Enhanced anticancer effect of gemcitabine by genistein in osteosarcoma: the role of Akt and nuclear factor-κB

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Genistein, a nontoxic flavonoid compound, has potent antitumor activity in various cancer cell lines. This study was designed to investigate whether combination therapy with gemcitabine and genistein enhances antitumor efficacy in osteosarcoma cell lines (MG-63 and U2OS). Our results show that significant reduction in cell viability and corresponding induction of apoptosis were observed with combination treatment in both cell lines. On the molecular level, we found that gemcitabine alone can activate nuclear factor κB (NF-κB) in osteosarcoma, suggesting the potential mechanism of acquired chemoresistance. In contrast, genistein reversed the cancer's resistance to gemcitabine through the downregulation of NF-κB activity and the suppression of Akt. These findings suggest that the combination of gemcitabine and genistein enhanced the antitumor efficacy by abrogating the Akt/NF-κB pathway.

The marked ability to induce apoptosis with a combination of gemcitabine and genistein suggests that this could be a rational and novel approach for osteosarcoma preclinical and clinical trials. *Anti-Cancer Drugs* 21:288–296 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Osteosarcoma (OS) is the most common malignant bone tumor, and mainly affects children and adolescents. The 5-year survival rate of patients with OS has increased up to 60% with current protocols [1]. However, although the prognosis of OS has improved significantly, the acquisition of resistance to the cytotoxic effects of chemotherapy has emerged as a significant impediment to effective cancer treatment.

Gemcitabine (dFdCyd, 2′2′-difluoro-2′-deoxycytidine) is an analog of cytosine arabinoside with antitumor activity [2]. It has been used in several solid tumors such as non-small cell lung cancer, pancreatic cancers, bladder cancer, ovarian cancer, and breast cancer [3–6]. In-vitro studies showed that gemcitabine could inhibit cell viability, growth, and metastasis of OS [7]. The clinical evidence indicated that the combination of gemcitabine and docetaxel was active in a variety of sarcomas including OS [8]. On the basis of this evidence, we suggest that gemcitabine may be a novel anticancer agent for OS. However, several clinical trials indicated that the effect of gemcitabine alone was disappointing [9,10]; this may be connected with drug resistance. Many anticancer agents, including gemcitabine, induce nuclear factor-κB (NF-κB) nuclear translocation and activation of its target genes, which impinge on cellular resistance to anticancer agents [11]. Therefore, a combination of an NF-κB inhibitor and gemcitabine may be a rational strategy for OS.

Genistein (4,5,7-trihydroxyisoflavone), a type of phytoestrogen in soybeans, has a heterocyclic diphenolic structure similar to that of estrogen. It is reported that genistein has potent antitumor activity in various cancer cell lines [12]. Epidemiological evidence suggests that the risk of cancer is reduced as a result of the increased consumption of phytoestrogens and lignans in a vegetarian diet [13]. Moreover, genistein can inhibit the activation of NF-κB, and block the Akt signaling pathway [14]. Some reports showed that Akt also regulates the NF-κB pathway through the activation of NF-κB, leading to the transcription of genes regulating growth, apoptosis, angiogenesis and invasion [15,16]. On the basis of these mechanisms, we used genistein, a nontoxic flavonoid compound, in combination with gemcitabine to test its efficacy against two OS cell lines: MG-63 and U2OS. Furthermore, the correlative molecular mechanisms were also evaluated.

Materials and methods

Reagents and antibodies

The following reagents were obtained commercially: genistein was purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA) and was dissolved in dimethyl sulfoxide (DMSO) to make 2 mmol/l of stock solution. Gemcitabine (Eli Lilly, Indianapolis, Indiana, USA) was dissolved in sterile PBS to make 10 mmol/l of stock solution. BAY11-7082 (Calbiochem, San Diego,

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California, USA) was reconstituted in DMSO as a 10 mmol/l stock solution. LY-294002 (Eli Lilly) was dissolved in DMSO to make 10 mmol/l of stock solution. Mouse monoclonal antibodies specific for Bcl-2, Bcl-xL, and β-actin and rabbit polyclonal antibody specific for survivin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Rabbit polyclonal antibody specific for phosphor-Akt (Thr308) was purchased from Cell Signaling Technology Inc. (Beverly, Massachusetts, USA). Rabbit polyclonal antibody specific for COX-2 was obtained from BioWorld Technology Ltd (Barrie, Ontario, Canada). Horseradish peroxidase-conjugated goat anti-mouse and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology Inc.

Cells and cell culture

The human OS cell line MG-63 was obtained from American Type Culture Collection (Manassas, Virginia, USA), and the U2OS OS cells were purchased from the ATCC (HTB-96; Rockville, Maryland, USA). MG-63 cells were maintained at 37°C with 5% CO_2 in an air atmosphere in Dulbecco's Modified Eagle's Medium with 10% (v/v) fetal bovine serum, and U2OS cells were grown in RPMI1640 medium containing supplements as above.

Cell viability assay

The viability of cells treated with gemcitabine or genistein was determined by the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) assay, which measures the conversion of a tetrazolium compound into formazan by a mitochondrial dehydrogenase enzyme in living cells. The amount of formazan is proportional to the number of living cells present in the assay mixture. MG-63 and U2OS cells were plated (6000–8000 per well) in 96-well plates and incubated overnight, and then the medium was removed and replaced with fresh medium containing different concentrations of gemcitabine (0–50 μmol/l) diluted from 10 mmol/l of stock or genistein (0–100 μmol/l) diluted from a 2 mmol/l stock. After 72 h of incubation, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well and incubated at 37°C for 4 h, and the medium was replaced with 150 μl of DMSO. The absorbance in control and treated wells was measured at 570 nm using the Dynatech MR7000 microplate reader (Dynatech Laboratories Inc., Chantilly, Virginia, USA). Each piece of experimental data represented the average obtained from five replicates, and each experiment was performed in triplicate.

The cells were plated as described above and allowed to attach overnight. They were then replaced with fresh medium containing gemcitabine (0.5 μmol/l), LY-294002 (10 μmol/l), BAY11-7082 (5 μmol/l), and the combination for 72 h. The effect on cell viability was examined by the MTT assay method as stated above.

Electron microscopy

The treated and untreated cells were fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epoxy resin. Thin sections were stained in uranyl acetate and lead citrate, and examined under a Philips TECNAI10 transmission electron microscope (FEI Company, Hillsboro, Oregon, USA).

DNA ladder analysis

After being treated with gemcitabine (0.5 μmol/l) and/or genistein (20 μmol/l) for 48 h, the MG-63 and U2OS cells were collected and lysed in lysis buffer consisting of 10 mmol/l Tris–HCl (pH 7.4), 10 mmol/l EDTA and 0.1% Triton X-100. They were then incubated with RNase A and proteinase K at 37°C for 60 min. After centrifugation, the soluble DNA fragments were precipitated by the addition of 0.5 volume of 7.5 mol/l ammonium acetate and 2.5 volumes of ethanol. DNA pellets dissolved in TE were loaded onto a 2% agarose gel and separated at 50 V for 90 min. The DNA fragments were visualized after staining with ethidium bromide by transillumination under UV light.

Quantification of apoptosis

For apoptosis detection, floating cells in the medium and adherent cells were collected after 72 h of treatment. Using an Annexin V–FITC Apoptosis Detection Kit (Sigma-Aldrich, St Louis, Missouri, USA), the cells were stained with Annexin V–FITC and propidium iodide according to the manufacturer's instructions. Untreated cells were used as the control. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, North Ryde, New South Wales, Australia).

Western blot analysis

Cells (2×10^6) treated with 20 μmol/l of genistein and/or 0.5 μmol/l of gemcitabine for 72 h were washed twice with ice-cold PBS, and resuspended in 200 μl of ice-cold solubilizing buffer [300 mmol/l of NaCl, 50 mmol/l of Tris–HCl (pH 7.6), 0.5% TritonX-100, 2 mmol/l of phenylmethanesulfonyl fluoride, 2 μl/ml of aprotinin and 2 μl/ml of leupeptin]. The cell resuspension was incubated at 4°C for 60 min. The lysates were centrifuged at 13 000 revolutions per minute for 20 min at 4°C. The proteins were quantified using a BCA protein assay kit (Pierce, Rockford, Illinois, USA) according to the manufacturer's specifications. Equivalent amounts of protein were loaded onto 8–12% sodium dodecylsulfate–polyacrylamide gel electrophoresis. The gels were transferred to polyvinylidene fluoride membranes, reacted with antibodies overnight at 4°C and then incubated with a horseradish peroxidase-coupled secondary antibody. The membranes were detected by enhanced chemiluminescence reagent and exposed to X-ray films.

Electrophoretic mobility shift assay for nuclear factor-κB activation

The cells were incubated with 20 μmol/l of genistein and/or 0.5 μmol/l of gemcitabine for 72 h. Nuclear proteins were prepared using nuclear and cytoplasmic extraction reagents according to the manufacturer's protocols (Pierce). A nonradioactive electrophoretic mobility shift assay was performed using an EMSA kit according to the manufacturer's instructions (Pierce). Four micrograms of nuclear protein was used to bind biotinylated oligonucleotides containing the NF-κB binding site for 30 min at room temperature. The samples were separated in a nondenaturing polyacrylamide gel (6%, with 2.5% glycerol) and blotted on a biodyne B (0.45 mm) positively charged nylon membrane (Pall Schweiz AG, Basel, Switzerland). The biotin was labeled with alkaline phosphatase-conjugated streptavidin, and alkaline phosphatase was detected with an enhanced chemiluminescence detection system (Santa Cruz Biotechnology Inc.).

Statistical analysis

The data were expressed as mean ± standard deviation. The mean values were calculated from data obtained from triplicates of each experiment. The Student's *t* test was used to evaluate the differences between the various experimental and control groups. *P* values less than 0.05 were considered statistically significant. All data were analyzed using Excel 2003.

Results

The effects of gemcitabine and genistein on the viability of osteosarcoma cells

To investigate the cytotoxicity of gemcitabine on OS, we tested the effect of various doses of gemcitabine on the viability of human OS cell lines: MG-63 and U2OS using the MTT assay. The cells were treated for 24, 48 and 72 h and it was found that cell inhibition was more significant at 72 h (data of 24 and 48 h was not shown). As shown in Fig. 1a, in MG-63 cell growth was inhibited by gemcitabine treatment in a dose-dependent manner. After treatment with 0.05, 0.1, 0.5, 1, 5,10, and 50 μmol/l of gemcitabine, the cell survival rate was 84.2, 74.3, 53.3, 38.8, 30.0, 32.8 and 30.7%, respectively. However, in U2OS, cell growth was inhibited in a nondose-dependent manner. The cell survival rate was 73.1, 70.7, 75.6, 88.7, 94.8, 100.0, and 94.9%, respectively, when exposed to the same concentrations of gemcitabine. These results indicated that MG-63 is sensitive to gemcitabine but U2OS is resistant to gemcitabine.

We subsequently evaluated the effect of genistein on cell growth. It was found that genistein was effective in inhibiting cell growth in both cell lines (Fig. 1b). These data suggested that genistein, as a single agent, was an effective inhibitor of OS cell growth, and that U2OS cells were more sensitive to genistein compared with MG63 cells.

We then assessed the effect of a combination of genistein and gemcitabine on cell viability by the MTT assay. The cells were treated with 0.5 μmol/l of gemcitabine, 20 μmol/l of genistein or the combination for 72 h. As shown in Fig. 1c, two kinds of OS cells were significantly inhibited when treated with gemcitabine and genistein. Subsequently, we used isobologram analysis [17] to examine whether there is a real sensitivation towards gemcitabine therapy by genistein in MG63, which is sensitive to gemcitabine; we found that the combination favors cell death by synergistic mechanisms (Fig. 1e).

Induction of apoptosis by gemcitabine and genistein

To examine whether the reduced viability of OS cells was caused by apoptosis, we carried out DNA electrophoresis and flow cytometry. As shown in Fig. 2, DNA electrophoresis showed a DNA ladder in OS cells treated with the combination of gemcitabine and genistein. Moreover, flow cytometry experiments (Fig. 3) achieved the same results that genistein can potentiate the OS cells apoptosis induced by gemcitabine. These results were consistent with cell growth inhibition by MTT. It is suggested that the loss of viable cells by gemcitabine and genistein is partly a result of the induction of an apoptotic cell death mechanism. In addition, the transmission electron microscope showed typical apoptotic morphological features (Fig. 4c and d): cell shrinkage, nuclear condensation, nuclear fragmentation, chromatin condensation, crescent nucleus, and cytoplasmic vacuoles.

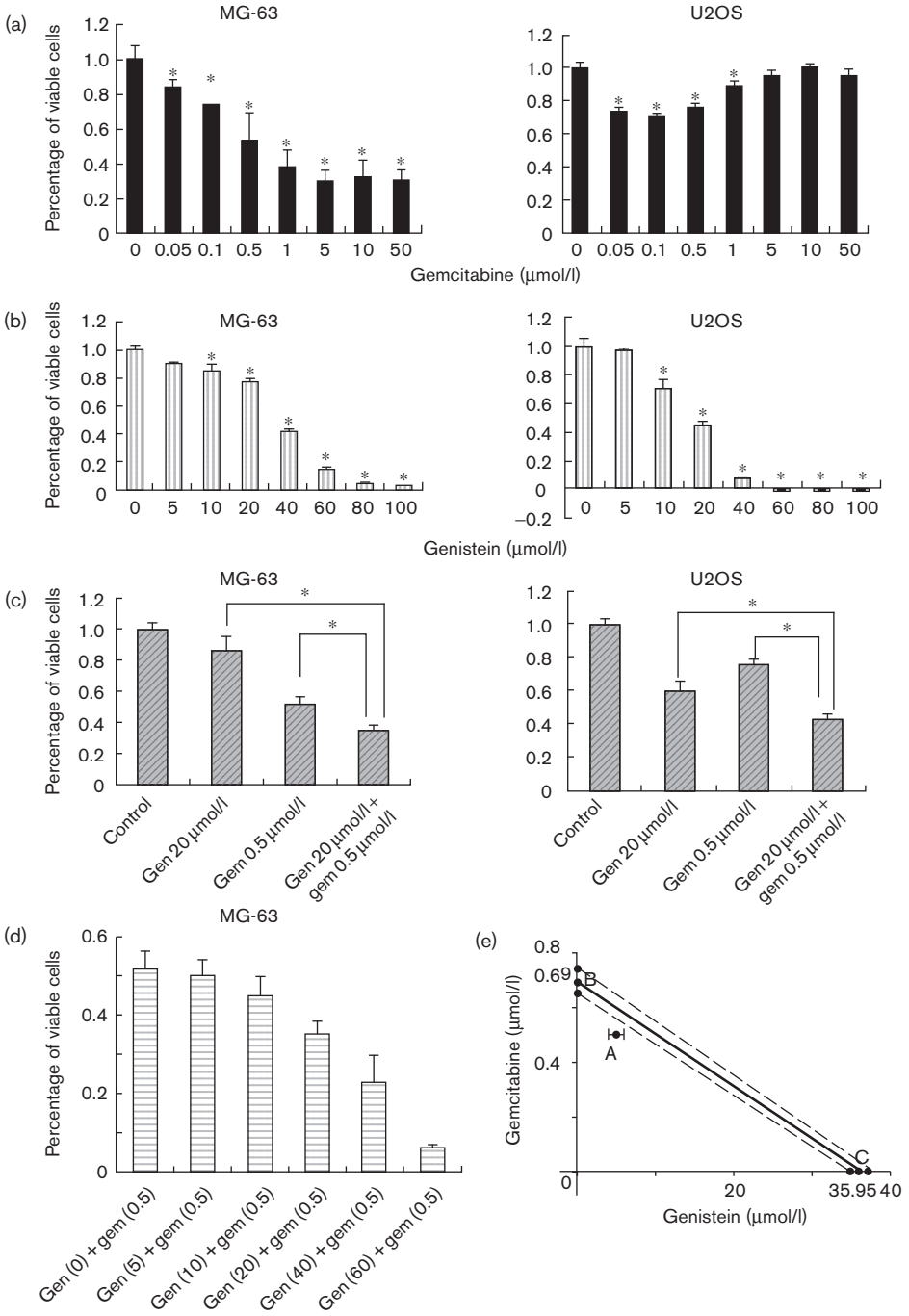
The effects of gemcitabine and genistein on nuclear factor-κB-DNA binding activity

As shown in Fig. 5a, constitutively active NF-κB-DNA binding activity was found in nuclear extracts from MG-63, whereas very weak basal NF-κB-DNA binding activity was detected in U2OS. Relative to the untreated control, gemcitabine treatment induced a shift of NF-κB-DNA binding activity in U2OS, while genistein treatment blocked the shift in both cells, and the combination treatment showed that it can block the shift in U2OS. This suggested that genistein abrogated the gemcitabine-induced activation of NF-κB-DNA binding activity in U2OS. These results suggest that genistein not only downregulates NF-κB-DNA binding activity in non-stimulating conditions, but also inhibits gemcitabine-induced NF-κB-DNA activation, which is believed to be responsible for better cell killing with the combination treatment.

The effects of gemcitabine and genistein on the Akt/nuclear factor-κB signal pathway

To confirm our hypothetical mechanism, we used western blot to detect the related protein in MG-63 and U2OS cells. As shown in Fig. 5b, our results indicated that the expression of Akt phosphorylation in both cell lines was not upregulated by gemcitabine. Genistein significantly downregulates the expression of Akt phosphorylation in

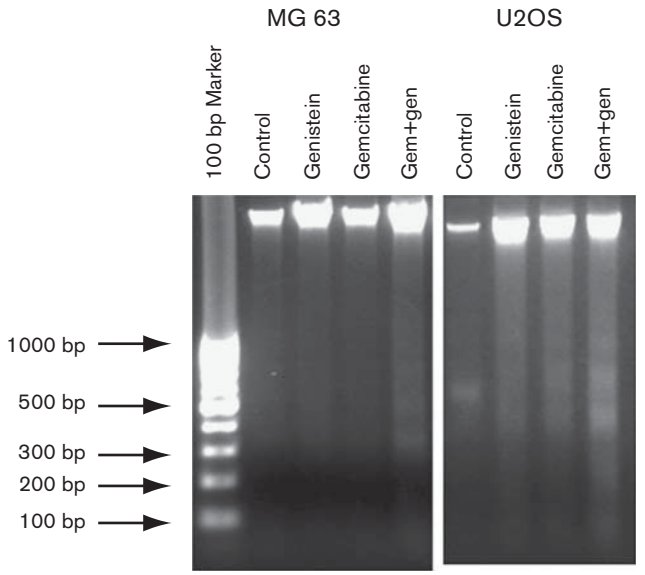
Fig. 1



Growth inhibition of human osteosarcoma cell lines (MG-63 and U2OS) treated with genistein (gen), gemcitabine (gem) and the combination for 72 h was evaluated by MTT assay. (a) The effect of various doses of gemcitabine on the viability of MG-63 and U2OS. (b) The effect of various doses of genistein on the viability of MG-63 and U2OS. (c) Cells were treated with 0.5 μmol/l of gemcitabine, 20 μmol/l of genistein or the combination for 72 h; a significant potentiation of the growth inhibition of gemcitabine by genistein was observed in both cell lines. (d) The effect of various doses of genistein with 0.5 μmol/l of gemcitabine on the viability of MG-63. (e) Results of isobologram analysis [17]. Points B and C are IC_{50} of gemcitabine and genistein alone with 95% confidence intervals; A (5 ± 0.82, 0.5) is IC_{50} of the combination of gemcitabine and genistein with 95% confidence intervals. The straight line connecting the single-drug IC_{50} is an additive line with 95% confidence intervals. Experimental data located below, on or above the line indicate synergy, additivity or antagonism, respectively. A is located below 95% confidence intervals of the additive line and indicate synergy; this means the combination favors MG63 cells death by synergistic mechanisms. Each piece of experimental data represented the average obtained from five replicates, and each experiment was performed in triplicate. $*P < 0.05$, statistical significance.

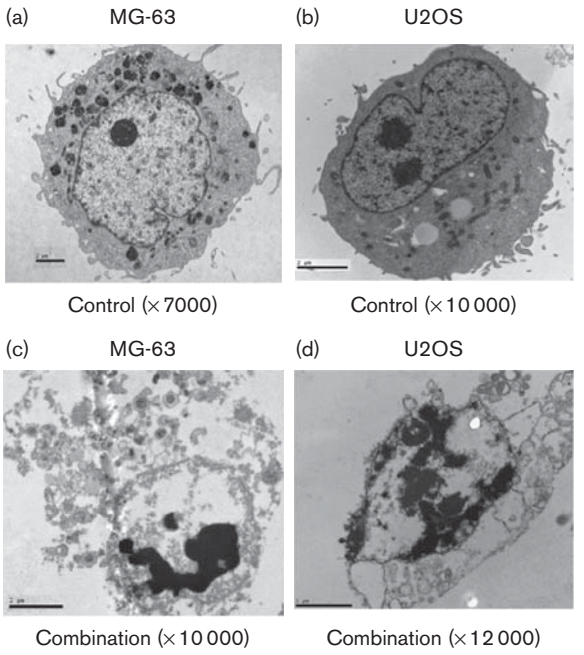
both cell lines as it acted in other cancers [12,13], and the combination treatment downregulated the expression of Akt phosphorylation significantly. Again the level of

Fig. 2



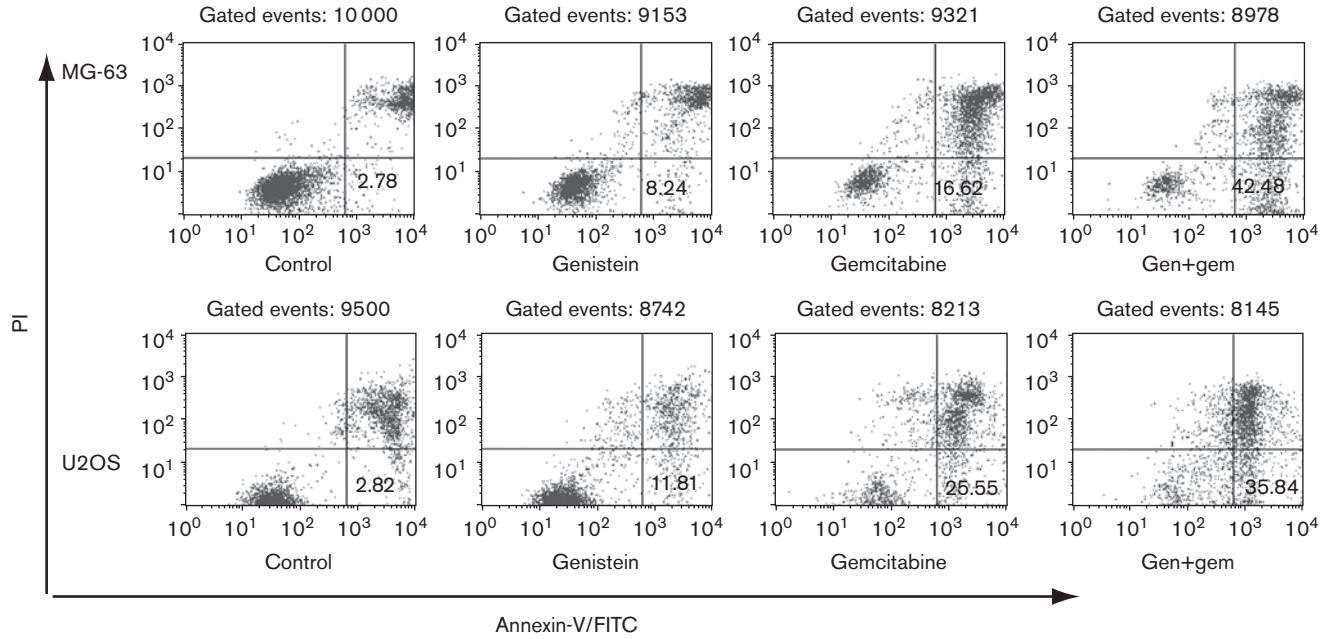
After 48 h treatment, the DNA ladder was shown in osteosarcoma cells treated with the combination of gemcitabine (gem) and genistein (gen).

Fig. 4



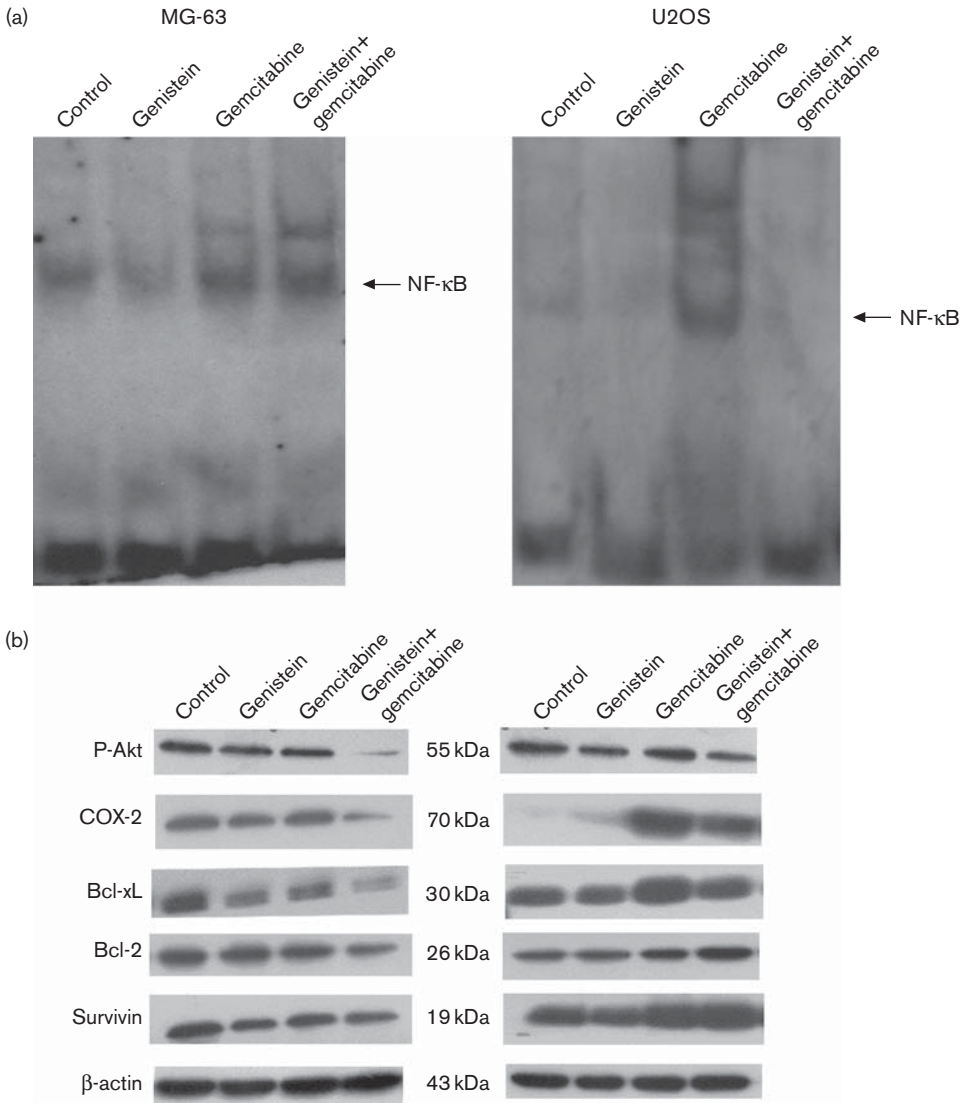
The normal appearance of MG63 and U2OS (a and b) in the transmission electron microscope. The morphological changes (c and d) were observed by transmission electron microscopy in MG-63 and U2OS treated with the combination for 48 h.

Fig. 3



Flow cytometry assay for apoptosis. The cells were stained with propidium iodide (PI) and Annexin V–fluorescein isothiocyanate (FITC) conjugate and measured by fluorescence activated cell sorting with an acquisition of 10 000 events. The gated events have been shown above all dot blots. Apoptotic cells (AV^+/PI^-) were monitored after treatment for 72 h. Genistein significantly potentiated osteosarcoma cell apoptosis induced by gemcitabine. The data shown here are from one of three different experiments.

Fig. 5



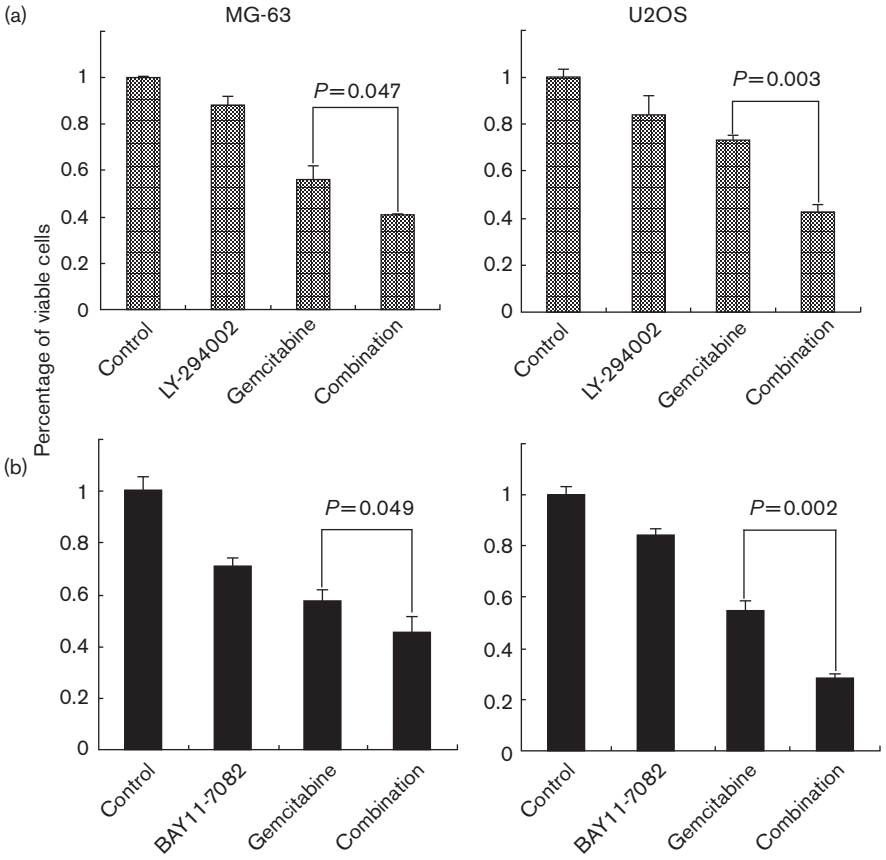
(a) Nonradioactive electrophoretic mobility shift assay for detecting nuclear factor κB (NF-κB)-DNA binding activity in MG-63 and U2OS after treatment with 0.5 μmol/l of gemcitabine, 20 μmol/l of genistein or the combination for 72 h. Gemcitabine treatment induced a shift of NF-κB-DNA binding activity in U2OS, while genistein treatment blocked the shift in both cells, and the combination treatment showed that it can block the shift in U2OS. This suggested that genistein abrogated gemcitabine-induced activation of NF-κB-DNA binding activity in U2OS. (b) Western blot analysis of P-Akt and antiapoptotic proteins, Bcl-xL, Bcl-2, survivin and COX-2, in whole cell lysates of MG-63 and U2OS after treatment with 0.5 μmol/l of gemcitabine, 20 μmol/l of genistein or the combination for 72 h. Downregulation of P-Akt and antiapoptotic markers such as Bcl-xL and COX-2 is evident in both cell lines treated with genistein and gemcitabine.

apoptosis-related proteins that are regulated by NF-κB was detected by western blot. The expression of Bcl-2, Bcl-xL and COX-2 was significantly reduced in MG-63 cells treated with the combination relative to the single-agent treatment and untreated control, while in U2OS cells, the expression of Bcl-xL and COX-2 decreased in the combination group compared with gemcitabine group.

Discussion

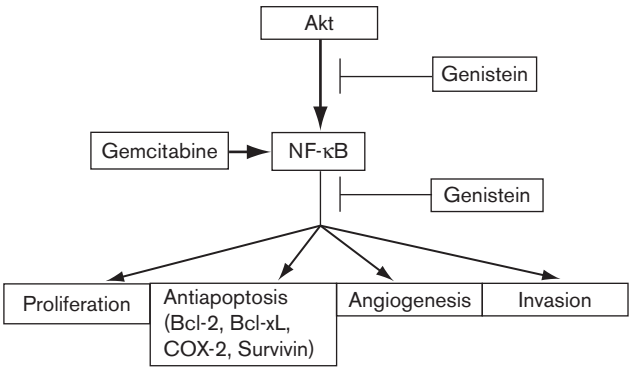
In the past decade, although the survival rate of patients with OS has increased as a result of rapid advancements in the fields of neoadjuvant chemotherapy and operative techniques, the effect of cytotoxic drugs on OS becomes less useful due to acquired chemoresistance. Therefore, a rational and effective approach should be explored to overcome the resistance to cytotoxic drugs. Combination treatment could potentially result in greater antitumor effectiveness at much lower drug doses, thus reducing the extent and severity of treatment-related toxicity. In our study, we use genistein, a nontoxic flavonoid compound, in combination with gemcitabine, against two kinds of OS cell lines: MG-63 and U2OS. Although each of the agents

Fig. 6



Cell viability of human osteosarcoma cell lines (MG-63 and U2OS) treated with gemcitabine (0.5 μmol/l), LY-294002 (10 μmol/l), BAY11-7082 (5 μmol/l) and the combination for 72 h was evaluated by the MTT assay. (a) The effect of LY-294002 and gemcitabine on the viability of MG-63 and U2OS. (b) The effect of BAY11-7082 and gemcitabine on the viability of MG-63 and U2OS. The results indicated that both the PI3K/Akt-pathway inhibitor (LY-294002) and the specific nuclear factor-κB inhibitor (BAY11-7082) can significantly potentiate growth inhibition of gemcitabine in both cell lines.

Fig. 7



Schematic diagram of potential mechanism of gemcitabine and genistein on Akt/NF-κB signal pathway.

alone was effective in inhibiting cell growth, the treatment of cells with genistein significantly enhanced cell killing induced by gemcitabine. Moreover, the observed cell growth inhibition with genistein treatment correlated well with apoptotic data; this suggested that the loss of viable cells with genistein treatment is partly a result of the induction of an apoptotic cell death mechanism.

Studies at the cellular level have provided insights into the ability of soybeans to prevent osteoporosis [18,19], and others showed the inhibitory action of genistein on OS differentiation and activity [20], but few mentioned apoptosis in OS. In our study, it is shown that genistein can induce OS cell apoptosis (Fig. 3). Earlier studies have shown that genistein can enhance the cytotoxicity of chemotherapeutic drugs such as docetaxel, cisplatin, campothecins, and taxotere [21–23]. In addition, genistein has also been shown to synergize with gemcitabine in exerting an antiproliferative effect in a variety of other tumor types, such as pancreatic cancer and ovarian cancer [24,25], our results show genistein can potentiate the growth inhibition and apoptotic effects of gemcitabine in OS.

Akt signaling is an important transduction pathway that plays a critical role in enhancing the survival rate of tumor

cells [26]. Evidence suggests that Akt also regulates the NF-κB pathway through phosphorylation and activation of molecules in the NF-κB pathway [15,27]. Thus, strategies to block the activity of Akt would ideally lead to the inhibition of proliferation and the induction of apoptosis. Moreover, genistein has been proven to abrogate the Akt pathway in other cancers [12]. Our results indicate that OS cells display constitutively active Akt, and that genistein treatment reduces the level of the phosphorylated Akt protein compared with control cells. It is therefore logical to speculate that genistein could reverse OS de novo resistance to gemcitabine by inhibiting the activation of Akt.

NF-κB plays important roles in the control of cell growth, differentiation, apoptosis, invasion, and angiogenesis by regulating downstream genes such as Bcl-2, Bcl-xL, survivin and COX-2 [28]. Under nonstimulating conditions, NF-κB is sequestered in the cytoplasm through tight association with the impeding IκB proteins. After stimulation, IκB protein is phosphorylated and degraded, allowing the NF-κB to translocate to the nucleus, bind to the NF-κB-specific DNA-binding sites or interact with other transcription factors, and thus regulate gene transcription [11]. It has been reported that some chemotherapeutic agents such as cisplatin, gemcitabine, and docetaxel induce the activation of NF-κB in some cancer cells, and that this causes drug resistance [24,29,30]. Our results show that gemcitabine activates NF-κB in OS, which is believed to confer chemoresistance and thus reduce the killing of OS cells. Genistein has been proven to inhibit the activation of NF-κB to potentiate the antitumor activity of chemotherapeutic agents [12]. Our results clearly show that genistein abrogates gemcitabine-induced NF-κB activation in U2OS. These findings correlate well with the increased apoptotic index, suggesting that inhibition of NF-κB by genistein may be one possible approach to sensitize OS cells to gemcitabine-induced apoptotic cell death. In addition, our hypothesis is further strengthened by the observation that genistein effectively downregulated the proteins expressed by NF-κB downstream target genes. Antiapoptotic molecules, such as Bcl-xL and COX-2, were substantially downregulated with treatment with the combination of genistein and gemcitabine. Moreover, we used a PI3K/Akt-pathway inhibitor (LY-294002) and a specific NF-κB inhibitor (BAY11-7082) to test the cell viability of MG-63 and U2OS treated with gemcitabine for 72 h, and both LY-294002 and BAY11-7082 can significantly potentiate the growth inhibition of gemcitabine in both cell lines (Fig. 6). These results strongly suggest that the resistance of OS cells treated with gemcitabine could be in part a result of the activation of NF-κB, and that the chemosensitization could be a result of genistein-induced inactivation of Akt/NF-κB signaling, resulting in the inhibition of cell proliferation and induction of apoptosis (Fig. 7).

In conclusion, in this study we find that genistein abrogates the Akt/NF-κB signaling pathway to potentiate OS cells apoptosis induced by gemcitabine. Our results prove that OS with *de novo* and acquired resistance to chemotherapeutic drugs such as gemicitabine could be reversed with genistein treatment. The marked ability to induce apoptosis by a combination of gemcitabine and genistein suggests that this could be a rational and novel approach for OS preclinical and clinical trials.

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