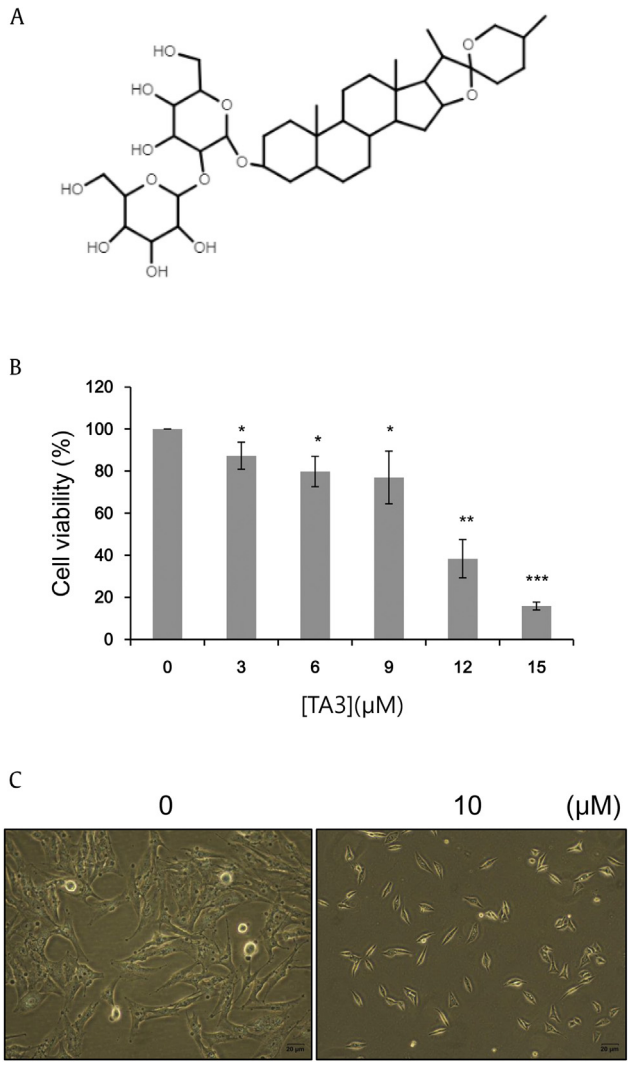

Fig. 1. Cytotoxic effect of TA3 on MG63 human osteosarcoma cells. (A) Timosaponin A III (TA3) chemical structure. **(B)** The viability of TA3-treated MG63 cells was assayed using CCK-8 viability assay. MG63 cells were treated with ascending dosages of TA3 (0, 3, 6, 9, 12, and 15 μM) for 24 h. **(C)** Morphological changes of MG63 cells after the treatment of TA3 (10 μM) for 24 h. Statistical analysis of results was performed by Student *t* test. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Multidrug resistance (MDR) is a major problem in the process of developing a new anticancer agent. Numerous studies have been identified MDR [9]. Ginseng has been used in oriental medicine in northeast Asia. Several ginseng-derived compounds stimulate apoptosis in various human cancers [10–12]. In addition, ginseng compounds have been studied to enhance effects of other compound [13] and overcome MDR effects [14].

In this study, we investigated the antitumor effects of TA3. Then, we tested the synergistic anticancer effect of TA3 with various ginsenosides which can be used to alleviate MDR in MG63 human osteosarcoma cancer cells.

2. Materials and methods

2.1. Cell culture

MG63 and U2OS osteosarcoma cells were cultured in Dulbecco's modified Eagles' medium (HyClone, South Logan, UT, USA) supplemented with mixed antibiotics (100 U/mL of penicillin plus 100 mg/mL streptomycin) (HyClone) and 10% fetal bovine serum (FBS) (HyClone). Cells were incubated at 37°C in a humidified atmosphere (5% CO_2 and 95% humidity). The stock solution of TA3 was prepared with dimethyl sulfoxide (DMSO).

2.2. Cell viability assay

MG63 cells were grown in 96-well plates at a density of 1.5×10^4 cells per well with 10% FBS and incubated overnight. U2OS cells were grown in 96-well plates at a density of 2.0×10^4 cells per well with 10% FBS and incubated overnight. Then, both cells were treated with TA3 and/or ginsenosides compound K (CK), Rb1, and Rc at indicated concentrations for 24 h. After treatment of TA3 and/or ginsenosides for 24 h, the medium was replaced with 10% of cell counting kit-8 (CCK-8) (Dojindo, Rockville, MD, USA) in Dulbecco's modified eagle medium (DMEM) containing 10% FBS and incubated for 1 h. The optical density was measured at 450 nm by spectrophotometer.

2.3. Quantitative real-time polymerase chain reaction

To extract RNA from MG63 and USOS cells, RNeasy kit (QIAGEN) was used following the manufacturer's protocol. cDNA was synthesized using Synthesis Kit (Philekorea, Daejeon, Korea). For quantitative real-time polymerase chain reaction, QuantiSpeed Sybr Kit (Philekorea) was used. Primers for real-time PCR were as follows: (1) GAPDH Forward *5′*-TGCACCACCAACTGCTTAGC- *3′*, GAPDH Reverse *5′*-GGCATGGACTGTGGTCATGAG-3′; (2) hMMP-2 Forward *5′*-TTGACGGTAAGGACGGACTC-*3′*, Reverse *5′*-ACT TGC AGT ACT CCC CAT CG-3′; and (3) hMMP-9 Forward *5′*-GAGACCGGT GAG CTG GAT-*3′*, Reverse *5′*-TAC ACG CGA GTG AAG GTG AG-*3′*. *The real-time PCR was conducted* by the ROTER GENE Q (QIAGEN) for the determination of Ct values. For the analysis of relative gene expression, we used the $2^{\Delta\Delta Ct}$ method.

2.4. Annexin V/PI staining apoptosis assay

To analyze TA3-induced apoptosis in MG63 cells, The FITC Annexin V apoptosis detection kit (BD Bio-Sciences, Franklin Lakes, NJ, USA) was used. The cells were seeded in a 6-well plate (1.5×10^5 cells/well) and incubated overnight. Then, MG63 cells were treated with TA3 and/or ginsenosides CK, Rb1, and Rc at indicated concentrations for 24h. Then, the cells were washed by Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 1x binding buffer with FITC Annexin V and PI reagent for 15 min at room temperature (RT, 25 °C) in the dark. Then, the MG63 cells were analyzed by flow cytometry (Beckman Coulter, USA).

2.5. Boyden chamber invasion assay

The ability of MG63 and U2OS cells to penetrate membrane-coated gelatin was assessed by the Boyden chamber invasion assay. In 0.1% acetic acid contained distilled water, gelatin (Sigma, St. Louis, MO, USA) was diluted down to 0.1 g/L. Then, the diluted gelatin solution was applied to the top side of the 8 mm pore polycarbonate membrane (Neuro Probe, Gaithersburg, MD, USA). Cells were collected by trypsin and resuspended in 0.1% FBS DMEM. 1% FBS containing medium with various dosages (0, 3 and 6 μM) of TA3 was applied to the lower chamber in 30 μl. 0.1% FBS was used for the negative control. Then, cells were seeded on the upper chamber (density of 5.0×10^4 cells/well in 50 μl). After incubation for 20 h at 37 °C, the upper chamber was carefully removed, and the lower side of the membrane were fixed by 4% formaldehyde in PBS and stained with crystal violet solution (1% crystal violet, 20% methanol in distilled water). The numbers of invaded cells were calculated by a Leica DM IL LED (Leica Microsystems).

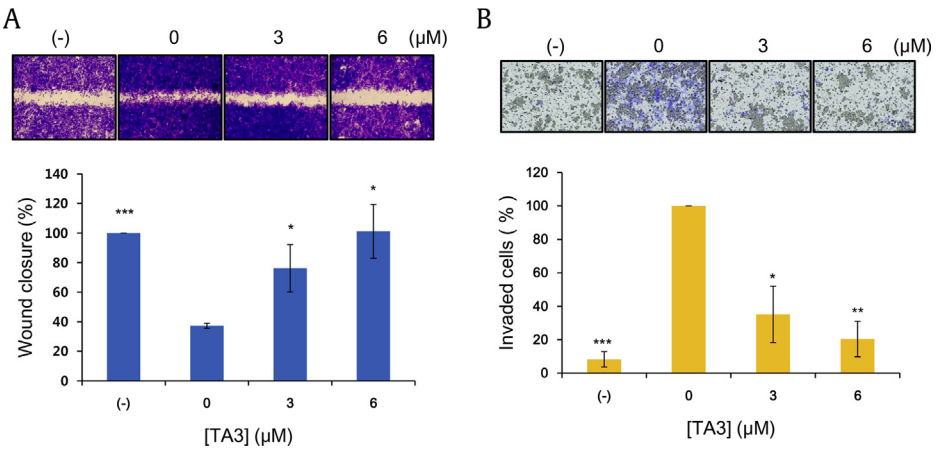

Fig. 2. Effects of TA3 on migration and invasion of MG63 human osteosarcoma cells. (A) TA3 inhibits migration of osteosarcoma MG63 cells. MG63 cells were treated with ascending concentrations (0, 3, and 6 μM) of TA3 for 24 h. The cells with 0.1% FBS were used for negative control. The recoveries of wound widths were reduced in a dosage dependent manner of TA3. The wound width was measured and used for the graphs. **(B)** TA3 suppresses invasion of osteosarcoma MG63 cells. The cells with 0.1% FBS were used as negative control. The cells, which had invaded across the membrane, were counted and plotted. Statistical analysis of results was performed by Student *t* test. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). TA3, timosaponin A III.

2.6. Wound healing migration assay

MG63 and U2OS human osteosarcoma cells were grown in 96-well plates at 1.5×10^4 cells/well and at 2.0×10^4 cells/well, respectively, with 10% FBS. Then, the middle of the cell surface was scraped by 200 μl micropipette tip to make a wound of constant width. Then, the debris was washed using PBS, and MG63 cells were exposed to TA3 (0, 3 and 6 μM) in DMEM containing 10% FBS, and 0.1% FBS was used for the negative control. The closures of wounds of MG63 and U2OS cells were monitored and photographed at 20 h and 30 h, respectively, by a Leica DM IL LED (Leica Microsystems, Wetzlar, Germany).

2.7. Gelatin zymography

MMP-2 and MMP-9 activities were evaluated by gelatin zymography. MG63 and USOS cells were seeded on 60-mm cell culture dish at 2.0×10^5 cells in DMEM containing 10% FBS. Then, both cells were incubated with TA3 (0, 3, and 6 μM) in presence of DMEM (+0.1% FBS) and incubated for 48 h. After the incubation, the culture media were harvested and concentrated by Amicon Ultra-15 centrifugal filter (Millipore). The samples were prepared in standard sodium dodecyl sulfate (SDS)-gel loading buffer (with 0.01% SDS) without dithiothreitol. Electrophoresis was conducted on 8% SDS polyacrylamide gel (with 0.1% gelatin). The gels were rinsed using washing buffer (2.5% Triton X-100 in distilled H_2O) three times for 10 min at room temperature (RT). Then, the gels were incubated in developing solution (50 mM Tris-HCl, pH 7.6, 5 mM $CaCl_2$) for 24 h at 37°C. The developed gels were stained in Coomassie Brilliant blue R Staining Solution for 30 min and washed with destaining solution (20% methanol, 10% acetic acid, 70% distilled H2O).

2.8. Western blotting

MG63 cells were seeded in 6-well dishes (1.5×10^5). Then, the cells were treated with TA3 (0, 5, and 10 μM) for 24 h. Then, MG63 cells were collected and lysed in RIPA buffer (with phosphatase inhibitor cocktail) (Gendepot, USA) and protease inhibitor cocktail (Gendepot, USA). The lysates were normalized by bicinchroninic acid (BCA) protein assay kit (Thermo Fisher, USA). The samples were resuspended in SDS-sample buffer [Tris-HCl (62 mM), pH 6.8, ethylenediaminetetraacetic acid (EDTA) (1 mM), glycerol (10 %), SDS (5%), dithiothreitol (50 mM)]. Then, SDS-PAGE was performed. The protein bands of the gel were transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were incubated in blocking buffer (5% nonfat skim milk in 1X tris-buffered saline (TBS)-T) for 1 h at 4°C on shaking incubator and incubated with specific primary antibodies at 4°C overnight. Then, the membranes were subjected to the incubation with secondary antibodies for 2 hrs. The membranes were washed with TBS-T three times. The immunoreaction was measured using Western blot detection kit (AbFrontier).

2.9. Statistical analysis

All quantitative data are marked as the mean ± SD from three independent times. Statistical analysis of results was performed by Student *t* test.

3. Results

3.1. Cytotoxicity of TA3 in MG63 human osteosarcoma cells

To determine the cytotoxic effect of TA3 on human osteosarcoma MG63 cells, MG63 cells were treated with ascending concentrations of TA3 (0, 3, 6, 9, 12, and 15 μM) for 24 h and subjected to the CCK-8 assay. As seen in Fig. 1B, about 80 % of cells were survived at 9 μM TA3 treatment. The viability of MG63 cells went down below 50% from 12 μM TA3 treatment. We also confirmed morphological changes, which are related apoptosis, in MG63 cells after treatment of TA3 (10 μM) (Fig. 1C). Morphological changes of TA3-treated MG63 cells implicate the possible induction of apoptosis by TA3.

3.2. Inhibitions of migration and invasion of MG63 human osteosarcoma cells by TA3

We performed wound healing assay to determine if TA3 affects migration of MG63 cells. MG63 cells were confluent on the culture dish, and the center part of the lawn of cells was scraped using a sterilized 200 μl tip to make a wound. Restoration of wound width means the migration of the cells. Our results showed that TA3 attenuated migration of human MG63 osteosarcoma cells (Fig. 2A). Then, we performed Boyden chamber invasion assay to confirm the

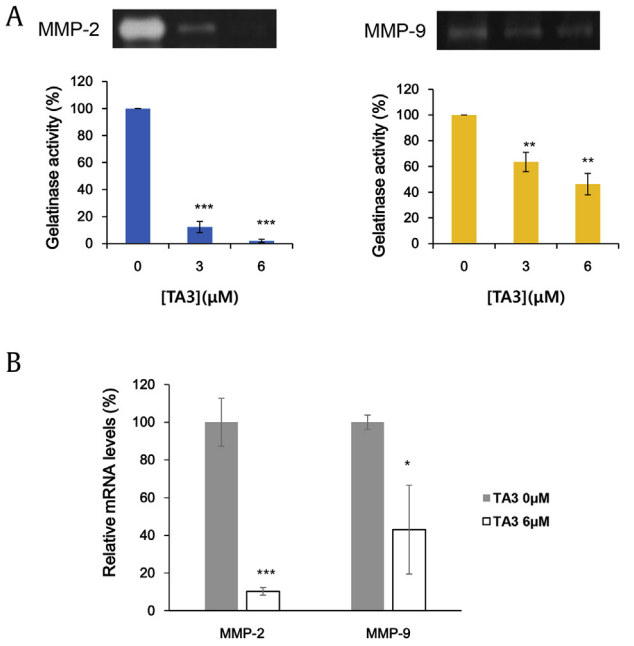

Fig. 3. Inhibitory effect of TA3 on MMP-2 and MMP-9 in MG63 human osteosarcoma cells. (A) Gelatinase activities of MMP-2 and MMP-9 were inhibited by TA3 (0, 3, and 6 μM) in MG63 cells. **(B)** Transcriptional expressions of MMP-2 and MMP-9 were reduced by TA3 (6 μM) in MG63 cells. quantitative Real Time-PCR (qRT-PCR) was conducted on the mRNA from the TA3-treated cells. Statistical analysis of results was performed by Student *t* test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). TA3, timosaponin A III; MMP, matrix metalloproteinase.

effect of TA3 on the invasive ability of MG63 cells. MG63 cells were placed in the upper chamber and various concentrations of TA3 (0, 3, and 6 μl) were placed in the lower chamber. The membrane, which is coated with gelatin, was sandwiched between the two chambers. The cells at the bottom of the coated membrane indicate the invaded cells. As seen in Fig. 2B, invasive ability of MG63 cells was reduced by the treatment with TA3 (3 and 6 μl) compared with the control. Then, we investigated the effects of TA3 on enzymatic activities of MMP-2 and MMP-9 by gelatin zymography assay (Fig. 3A). The results showed that TA3 inhibited activities of both enzymes. To determine whether the decreased gelatin degradation is related with the transcriptional expressions of the two enzymes, we performed quantitative real time PCR (Fig. 3B). Transcriptional expression levels of MMP-2 and MMP-9 were reduced by the treatment of TA3 (6 μM).

3.3. Effects of TA3 on signaling proteins related with cell migration and invasion in MG63 human osteosarcoma cells

We performed Western blot analysis to identify if TA3 affects the activations of the two signaling molecules Src and FAK which are related with migration of cells. The phosphor forms of the two proteins, which represent the active forms of the proteins, were decreased by the treatment of TA3 (0, 5, and 10μM) in MG63 cells (Fig. 4A). We also investigated the effect of TA3 on the three mitogen-activated protein kinases (MAPKs) JNK, p38, and ERK1/2. The phosphor forms of ERK1/2, JNK, and p38 were reduced in a dose-dependent manner by the treatment with TA3 (Fig. 4B). This indicates that TA3 inhibits the activations of those three MAPKs. cAMP response element binding (CREB) protein, which is classified in leucine zipper protein, has been reported to be a transcription factor that induces MMP-2 and MMP-9 [15]. β-catenin has also been reported to enhance MMP-2 and MMP-9 expressions as a transcription factor [16]. Therefore, we investigated the effect of TA3 on the two transcription factors CREB and β-catenin. β-catenin level was decreased by the treatment with TA3 (0, 5, and 10 μM) (Fig. 4C). Also, activation of CREB was downregulated as indicated by the decreased Western blot band intensities of phosphor forms of CREB (Fig. 4C).

3.4. Apoptotic effect of TA3 on MG63 human osteosarcoma cells

We conducted Annexin V/PI apoptosis assay to examine whether TA3 induces apoptosis in MG63 cells. The results indicated that the proportion of total apoptotic cells (early and late cell death) was increased by the treatment with TA3 (6 and 12 μM) (Fig. 5A). We also performed Western blot analysis to examine the levels of PARP, caspase-3, and caspase-7, which are deeply related with the apoptosis, in MG63 cells. Caspases are protease enzymes that induce programmed cell death. Among them, caspase-3 and capsase-7 can be activated by the proteolytic cleavage and induce apoptosis [17]. We treated various dosages of TA3 (0, 5, and 10 μM) to MG63 cells for 24 h. We were able to confirm that the protein levels of uncleaved forms of caspase-3 and caspase-7 were decreased by TA3 (Fig. 5B). Also, the uncleaved PARP was decreased, and the cleaved PARP was increased by the treatment of TA3 in a dose-dependent manner (Fig. 5C).

3.5. Antimetastatic effects of TA3 in U2OS osteosarcoma cells

We also conducted the assays that are related with cancer metastasis in U2OS cells to investigate whether the antimetastatic effects of TA3 are limited to the MG63 cells. TA3 also showed cytotoxic effect on U2OS cells in a dose-dependent manner (Fig. 6A). Migration and invasion of U2OS cells were also downregulated by the treatment of TA3 in a dose-dependent manner (Fig. 6B and C). In addition, we confirmed the effect of TA3 on activities of MMP-2 and MMP-9 in U2OS cells. The gelatinase activities of MMP-2 and MMP-9 were attenuated by the treatment of TA3 (0, 3, and 6 μM) (Fig. 6D). Taken together, antimetastatic effects of TA3 may not be limited to the MG63 cells.

3.6. Screening of ginsenosides for the synergistic effect on TA3-induced apoptosis in MG63 human osteosarcoma cells

To determine the ginseng derived compounds that can be used in combination with TA3 for the synergistic anticancer effect in human osteosarcoma, we performed CCK-8 cell viability assay (Fig. 7A). TA3 was treated with the three ginsenosides, CK, Rb1, and Rc for 24 h. The results showed that all the ginseng compounds tested enhance the cytotoxicity of TA3 (Fig. 7A). In addition, we conducted annexin V/PI apoptosis assay to examine whether the ginseng compounds improve TA3-induced apoptosis. We confirmed that the ginsenosides Rb1 and Rc stimulate the TA3-induced apoptosis (Fig. 7B and C). However, CK does not have synergistic effect with TA3. Notably, ginsenoside Rc has a significant apoptotic effect by itself.

4. Discussion

There are various anticancer agents for osteosarcoma developed so far. However, the survival rate of osteosarcoma patients is still low. Thus, it is worth to develop new anticancer drugs to treat osteosarcoma patients. *A. asphodeloides* is used as a traditional herb. TA3 is one of the major compounds extracted from *A. asphodeloides*. In this study, we investigated TA3 as an anticancer candidate molecule for the osteosarcoma and tried to screen ginsenosides for

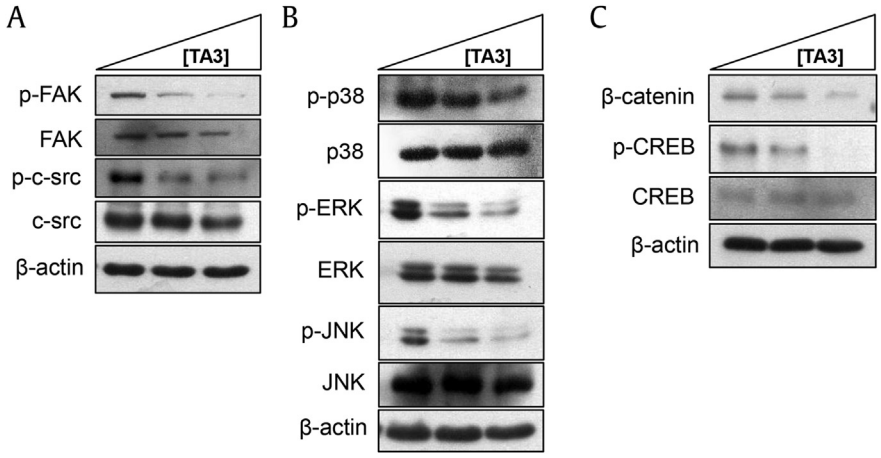

Fig. 4. Effect of TA3 on signalings in MG63 human osteosarcoma cells. (A) Activations of Src and FAK were attenuated by the treatment of TA3. MG63 cells were treated with TA3 (0, 5, and 10 μM) for 24 h, and Western blot analysis was performed. **(B)** Effect of TA3 on p38, JNK, and ERK1/2 in MG63 human osteosarcoma cells were investigated by Western blot analysis. p38, JNK, and ERK1/2 were inactivated by the treatment of TA3. MG63 cells were treated with TA3 (0, 5, and 10 μM) for 24 h. (C) β-catenin and active CREB (p-CREB) were decreased by TA3 (0, 5, and 10 μM). TA3, timosaponin A III; FAK, focal adhesion kinase; CREB, cAMP response element binding.

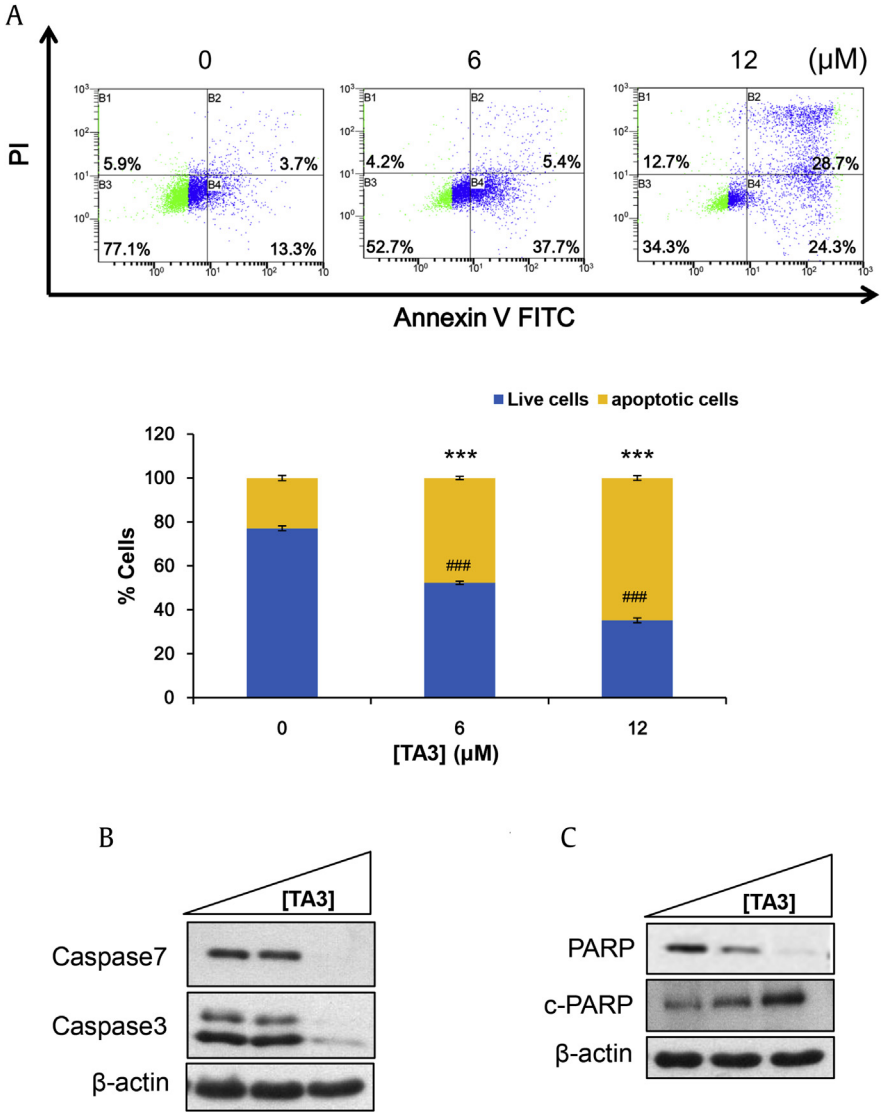

Fig. 5. Apoptosis-inducing effect of TA3 in MG63 human osteosarcoma cells. (A) TA3 induces apoptosis of MG63 human osteosarcoma cells. Cells were treated with TA3 (0, 6, and 12 μM) for 24 h. Apoptosis of MG63 cells was analyzed by flow cytometry. **(B)** Uncleaved forms of caspase-3 and caspase-7 were diminished by TA3 treatment (0, 5, and 10 μM) for 24 h. (C) Uncleaved form of PARP was decreased while cleaved form of PARP was increased by TA3 treatment (0, 5, and 10 μM) for 24 h. Statistical analysis of results was performed by Student *t* test. ($^{***}p < 0.00$ versus control of total apoptotic cells, $^{###}p < 0.001$ versus control of live cells). TA3, timosaponin A III.

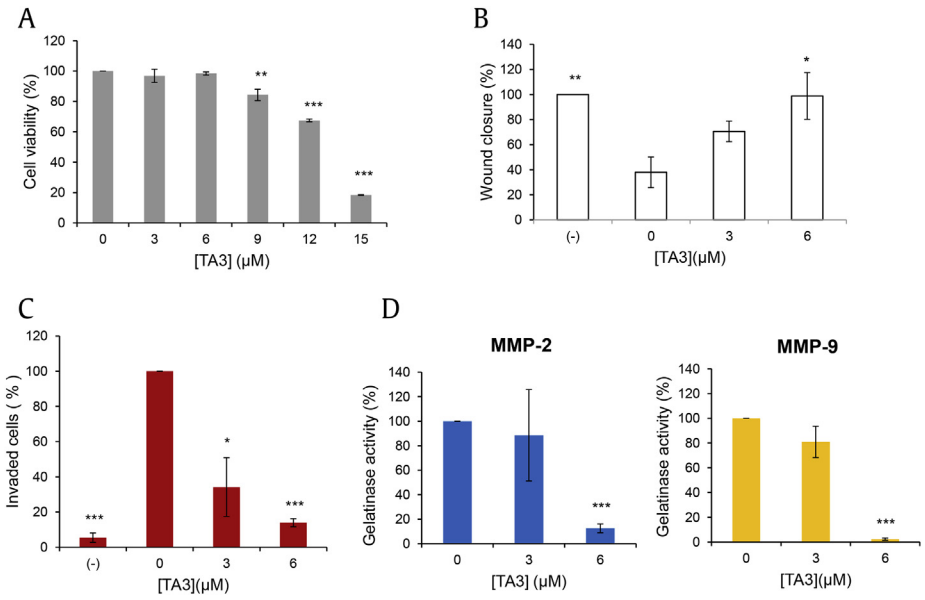

Fig. 6. Antimetastatic effects of TA3 in U2OS human osteosarcoma cells. (A) Cytotoxicity of TA3 in U2OS human osteosarcoma cells was measured by CCK-8 viability assay. U2OS cells were treated with TA3 (0, 3, 6, 9, 12, and 15 μM) for 24 h. **(B)** TA3 inhibits migration of U2OS human osteosarcoma cells. The cells were treated with several concentrations (0, 3, and 6 μM) of TA3 for 24 h. The cells with 0.1% FBS were used as negative control. The recoveries of wound widths were reduced by TA3 treatment. The wound widths were measured and used for the graphs. **(C)** TA3 attenuated invasion of U2OS human osteosarcoma cells. The cells were treated with TA3 (0, 3, and 6 μM) for 20 h. The cells with 0.1% FBS were used as negative control. The invaded cells were counted and plotted. **(D)** Gelatinase activities of MMP-2 and MMP-9 were inhibited by TA3 treatment (0, 3, and 6 μM) in U2OS cells. Statistical analysis of results was performed by Student *t* test. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). TA3, timosaponin A III, MMP, matrix metalloproteinase; FBS, fetal bovine serum.

the combinatorial use with TA3 to drive synergistic anticancer effect.

Cancer metastasis is the multistep processes, such as cell migration/invasion, epithelial into mesenchymal transition and angiogenesis. Among them, cellular migration and invasion are initial steps for the cancer cells to extend into the other tissues through the blood and/or lymphatic vessels [18]. We were able to conclude that TA3 has antimigratory and antiinvasive effects on human osteosarcoma in this study. In consistent with this study, earlier reports also indicated that TA3 has inhibitory effects on migratory and invasive abilities in non−small cell lung cancer cells and melanoma cancer cells [8,19]. MMPs are the key enzymes to degrade extracellular matrix (ECM). The ECM is a matter of multicomponents such as gelatin, collagen, elastin, and laminin. It supports the surrounding cells structurally [20]. The ECM must be degraded to allow cell migration and invasion. Both MMP-2 and MMP-9 contribute to the cellular migration/invasion via enzymatic and nonenzymatic ways [21,22]. In this study, we identified that TA3 inhibits MMP-2 and MMP-9 via downregulation of transcriptional expressions. There are many signalings associated with migration/invasion of cells. Src and FAK signaling pathways are well known to regulate MMP-2 and MMP-9 [21,23−25]. FAK and c-Src are dual kinases which act as a complex. This complex can phosphorylate various signaling proteins such as MAPKs, which are key signaling molecules regulating cell metastasis [26]. Our investigation indicated that TA3 downregulates these signaling molecules significantly. We also investigated transcription factors such as β-catenin and CREB. These transcription factors are well known for stimulation of MMP-2 and MMP-9 [27,28]. β-catenin and active CREB levels were decreased by TA3. These results may indicate that TA3 may inhibit migration and invasion of MG63 cells by the attenuations of FAK/c-Src, ERK1/2, JNK, p38, β-catenin, and CREB signaling pathways.

Various mutant genes block apoptosis and lead to cancer metastasis and stimulation of tumor proliferation [29]. TA3 induces apoptosis in many types of cancer cells [7,30,31]. Our data from this study indicate that TA3 induces apoptosis in MG63 cells. Caspase-3 and caspase-7 are initial signals that trigger apoptosis [32]. In earlier reports, caspase-3 and caspase-7 amplify cytochrome c release which induces apoptosis [33]. We observed that inactive forms of caspase-3 and caspase-7, which are procaspase-3 and procaspase-7, were decreased by the treatment of TA3 in MG63 cells. This may indicate that TA3 stimulated the proteolytic cleavages of caspase-3 and caspase-7 to generate active forms and contribute to the stimulation of apoptosis. PARP plays a role in repair of damaged DNA. When cleaved by caspase-3 and caspase-7, PARP loses its function and stimulates signals for cell death [34,35]. Here, we found that TA3 stimulates PARP cleavage and induces apoptosis in MG63 cells.

For cancer chemotherapy, MDR is one of the major problems. Recent studies reported that the ginseng compounds exert antimultidrug resistance [14,36]. Also, the combinatorial use of a natural compound maclurin with ginsenoside CK shows the significant synergistic effect [13]. To find the ginseng saponins for the combinatorial use with TA3, we tested synergistic effect of ginsenosides CK, Rb1, and Rc on TA3-induced apoptosis. Results indicate that ginsenosides Rb1 and Rc stimulated TA3-induced apoptosis, but ginsenoside CK did not show the synergistic effect. In addition, we found out that ginsenoside Rc has a significant apoptosis-inducing effects on its own in MG63 cells.

In conclusion, we have shown that TA3 exerts anticancer effects in MG63 human osteosarcoma cells. The anticancer effects of TA3 are not limited to the MG63 cells as shown and are also effective in U2OS cells. Especially, the ginsenosides Rb1 and Rc showed the synergistic effect on TA3-induced anticancer effect in human osteosarcoma cells. Therefore, we suggest that the combinatorial use of ginsenosides Rb1 and Rc with TA3 may be the promising way to develop a strong candidate for the effective novel antiosteosarcoma agent

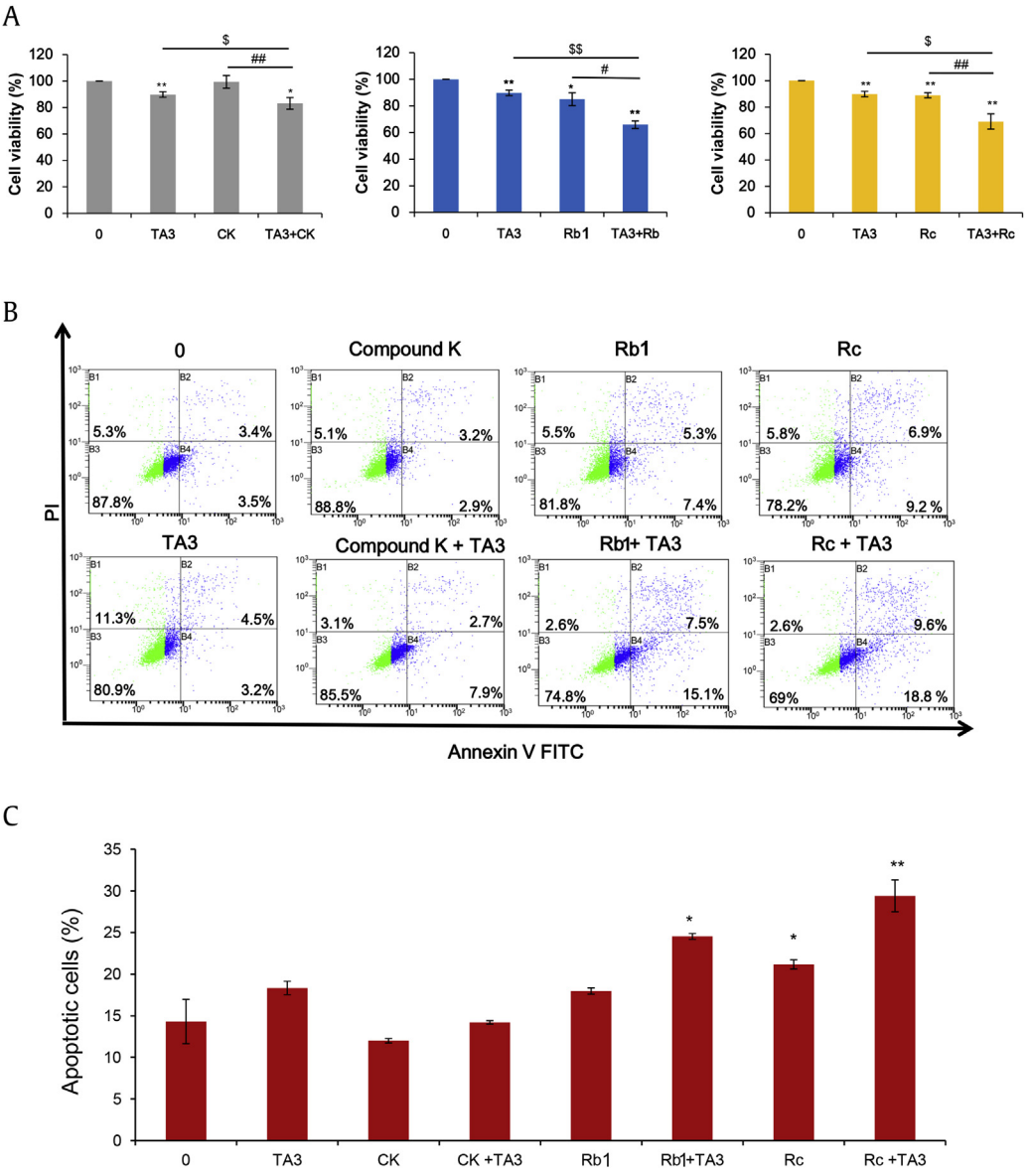

Fig. 7. Synergistic effects of ginseng saponins Rb1 and Rc on TA3-induced apoptosis in MG63 human osteosarcoma cells. (A) Cytotoxic effects of several ginseng compounds with TA3 were tested in MG63 cells. Cells were subjected to the following treatment for 24 h; TA3 (6 μM), Compound K (20 μM), TA3 (6 μM) plus Compound K (20 μM), Rb1 (250 μM), TA3 (6 μM) plus Rb1 (250 μM), Rc (250 μM), and TA3 (6 μM) plus Rc (250 μM) for 24 h and subjected to CCK-8 assay. **(B)** Stimulations of TA3-induced apoptosis by the treatment of Rb1 and Rc in MG63 cells. Cells were treated with TA3 (6 μM) and/or Compound K (20 μM), Rb1 (250 μM), and Rc (250 μM) for 24 h and subjected to annexin V/PI apoptosis assay. **(C)** Quantifications of apoptotic cells from annexin V/PI apoptosis assay. Statistical analysis of results was performed by Student *t* test. ($^{*}p < 0.05$, $^{**}p < 0.01$ versus control, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ versus treatment of TA3, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus treatment of ginseng compounds). TA3, timosaponin A II.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgment

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) and is funded by the Ministry of Education (NRF-2018R1D1A1B07040998).

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