Novel Quinazoline HMJ-30 Induces U-2 OS Human Osteogenic Sarcoma Cell Apoptosis Through Induction of Oxidative Stress and Up-Regulation of ATM/p53 Signaling Pathway

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ABSTRACT: Human osteogenic sarcoma is the most common primary bone tumor. Despite of the success of frontline therapy, about 40% of patients have disease progression and further therapy is palliative and toxic. In this study, we developed a novel quinazoline HMJ-30 to investigate the cell growth inhibition and apoptotic responses in U-2 OS human osteogenic sarcoma cells. Our results demonstrated that HMJ-30 significantly reduced cell viabilities of U-2 OS, HOS, and 143B cells in a dose-dependent manner, but it exhibited low cytotoxicity in normal hFOB cells. HMJ-30 induced DNA damage and apoptosis in U-2 OS cells as revealed by morphologic changes, comet assay and DAPI staining. Immuno-staining, colorimetric assays, and Western blotting analyses indicated that activities of caspase-8, caspase-9, and caspase-3 and the levels of Bcl-2 family-related proteins (Bcl-2, Mcl-1, Bax, BAD, and t-Bid) were altered in HMJ-30-treated U-2 OS cells. Pretreatment of cells with caspase-8, -9, and -3 specific inhibitors significantly reduced the cell growth inhibition. HMJ-30-induced apoptosis was mediated through both death-receptor and mitochondria-dependent apoptotic pathways in U-2 OS cells. HMJ-30 induced early phosphorylation of $p53^{Ser18}$ was through the activation of ataxia telangiectasia mutated (ATM) in U-2 OS cells. The cell growth inhibition by HMJ-30 was substantially attenuated either by the pre-incubation of U-2 OS cells with *N*-acetylcysteine (NAC, an antioxidant) and caffeine (an ATM kinase inhibitor) or by p53 knockdown via RNAi. In conclusion, ROS dependent-ATM/p53 signaling pathway is involved in HMJ-30-induced apoptosis in U-2 OS cells. © 2011 Orthopaedic Research Society Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: HMJ-30; human osteogenic sarcoma U-2 OS cells; apoptosis; ATM; p53

Human osteogenic sarcoma is the most common primary malignancy of bone in adolescents and young adults, accounting for about 60% of malignant bone tumors diagnosed in the first two decades of life. In Taiwan, the mortality rate in bone cancer is about four per million annually based on the 2008's report from the Department of Health, Taiwan. Current treatment for human osteosarcoma includes surgery resection and systemic chemotherapy. The prognosis has significantly improved from 11% with surgery alone to approximately 70% when combined with chemotherapy.[1] Despite of the success of frontline therapy, about 40% of patients have disease progression and further chemotherapy is palliative and toxic. Long-term outcomes have reached a plateau over the past 20 years.[2] Most current chemotherapeutic agents are highly toxic to normal cells and cause severe side effects. Furthermore, drug-resistance also poses serious problems to the therapy. Therefore, recent studies focus on the discovery of novel targets for drug design to treat human osteogenic sarcoma and the induction of cell death in cancer cells through apoptotic signaling pathway is a good strategy.

Apoptosis is the process of programmed cell death that may occur in multi-cellular organisms, playing an important role in normal physiology in animals. However, impairment of apoptotic function is associated with several diseases, including cancer genesis.[3] Perturbation of this process is considered a crucial part of cancer prevention and therapy. Two major pathways lead to apoptosis. The death receptor pathway is triggered by the binding of extrinsic signals to surface receptors including CD95/Fas, tumor necrosis factor (TNF) and death receptors, resulting in activation of caspase-8, followed by the activation of caspase-3 and -7. The mitochondrial pathway is triggered by various damage stimuli inside the cell which increase reactive oxygen species (ROS) and subsequent damage DNA. These stresses decrease mitochondrial membrane potential and cause cells undergoing apoptosis cascade.[4] To induce apoptosis through novel target in these pathways is an attractive approach to fight with cancer cells.

Quinazoline derivatives have diverse pharmacological properties, including anti-fungal, anti-malarial, anti-inflammatory, and anti-cancer activities.[5] We have recently designed and synthesized quinazoline series compounds in our laboratory with fluorine as anti-human osteogenic sarcoma candidates. Once inside the cells through circulation, the fluorine would be recognized by macromolecular recognition sites and increase its metabolic stability.[6] Here we reported that

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one of these compounds, 6-fluoro-2-(3-fluorophenyl)-4-(cyanoanilino) quinazoline (HMJ-30) which chemical structure shown in Figure 1A, can substantially inhibit the cell growth and trigger apoptosis of human osteogenic sarcoma cells.

MATERIALS AND METHODS

Chemicals and Reagents

HMJ-30 was obtained from Mann-Jen Hour Ph.D. (School of Pharmacy, China Medical University). Agarose, caffeine, DAPI (4,6-diamidino-2-phenylindole dihydrochloride), dimethyl sulfoxide (DMSO), *N*-acetylcysteine (NAC), potassium phosphate, trypan blue, propidium iodide (PI), Triton X-100, Tris–HCl, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide), and ribonuclease-A were obtained from Sigma–Aldrich Corp. (St. Louis, MO). H_2DCF-DA was obtained from Invitrogen Life Technologies (Carlsbad, CA). McCoy's 5a medium, DMEM/Ham's F12 medium, L-glutamine, fetal bovine serum (FBS), trypsin-EDTA, penicillin, and streptomycin were obtained from Invitrogen. Materials and chemicals for electrophoresis were obtained from BioRad (Hercules, CA). Caspase-3, -8, and -9 activity assay kits, caspase-3 specific inhibitor (Z-DEVD-FMK), caspase-8 specific inhibitor (Z-IETD-FMK) and caspase-9 specific inhibitor (Z-LEHD-FMK) were bought from R&D Systems (Minneapolis, MN). Primary antibodies (anti-Bcl-2, anti-Mcl-1, anti-Bcl-xL, anti-Bax, anti-BAD, anti-Bid, anti-Fas/CD95, anti-FADD, anti-cytochrome *c*, anti-ATM, anti-p53, and anti-β-actin), and second antibodies for western blotting were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies (anti-caspase-8, anti-caspase-9, anti-caspase-3, anti-phosphorylated ATM (Ser1981), and anti-phosphorylated p53 (Ser15)) for Western blotting were obtained from Santa Cruz Biotechnology. The p53 small interfering RNA (siRNA) and Lipofectamine 2000 was obtained from Invitrogen.

Cell Culture

Human osteogenic sarcoma cell line U-2 OS, HOS, 143B, and conditionally immortalized normal human fetal osteoblast progenitor hFOB cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). U-2 OS cells were cultured in 75 cm^2 tissue culture flasks with McCoy's 5a medium. HOS, 143B, and hFOB cells were cultured in Dulbecco's modified Eagle's medium and DMEM/Ham's F12 medium. All cell culture media contained 2 mM L-glutamine and supplemented with 10% FBS, 100 Units/mL penicillin and 100 mg/mL streptomycin and maintained at 37°C under a humidified 5% CO_2 atmosphere.

Cell Viability and Morphological Changes

To determine the cytotoxicity effects of HMJ-30, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used. U-2 OS, HOS, 143B, and hFOB cells were placed in 96-well plates with an initial concentration of 1×10^4 cells/well and exposed to HMJ-30 at various concentrations (25, 50, 75, and 100 μM) or 0.1% DMSO as a vehicle control for 24 h. Each concentration was repeated three times. After a 24 h-incubation period, MTT solution (0.5 mg/mL) was added into the wells for 4 h. The growth medium was removed, and the formazan crystals formed by oxidation of the MTT solution were dissolved with isopropanol in 0.04 N HCl and measured at 570 nm wavelength by ELISA reader. The cell survival ratio was expressed as % of the control as previously described.[7] For morphological changes, U-2 OS cells were cultured in 12-well plates at a density of 2.5×10^5 cells/well and treated with 50 μM of HMJ-30 for 0, 12, 24, and 48 h. Morphological changes in

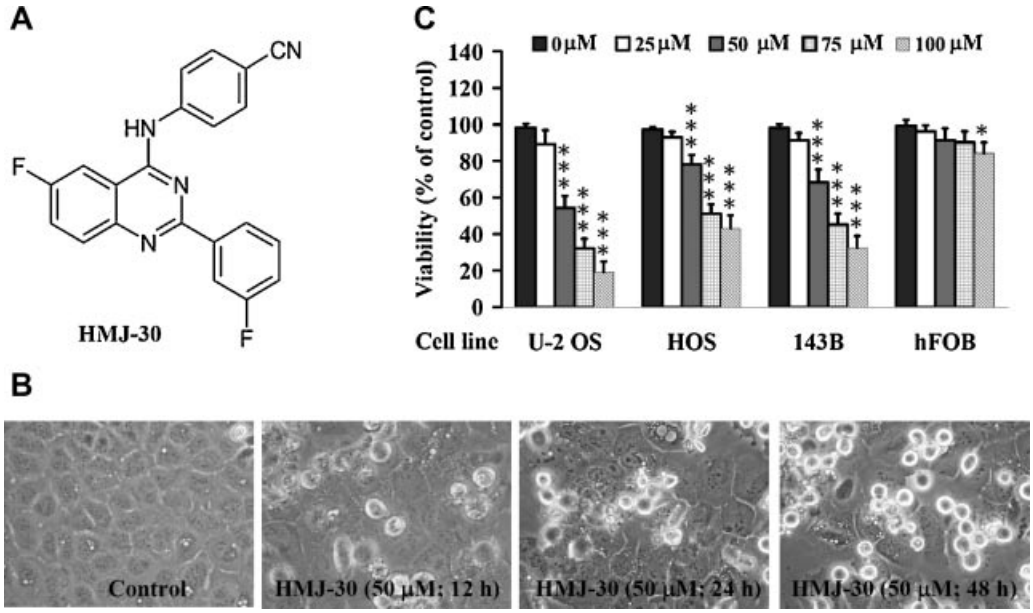

Figure 1. Effects of HMJ-30 on cell viability and morphological changes in human osteogenic sarcoma cell lines. (A) The chemical structure of HMJ-30. (B) After treatment with various concentrations of HMJ-30 for 24 h, the cell viabilities of human osteogenic sarcoma cell lines (U-2 OS, HOS, and 143B) and the conditionally immortalized human normal fetal osteoblast progenitor hFOB cells were shown. (C) U-2 OS cells in response to 50 μM of HMJ-30 showed morphological changes in a time-dependent manner. The values presented are the mean ± SD ($n = 3$) from three independent experiments. $^{*}p < 0.05$, $^{***}p < 0.001$, significantly different from vehicle control treated cells.

HMJ-30-treated cells were examined by a phase-contrast microscope. All results were obtained from three independent experiments.

DAPI Staining

U-2 OS cells were cultured in 24-well plates at a density of 2.5×10^5 cells/well and incubated with 50 μM of HMJ-30 for 0, 12, 24, and 48 h. Cells were harvested by centrifugation and stained by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and assessed by fluorescence microscopy as previously described.[7,8]

Comet Assay

U-2 OS cells were cultured in 24-well plates at a density of 2.5×10^5 cells/well and incubated with 50 μM of HMJ-30 for 0, 12, 24, and 48 h. After incubation, cells were harvested and mixed with low melting point agarose at 37°C. This mixture was placed on the top of previous layer of 0.5% agarose (normal melting point) on the slide, and then covered with a covership at 4°C until solid. Subsequently, the covership was removed gently and some agarose was added onto this slide, and then covered with the covership again. The slide was placed at 4°C until the mixture was solid, and put in chilled alkaline lysis buffer for electrophoresis. Afterwards, the slide was gently washed with neutralized buffer, and the DNA was stained with DAPI as previously described.[8,9] All results were obtained from three independent experiments.

Immuno-Staining for Caspase-3 and Confocal Laser Microscopy

U-2 OS cells were plated on 4-well chamber slides at a density of 2.5×10^5 cells/well and treated without and with 50 μM of HMJ-30 for 24 h. Cells were then fixed in 4% ice-cold formaldehyde for 5 min and placed in 1% bovine serum albumin (BSA) containing 0.1% Triton X-100 at 37°C for 30 min. Subsequently, the cells were washed twice with PBS for 5 min and incubated with anti-caspase-3 antibody overnight and then exposed to FITC-conjugated anti-rabbit IgG secondary antibodies, followed by DNA staining with PI. Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope.[10,11] All results were obtained from three independent experiments.

Assays for Caspase-3, Caspase-8, and Caspase-9 Activities

Caspase-3, -8, and -9 activities were assessed according to manufacturer's instruction of caspase colorimetric kit (R&D system Inc.). U-2 OS cells (5×10^6 cells) were incubated in 75-T flasks and treated with 50 μM of HMJ-30 for 0, 12, 24, and 48 h. Then, cells were harvested in 50 μl lysis buffer contained 2 mM DTT. After centrifugation, the supernatant containing 100 μg protein was incubated with caspase-3 substrate (Ac-DEVD-pNA), caspase-8 substrate (Ac-IETD-pNA) and caspase-9 substrate (Ac-LEHD-pNA) in the reaction buffer. Then all samples were incubated in 96-well flat bottom microplate at 37°C for 1 h. Levels of released pNA were measured with ELISA reader (Anthos 2001). All results were obtained from three independent experiments.[4,12]

Quantification of Reactive Oxygen Species (ROS) by Flow Cytometry

U-2 OS cells were cultured in 24-well plates at a density of 2.5×10^5 cells/well and incubated with 50 μM of HMJ-30 for 0, 2, and 4 h. The cells were treated with ROS indicator, 5 μM of 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) at 37°C for 30 min. ROS was analyzed for fluorescence intensity by using flow cytometry. The median fluorescence intensity was quantified by BD Pro CellQuest software (Becton-Dickinson, Franklin, NJ) as previously described.[13,14] All results were obtained from three independent experiments.

Small Interfering RNA Transfection

U-2 OS cells were cultured in 6-well plates, grown to 70% of confluency, and transfected with 100 nM of p53 siRNA or control siRNA molecules by using Lipofectamine 2000 (Invitrogen) for 48 h. Transfected cells were treated with 50 μM of HMJ-30, then the cell viability was analyzed using MTT assay, and cell lysate was subjected to Western blotting analysis. All results were obtained from three independent experiments.[10]

Western Blot Analysis

U-2 OS cells (1×10^7 cells) were incubated in 75-T flasks and treated with 50 μM of HMJ-30 for 0, 6, 12, and 24 h. Isolation of total and cytosolic proteins and protein quantification were as described previously.[8,14] Equal amounts of protein lysate were run on 10–12% SDS–PAGE and electrotransferred to a nitrocellulose membrane by using iBotTM Dry Blotting System (Invitrogen). The blot was soaked in blocking buffer (5% non-fat dry mik/0.05% Tween 20 in 20 mM TBS at pH 7.6) at room temperature for 1 h and then were incubated with primary antibodies in blocking buffer at 4°C overnight. Primary antibodies were used including anti-Bcl-2, anti-Mcl-1, anti-Bcl-xL, anti-Bax, anti-BAD, anti-Bid, anti-Fas/CD95, anti-FADD, anti-cytochrome *c*, anti-caspase-8, anti-caspase-9, anti-caspase-3, anti-ATM, anti-phosphorylated ATM (Ser1981), anti-p53, anti-phosphorylated p53 (Ser15), and anti-β-actin antibodies. Membranes were washed three times with Tris-buffered saline/Tween 20 for 10 min and incubated with secondary HRP-conjugated antibody for additional 1 h. Protein signals were revealed by ECL and the blot was exposed to Kodak Bio-MAX MR film (Eastman Kodak, Rochester, NY). All results were obtained from three independent experiments.[10]

Statistical Analysis

All data were expressed as mean ± SEM from at least three separate experiments. Statistical calculations of the data were obtained using an unpaired Student's *t*-test. Statistical significance was set at $^{*}p < 0.05$, $^{***}p < 0.001$ were taken as significant.

RESULTS

HMJ-30 Inhibited Cell Growth and Induces Cell Morphological Changes in Human Osteogenic Sarcoma Cells

To determine the growth inhibition effects of HMJ-30, human osteogenic sarcoma U-2 OS, HOS, 143B, and human normal fetal osteoblast progenitor hFOB cells were treated with different concentrations (0, 25, 50, 75, and 100 μM) of HMJ-30 for 24 h. The viability of the cells was determined by the MTT assay. As shown in Figure 1B, the viable cells were significantly reduced in the HMJ-30-treated human osteogenic sarcoma cells (U-2 OS, HOS, and 143B), but no effect

on hFOB cells (IC_{50} > 100 μM). The IC_{50} of HMJ-30 for U-2 OS cells was 50 ± 6.59 μM after a 24 h-treatment. HMJ-30-induced reduction of cell viability could be medicated through DNA damage and apoptotic cell death. The apoptotic morphological changes, including the cell surface with some debris, cell rounding and cell shrinking after 24 and 48-h incubation with 50 μM of HMJ-30 (Fig. 1C). Our results indicated that HMJ-30 inhibited cell growth of human osteogenic sarcoma cells in a dose-dependent manner, but exhibited less cytotoxicity on normal hFOB cells. Thus, 50 μM of HMJ-30 was chosen for all the following experiments.

HMJ-30 Stimulated the Activities of Caspase-8, Caspase-9, and Caspase-3 in U-2 OS Cells

To investigate the mechanism of HMJ-30-induced apoptosis, we investigated the activities of caspase-9, caspase-8, and caspase-3, respectively, in HMJ-30-treated U-2 OS cells. As shown in Figure 3A, the activities of caspase-8, caspase-9, and caspase-3 increased after treatment with 50 μM of HMJ-30 in a time-dependent manner. The amount of the active forms of caspase-8, -9, and -3 also elevated (Fig. 3B). Pre-treatment of cells with specific inhibitors to caspase-8, caspase-9, and caspase-3 significantly prevented the HMJ-30-induced cell growth inhibition and cell apoptosis (Fig. 3C). Our results suggest that the caspase-dependent signaling pathway plays a key role in HMJ-30-induced apoptotic cell death in U-2 OS cells.

HMJ-30-Induced Apoptosis is Involved in Death Receptor and Mitochondria-Mediated Pathways in U-2 OS Cells

To elucidate the possible signaling pathways involved in HMJ-30-induced apoptosis in U-2 OS cells, the levels of apoptosis-associated and Bcl-2 family proteins were evaluated by western blot analysis. Results shown in Figure 4A indicated that HMJ-30 promoted the pro-apoptotic protein levels of Bax, Bid, t-Bid and BAD, and inhibited the anti-apoptotic protein levels of Bcl-2 and Mcl-1. As shown in Figure 4B, HMJ-30 elevated the death receptor pathway-associated proteins, Fas/CD95 and FADD. Furthermore, apoptosome-related protein, cytochrome *c*, was also increased in cytosolic fraction of U-2 OS cells (Fig. 4C). These results suggest that HMJ-30 induced apoptosis in U-2 OS cells through the death receptor and mitochondria-dependent pathways.

HMJ-30 Increased Reactive Oxygen Species (ROS) and Induced ATM, p53 Activation in U-2 OS Cells

Previous studies have shown that intracellular ROS induced by anticancer drug plays an important role in

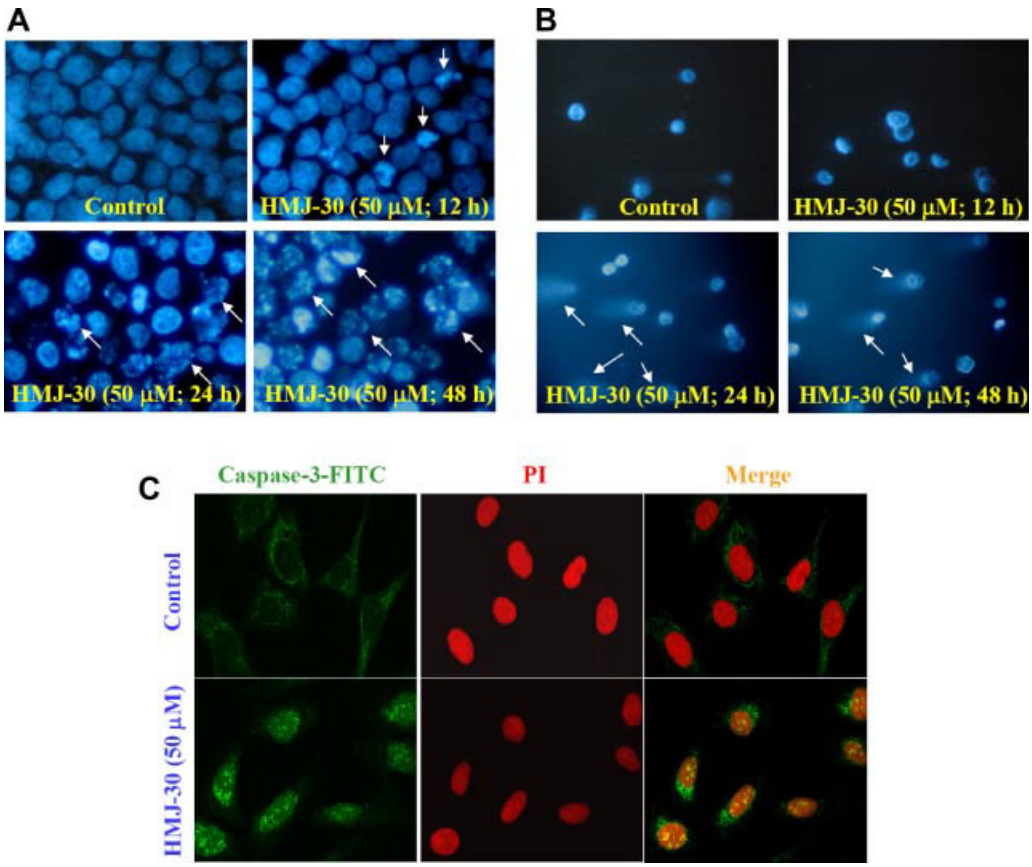

Figure 2. Effects of HMJ-30 on DNA damage and caspase-3 protein expression in U-2 OS cells. Cells were treated with 50 μM of HMJ-30 for 0, 12, 24, and 48 h. (A) DAPI staining and (B) comet assay revealed that HMJ-30-induced DNA damage and fragmentations. (C) Immuno-staining showed that HMJ-30 increased caspase-3 protein expression in U-2 OS cells.

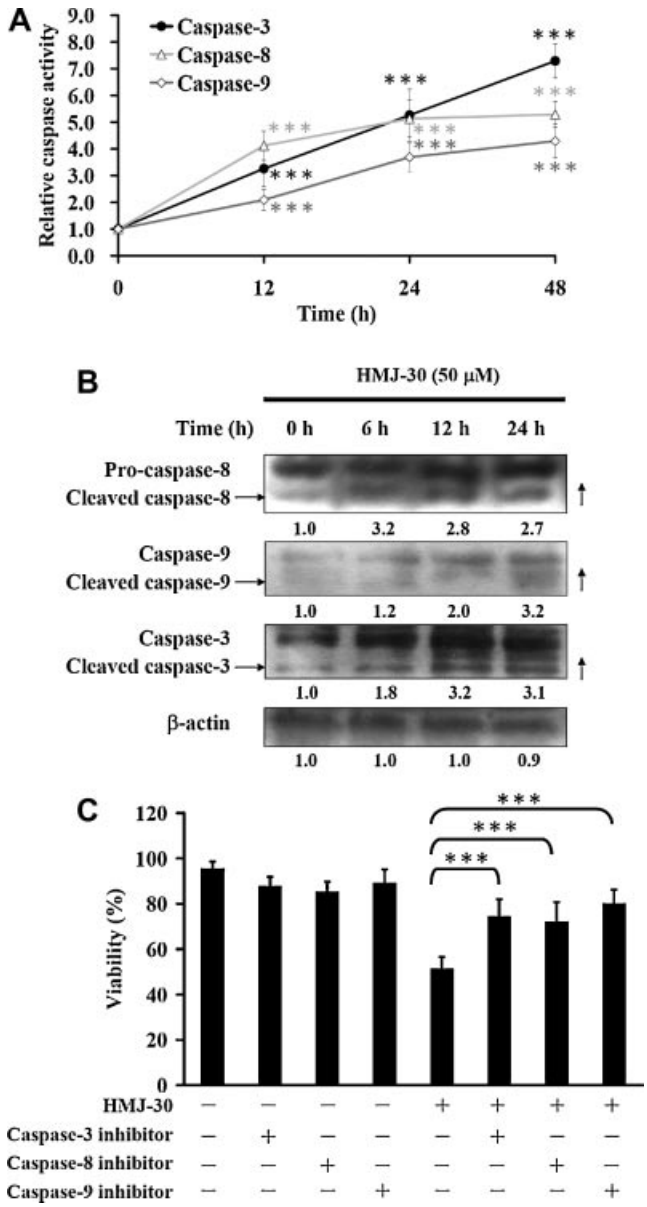

Figure 3. Effects of HMJ-30 on caspase-dependent apoptotic cell death in U-2 OS cells. Cells were treated with 50 μM of HMJ-30 for the indicated time, (A) caspase-8, caspase-9, and caspase-3 activities were analyzed by caspase activity assay. (B) The protein levels of caspase-8, caspase-9, and caspase-3 were determined by Western blotting. (C) Cells were also pretreated with the specific inhibitors of caspase-8, caspase-9, and caspase-3 for 1 h, and then exposure to HMJ-30 for 24 h. Cells were collected to determine the percentage of viable cells. The values presented are the mean ± SD ($n = 3$) from three independent experiments. $^{***}p < 0.001$, significantly different from vehicle control 0-h treated cells.

eliciting an early response in apoptosis.[15] To examine the effect of HMJ-30 on ROS production in U-2 OS cells, a specific ROS fluorescence probe, H_2DCFDA, was used to detect the level of ROS. In Figure 5A, HMJ-30 increased intracellular ROS level in U-2 OS cells. Cells pretreated with *N*-acetylcysteine (NAC, an antioxidant) and caffeine (an ATM kinase inhibitor) significantly reduced HMJ-30-induced growth inhibition effect and cell death (Fig. 5B). It is also reported that p53-inducible proapoptotic genes triggered apoptosis through both death-receptor and mitochondrial apoptotic pathways.[16] Fas/CD95 (a death-receptor family protein) is increased by p53-dependent transcriptional activation.[17] Phosphorylation of p53 at the Ser15 residue is closely related to its transcriptional activation.[18] To elucidate the role of p53 in HMJ-30-induced growth inhibition, western blot analysis was used to detect ATM, phosphorylated ATM (Ser1981), p53, and phosphorylated p53 (Ser15) protein levels. As shown in Figure 5C, treatment with HMJ-30 increased the ATM, phosphorylated ATM (Ser1981), p53, and phosphorylated, and p53 (Ser15) protein levels. Then, to elucidate if p53 plays an important role in HMJ-30-induced growth inhibition and cell death, we knockdown the protein level of p53 by using p53 siRNA. The results showed that knockdown of p53 significantly prevented the apoptotic cell death in U-2 OS cells after exposure to HMJ-30 (Fig. 5D). We hypothesized that HMJ-30 induces apoptosis through the increase in Fas/CD95 by p53-dependent transcriptional activation. Therefore Fas/CD95 protein level was examined in HMJ-30-treated p53-knockdown U-2 OS cells by Western blotting. The result indicated that HMJ-30 treatment decreased the protein level of Fas/CD95 in p53-knockdown U-2 OS cells (Fig. 5E). Based on the results, we concluded that HMJ-30 is able to increase the p53 transcriptional activation and hence the Fas/CD95 protein level in U-2 OS cells. These results suggest that HMJ-30-induced apoptosis in U-2 OS cells is through the induction of oxidative stress and up-regulation of ATM/p53 signaling pathways.

DISCUSSION

Quinazoline is an attractive scaffold for designing drugs because of its diverse pharmacological properties, including anti-fungal, anti-malarial, anti-inflammatory, and anti-cancer activities.[19–24] Previous studies have shown that quinazoline derivatives defeat tumor cells via inhibiting dihydrofolate reductase, thymidylate synthase, tyrosine kinase, microtubule polymerization or causing intracellular stress leading to apoptotic signaling cascades.[10,25–27] The novel quinazoline derivative, 6-fluoro-2-(3-fluorophenyl)-4-(cyanoanilino)quinazoline (HMJ-30), was active against human osteosarcoma U-2 OS, HOS and 143B cell lines in vitro. In Figures 1 and 2C, HMJ-30 triggered the shrinking and the detaching of some debris from the surface of U-2 OS cells. The protein levels of caspases were also increased, suggesting that they might play a crucial role in the execution-phase of apoptosis.[28] These results suggest that HMJ-30-induced apoptosis in U-2 OS cells is through the caspases-dependent signaling pathway. Low toxicity to normal cells and few side effects are essential for the success of drug development. Previously, numerous quinazoline derivatives have been accepted by FDA and prescribed

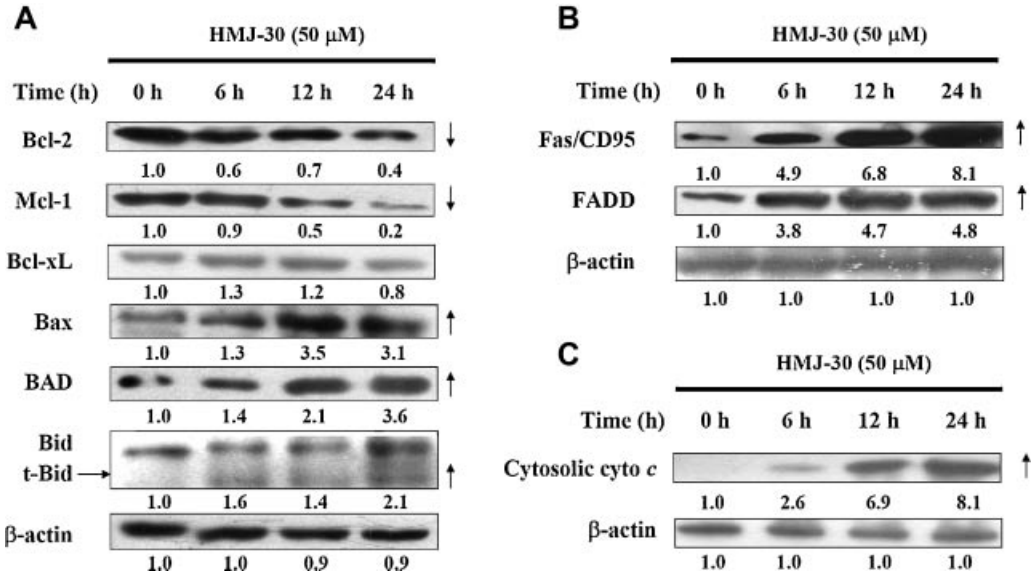

Figure 4. Effects of HMJ-30 on Bcl-2 family proteins, Fas/CD95 and FADD, cytosolic cytochrome *c* protein levels in U-2 OS cells. Cells were treated with 50 μM of HMJ-30 for 0, 6, 12, and 24 h, cytosolic or total protein lysate was prepared and subjected to Western blotting. (A) HMJ-30 elevated the pro-apoptotic protein levels of Bax and BAD and decreased the anti-apoptotic protein levels of Bcl-2 and Mcl-1. (B) Death receptor-associated proteins, Fas/CD95 and FADD, were increased. (C) Alteration in the ratio of Bax/Bcl-2 initiated mitochondrial outer membrane permeabilization, stimulating the release of cytochrome *c* from mitochondria into the cytosol. $^{***}p < 0.001$, significantly different from vehicle control 0-h treated cells.

for years without severe side effects.[23–26] In this study, HMJ-30 was actively against osteosarcoma cells, but it exhibited low toxicity to human fetal osteoblast progenitor hFOB cells ($IC_{50} > 100$ μM) in vitro. The IC_{50} for 24 h-treatment of HMJ-30 in U-2 OS, HOS, 143B, and hFOB cells were 50 ± 6.59, 76.35 ± 6.25, 83.26 ± 3.28, and over 100 μM, respectively. The reasons for the differences in sensitivities in IC_{50} of different cell lines may be due to the inherent different doubling time in different cell lines. The doubling time of U-2 OS is 16.8 h.[28] However, the hFOB cells are also a normal fetal osteoblast cell line and the doubling time of those cells is around 34 h.[29] Compared with U-2 OS cells, the two normal cell lines need a long time for entrance apoptosis.

Caspases are essential in cells for apoptosis. Activation of both the extrinsic initiator caspase-8 and the mitochondria-associated caspase-9 would activate downstream caspases, such as caspase-3, leading to induced cell apoptosis.[3] Our results in Figure 3 showed that the activities and protein levels of caspase-8, -9, and -3 were increased in HMJ-30-treated U-2 OS cells in a time-dependent manner. Furthermore, pre-incubation with the respective caspase inhibitors increased the cell viability. It is suggested that caspases played key roles in HMJ-30-induced apoptosis. In Figure 4, the death receptor and mitochondria-associated apoptotic protein levels were evaluated by Western blotting, which are shown that the death receptor pathway-associated proteins (Fas/CD95 and FADD) were increased, which subsequently promoted caspase-8 activation to activate downstream effectors caspase-3 and the cleavage of Bid to truncated Bid (tBid).[30] Therefore, HMJ-30 elevated the pro-apoptotic protein levels of Bax and BAD and decreased the anti-apoptotic protein levels of Bcl-2 and Mcl-1. Alteration in the ratio of Bax/Bcl-2 initiated mitochondrial outer membrane permeabilization, resulting in the loss of $\Delta\Psi m$ and stimulating the release of cytochrome *c* from mitochondria into the cytosol.[31,32] Then, cytosolic cytochrome *c* bond to Apaf-1, ATP, and pro-caspase-9, creating a protein complex (apoptosome) which induced caspase-dependent apoptosis pathway.[32] These accumulating data suggest that HMJ-30 induced apoptosis in U-2 OS cells via both death receptor and mitochondria-dependent signaling pathways.

DNA damage stress triggered the activation of ataxia telangiectasia mutated (ATM) signaling pathways, including ATM autophosphorylation and phosphorylation of ATM target substrates, p53, Nbs1, and Chk2.[33] Previous studies showed that ATM was autophosphorylated on Ser1981 in vivo after treatment of ionizing radiation or neocarzinostatin in mammalian cells[34] and in vitro during incubation with γ^{32}-ATP.[35] In agreement with previous studies, our results showed that HMJ-30 increased the ROS level in U-2 OS cells (Fig. 5) and induced DNA damage, as revealed by DAPI staining and comet assay. The stress increased the protein levels of ATM and phosphorylated ATM (Ser1981) subsequently leading p53 phosphorylation. Pretreated with NAC, a scavenger of ROS, or caffeine, an inhibitor of ATM, in HMJ-30-treated U-2 OS cells restored the cell viability, suggesting the functional involvement in ROS and ATM/p53-dependent pathways. The p53 tumor suppressor gene has many anticancer mechanisms and

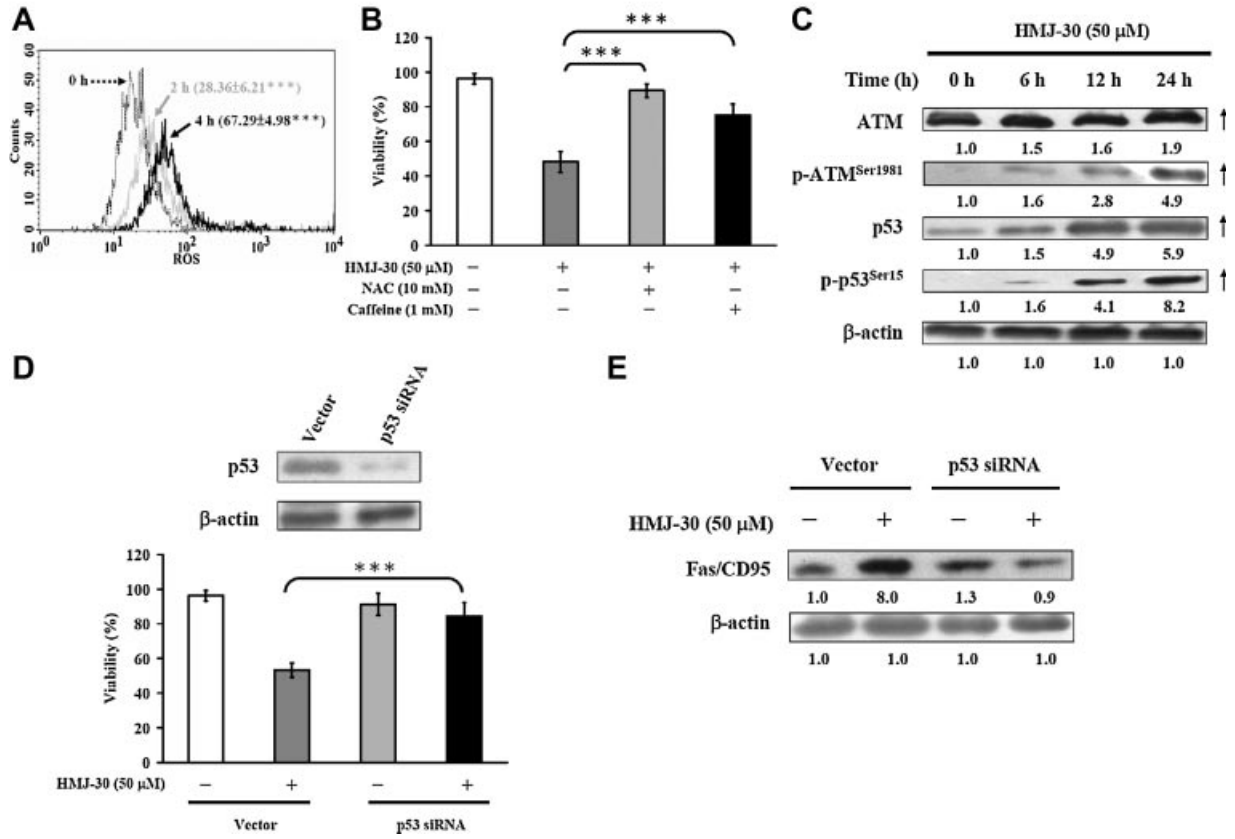

Figure 5. Effects of HMJ-30 on DNA damage stress and HMJ-30 induced ATM, p53 activation in U-2 OS cells. (A) The ROS level in HMJ-30-treated U-2 OS cells from 0, 2, and 4 h were measured by flow cytometric analysis. (B) Pretreated with NAC, a scavenger of ROS, or caffeine, an inhibitor of ATM, in HMJ-30-treated U-2 OS cells restored the cell viability. (C) HMJ-30 elevated the protein levels of ATM, phosphorylated ATM (Ser1981), p53, phosphorylated and p53 (Ser15). (D) Knockdown of p53 led to significant abolishment of HMJ-30-induced growth inhibition. $^{***}p < 0.001$, significantly different from vehicle control 0-h treated cells. (E) Fas/CD95 protein expression was analyzed by Western blotting in p53 siRNA or control siRNA-transfected U-2 OS cells after treatment with HMJ-30.

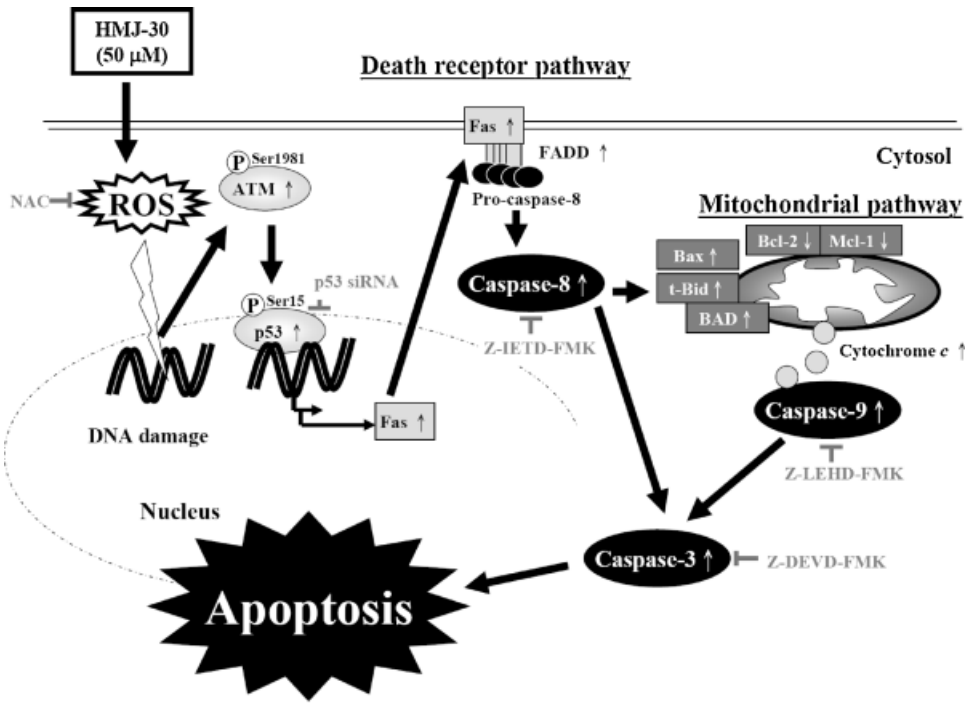

Figure 6. A proposed model illustrates the molecular mechanism and the overall possible signaling pathways of HMJ-30-induced apoptosis in U-2 OS human osteogenic sarcoma cells.

plays a major role in cellular response to DNA damage, including activating DNA repair, inducing growth arrest and apoptosis initiation.[36] As high levels of DNA double-strand broken, ATM/p53 activation induces pro-apoptotic genes transcription such as Bax (Bcl2-associated X protein), PUMA (p53 up-regulated modulator of apoptosis) and Fas/CD95 receptor.[36–38] Our results showed that the protein level of p53 and phosphorylated and p53 (Ser15) increased after HMJ-30 treatment. Furthermore, knockdown of p53 led to significant abolishment of HMJ-30-induced viability inhibition. In Figure 5E, we examined the Fas/CD95 protein level in the HMJ-30-treated p53 knockdown U-2 OS cells by Western blotting and the results showed that HMJ-30 treatment decreased the protein level of Fas/CD95 in p53 knockdown U-2 OS cells. These results suggest that HMJ-30 induced the p53-dependent, DNA damage-induced apoptotic cascades in U-2 OS cells.

In conclusion, this study reported that HMJ-30 triggered ATM/p53-dependent DNA damage-induced apoptosis is through mitochondria and death receptor-mediated pathways in a dose- and time-dependent manner. However, HMJ-30 has less toxic to hFOB normal cells. As shown in Figure 6, we proposed a model to depict the signaling pathways of HMJ-30-induced apoptosis in human osteosarcoma U-2 OS cells. Taken together, our findings provide important possible molecular mechanisms of the anti-human osteosarcoma of HMJ-30 and confirm that HMJ-30 may be a promising anti-osteosarcoma drug in the future.

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