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Sorafenib Induces Apoptosis and Inhibits NF-κB-mediated Anti-apoptotic and Metastatic Potential in Osteosarcoma Cells

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Abstract. *Background/Aim: Sorafenib, an oral multi-kinase inhibitor, has been shown to improve the outcome of patients with osteosarcoma (OS). However, the anti-OS effect and mechanism of sorafenib has not yet been fully understood. The main purpose of this study was to investigate the effect of sorafenib on apoptotic signaling and Nuclear Factor-κB (NF-κB)-mediated anti-apoptotic and metastatic potential in OS in vitro. Materials and Methods: The effect of sorafenib on apoptotic signaling transduction, anti-apoptotic, and metastatic potential of OS U-2 cells was verified with flow cytometry, trans-well invasion/migration, and western blotting assay. Results: Sorafenib induced the extrinsic and intrinsic apoptotic pathways. In addition, sorafenib reduced the invasion and migration ability of OS cells, induced NF-κB activation, and the expression of anti-apoptotic proteins and metastasis-associated proteins encoded by NF-κB target genes. Conclusion: Sorafenib led to stimulation of extrinsic/intrinsic apoptotic pathways and NF-κB inactivation in U-2 OS cells.*

Osteosarcoma (OS) is the common form of malignant bone tumor that frequently occurs in children and young adults (1). Conventional treatment strategies for osteosarcoma usually consist of surgery, chemotherapy, and radiotherapy, but the anti-OS efficacy of conventional treatment strategies is limited by chemo-radioresistance and metastasis (2, 3). Nuclear factor-κB (NF-κB), an oncogenic transcription factor, mediates chemo-radioresistance and metastasis by modulating the expression of anti-apoptotic and invasion-associated proteins encoded by NF-κB target oncogenes (4, 5). Suppression of NF-κB activation not only increases chemo-radiosensitivity but also reduces the metastatic potential of OS cells (6-9).

In addition to improving chemo-radio resistance, how to effectively elicit cell death is also crucial for the treatment of OS. Apoptosis, a form of programmed cell death, is initiated by extrinsic (death receptor) and intrinsic (mitochondrial) signaling pathways, and is carried out by caspases (10). Treatment of OS cells and animal models with anticancer agents leads to

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activation of apoptotic signaling pathways resulting in growth inhibition (11-14). For instance, regorafenib, an oral multikinase inhibitor derived from sorafenib, was shown to inhibit the growth of OS cells by inducing apoptosis via extrinsic and intrinsic signaling pathways (15).

Several oral multi-kinase inhibitors such as sorafenib and regorafenib have been recognized as novel therapeutic agents that improve the outcome of patients with OS (16, 17). Sorafenib blocks tumor angiogenesis and growth by targeting angiogenic and oncogenic kinases (18, 19). Ymera *et al*. found that sorafenib reduced tumor growth, angiogenesis, and metastatic ability through blockage of ERK1/2, MCL-1 and ezrin pathways (20). However, the anti-OS effect and mechanism of sorafenib has not yet been elucidated. The main purpose of this study was to verify the effects of sorafenib on apoptotic signaling transduction and NF-κB-mediated anti-apoptotic and metastatic potential in OS cells.

Materials and Methods

Chemical drugs and reagents. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and dimethyl sulfoxide (DMSO) were purchased from Sigma- Aldrich (St. Louis, MO, USA). Sorafenib was purchased from LC Laboratories® (Woburn, MA, USA) and dissolved in DMSO at 10 mM stock. CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit, CaspGLOW™ Red Active Caspase-8 Staining Kit and Active Caspase-9 were all obtained from Biovision (Mountain View, CA, USA). Annexin-V/PI, Fas, Fas-L and PI/RNase were all obtained from BD Biosciences (Franklin Lakes, NJ, USA). Dihexyloxacarbocyanine Iodide ($DiOC_6$) was bought from Enzo Life Sciences (Farmingdale, NY, USA).

Cell culture. U-2 OS cells were purchased from Bioresource Collection and Research Center, Hsinchu, Taiwan. Cells were maintained in 90% McCoy's 5a medium with 1.5 mM L-glutamine, 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS, 100 Units/ml and 100 μg/ml). Cells were maintained in a humidified incubator, in a 5% CO_2 atmosphere at 37˚C (21). McCoy's 5A medium, FBS, L-glutamine, and PS were obtained from Hyclone, GE Healthcare Life Sciences (Logan, UT, USA) and Gibco/Life Technologies (Carlsbad, CA, USA), respectively.

Cell viability assay. U-2 OS cells were plated at a density 2×10^4 cells/well in a 96-well plate overnight. After 80% confluency of cells was reached, cells were treated with sorafenib at the final concentrations of 0, 10, 15, 20, 25, and 30 μM or 0.1% DMSO as a vehicle for 48 h. Media were then washed out, replaced by MTT (5 mg/ml) for 4 h incubation and dissolved by DMSO for further absorbance detection. The percentage of viable cells was then quantified by measuring the absorbance value (OD) at 570 nm (22).

Invasion/migration assay. Trans-well cell culture chambers (8 mm pore size; Corning Life Sciences, Tewksbury, MA, USA) which coated with or without matrigel (Selleck Chemicals, Houston, TX, USA) were used for cell invasion and migration assay, respectively. U-2 OS cells were plated at a density of 3×10^6 cells/well in a 10 cm dish overnight and treated with 0, 10, 20 μM sorafenib for 48 h. One million of viable cells were resuspended in 100 μl McCoy's 5a medium (serum free) and added onto the upper chamber of a transwell insert. The lower chamber was filled with complete medium (90% McCoy's 5a medium containing 10% FBS). Cells were allowed to migrate at 37˚C for 24 h. Staining procedure was described in a previous study (23). Crystal violet staining transwell membranes were photographed by Nikon ECLIPSE Ti-U microscope (Tokyo, Japan). Five bright field photographs of each group were used to quantify the number of cells that invaded and migrated by ImageJ software version 1.50 (National Institutes of Health, Bethesda, MD, USA).

Apoptosis analysis. U-2 OS cells were plated at a density of 2×10^5 cells/well in a 12-well plate overnight and treated with 0, 10, 20 μM sorafenib for 48 h. After sorafenib treatment, cells were harvested and stained with cleaved caspase-3, -8, -9, Annexin-V/PI, Fas, Fas-L, and $DiOC_6$ reagents as described in Hsu's *et al*. study (22, 24). The fluorescence signal emitted by cells was detected and quantified by NovoCyte flow cytometry and NovoExpress® software (Agilent Technologies Inc., Santa Clara, CA, USA).

Cell cycle analysis. U-2 OS cells were plated at a density of 2×10^5 cells/well in a 12-well plate overnight and treated with 0, 10, 20 μM sorafenib for 48 h. Cells were then collected, evenly fixed with 75% ethanol using vortex equipment, and stained with PI/RNase solution (cat: 550625, BD Biosciences) at 37˚C for 1 h. Percentage of cells in the subG1 phase was evaluated and quantified by NovoCyte flow cytometry and NovoExpress® software (25).

Western blotting. U-2 OS cells were plated at a density of 3×10^6 cells/well in a 10 cm dish overnight and treated with 0, 10, 20 μM sorafenib for 48 h. Forty micrograms of total protein was then analyzed using western blotting as previously described (25). Primary antibodies against C-FLIP, XIAP, CyclinD1, MCL-1, MMP-2, MMP-9, VEGF, NF-κB p65 (Ser536), NF-κB p65 were used to identify any changes in the levels of these proteins after sorafenib treatment (22, 26).

Statistical analysis. All results are shown as mean±S.D from at least three experiments. Statistically significant differences between sorafenib treated and untreated (control) cells were tested by one-way ANOVA using 2016 Microsoft excel (a^1 and b^1 were defined as $p<0.05$; a^2 and b^2 were defined as $p<0.01$).

Results

Sorafenib markedly induced cytotoxicity and apoptosis in U-2 OS cells. MTT assay showed that sorafenib induced cytotoxicity of U-2 OS cells in a dose-dependent manner (Figure 1). We further investigated whether sorafenib induced cytotoxicity by inducing apoptosis. As illustrated in Figure 2A, sorafenib increased the levels of cleaved caspase-3. In addition, Annexin-V/PI staining results indicated that sorafenib not only induced late apoptosis but also early apoptosis in U-2 OS cells (Figure 2B). Furthermore, cell cycle analysis by flow cytometry showed that the population of cells in subG1 was also significantly increased after

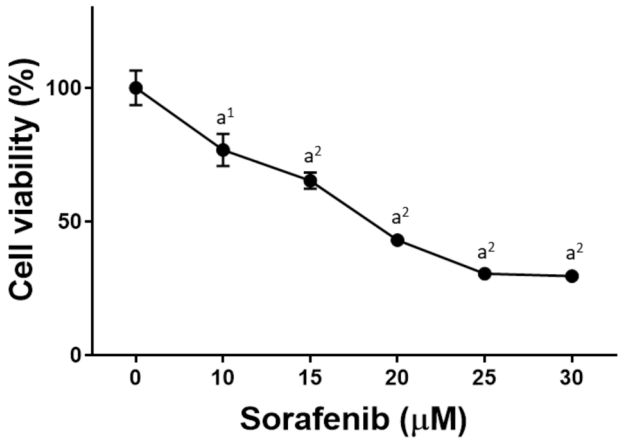

Figure 1. *Effect of sorafenib on cell viability in U-2 OS cells. U-2 OS cells were treated with 0-30 μM sorafenib for 48 h and the viability of cells was then assayed by MTT (a1 p<0.05, a2 p<0.01 vs. 0 μM sorafenib).*

sorafenib treatment (Figure 2C). To further investigate the effect of sorafenib in the proliferation and apoptosis of U-2 OS cells, we analyzed the expression of anti-apoptosis related proteins by western blotting. The protein levels of C-FLIP, XIAP, CyclinD1 and MCL-1 were all markedly decreased after treatment with sorafenib (Figure 2D). In summary, sorafenib-induced cytotoxicity of U-2 OS cells was correlated to the induction of apoptosis and the inhibition of expression of anti-apoptotic proteins.

Sorafenib effectively triggered the extrinsic apoptosis pathway in osteosarcoma U-2 OS cells. To identify the possible mechanism of sorafenib-induced apoptosis, we investigated the activation of three death receptor-dependent extrinsic apoptosis markers by flow cytometry. As illustrated in Figure 3A, the activation of Fas ligand (Fas-L) was increased 30-40% after sorafenib treatment as compared to non-treated control cells (0 μM sorafenib). In Figure 3B, the activation of Fas was also induced by sorafenib 40-50% as compared to non-treated control cells. Furthermore, we also investigated whether the downstream extrinsic apoptosis marker cleaved caspase-8 was also induced by sorafenib. As shown in Figure 3C, induction of cleaved caspase-8 was only found in sorafenib-treated cells. Thus, sorafenib may effectively trigger the extrinsic apoptosis pathway in U-2 OS cells.

Sorafenib markedly activated the intrinsic apoptosis pathway in U-2 OS cells. After confirming the activation of the extrinsic apoptosis pathway by sorafenib, we further investigated whether sorafenib may also trigger the intrinsic apoptosis pathway in U-2 OS cells. As shown in Figure 4A, the activation of cleaved caspase-9 was induced in sorafenib-treated cells. Additionally, the loss of the mitochondria membrane potential was also enhanced 30-40% by sorafenib (Figure 4B). In summary, the mitochondria-dependent intrinsic apoptotic pathway was effectively induced by sorafenib.

Sorafenib suppressed NF-κB phosphorylation, invasion/migration ability and the expression of related proteins in U-2 OS cells. We examined whether sorafenib suppresses the invasion and migration potential of U-2 OS cells by invasion/migration trans-well assay. The invasion and migration of cells on a trans-well membrane were significantly decreased by sorafenib as compared to non-treated control cells (Figure 5A-C). We then investigated the changes in the expression of invasion- and migration-related proteins, such as MMP-2, MMP-9, and VEGF, by sorafenib treatment. As shown in Figure 5D, the protein expression of MMP-2, MMP-9, and VEGF was decreased by sorafenib. Moreover, the phosphorylation of NF-κB was also reduced by sorafenib in U-2 OS cells. These results suggest that the anti-metastatic potential of sorafenib was associated with NF-κB inhibition in OS cells.

Discussion

Evasion of apoptosis is involved in tumor resistance to conventional therapies. Two common strategies for induction of apoptosis include the stimulation of apoptotic signaling pathways and the inhibition of anti-apoptotic protein expression (10, 27).

Both the loss of the mitochondria membrane potential (MMP) and the expression of cleaved caspase-9 are characteristics of the intrinsic apoptotic pathway (28). Death ligand binding to a death receptor activates apoptotic signaling through conversion of procaspase-8 to caspase-8 (29). Our results showed that sorafenib induced the expression of cleaved-caspase-3, -8, -9, and the loss of MMP (Figures 2, 3 and 4). Furthermore, activation of death receptor Fas (CD95) and Fas ligand (FasL) was also significantly induced by sorafenib treatment (Figure 3).

Anti-apoptotic proteins such as B-cell lymphoma-2 (BCL-2), MCL-1, C-FLIP, and XIAP mediate tumor cell resistance to chemo-radiotherapy through blockage of apoptotic signaling pathways and inactivation of caspases. Suppression of anti-apoptotic protein expression sensitizes OS cells to apoptosis induced by therapeutic agents (10, 22, 30-32). Our data indicated that the protein levels of MCL-1, C-FLIP, and XIAP were diminished by sorafenib treatment (Figure 2D). Sorafenib has been demonstrated to inhibit MCL-1 expression, leading to the apoptosis of OS cells (20). Previous studies have shown that NF-κB inactivation may down-regulate the expression of anti-apoptotic proteins in cancers (33, 34). Our results demonstrated that the protein levels of NF-κB p65 (Ser 536) were decreased by sorafenib treatment (Figure 5D).

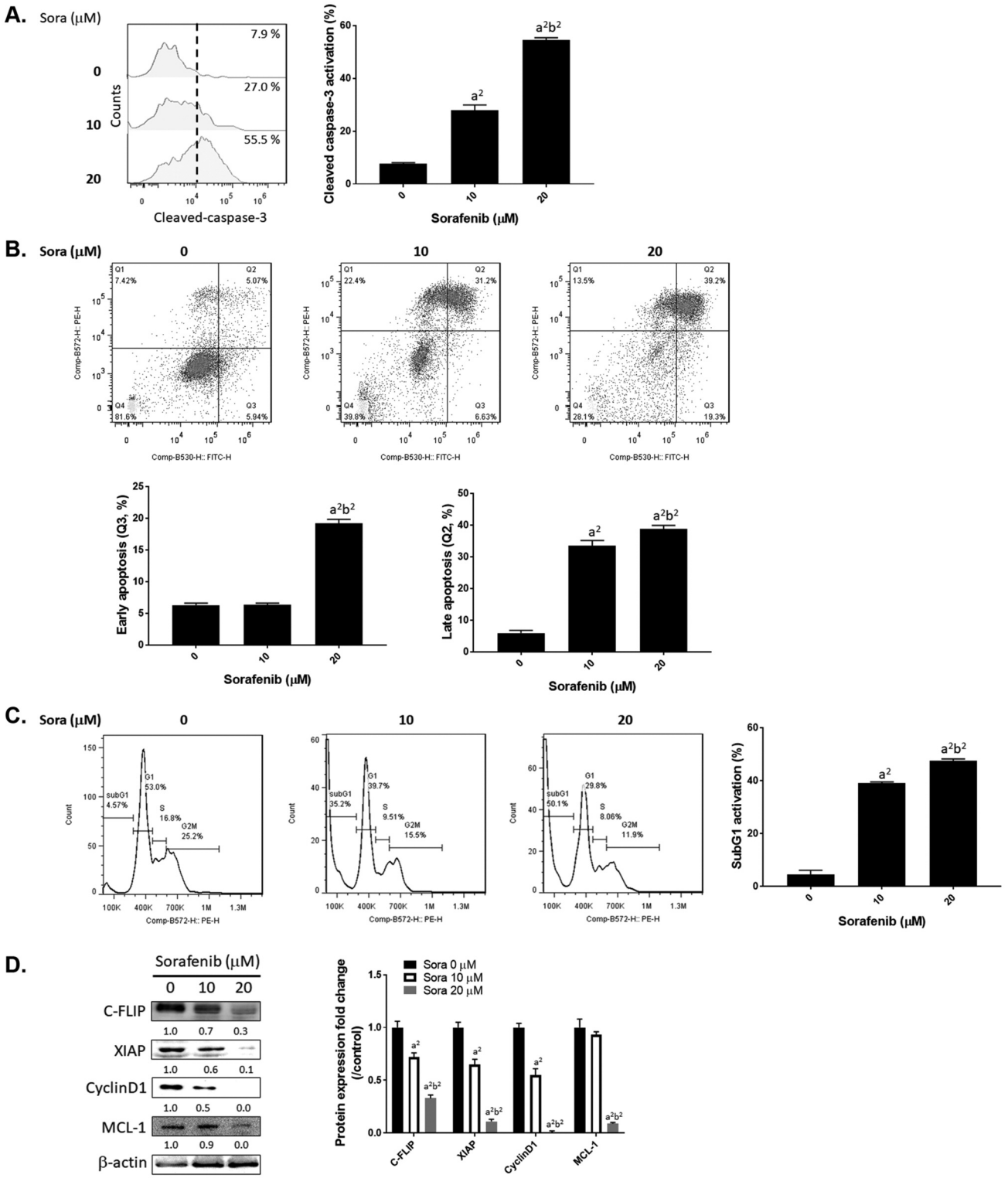

Figure 2. *Sorafenib induced apoptosis in U-2 OS cells. U-2 OS cells were treated with 0, 10 or 20 μM sorafenib for 48 h and its effect on apoptosis was examined by flow cytometry after staining for cleaved caspase-3 and Annexin-V/PI. (A) Histogram patterns from each group and quantification results of cleaved caspase-3 after sorafenib treatment. (B) Annexin-V/PI double staining from each group of cells and quantification results after sorafenib treatment. (C) Distribution of cells in the cell cycle in each group and quantification results of the subG$_1$ population. (D) Protein expression pattern and quantification results of C-FLIP, XIAP, CyclinD1 and MCL-1 (a^2 $p<0.01$ vs. 0 μM sorafenib; b^2 $p<0.01$ vs. 10 μM sorafenib).*

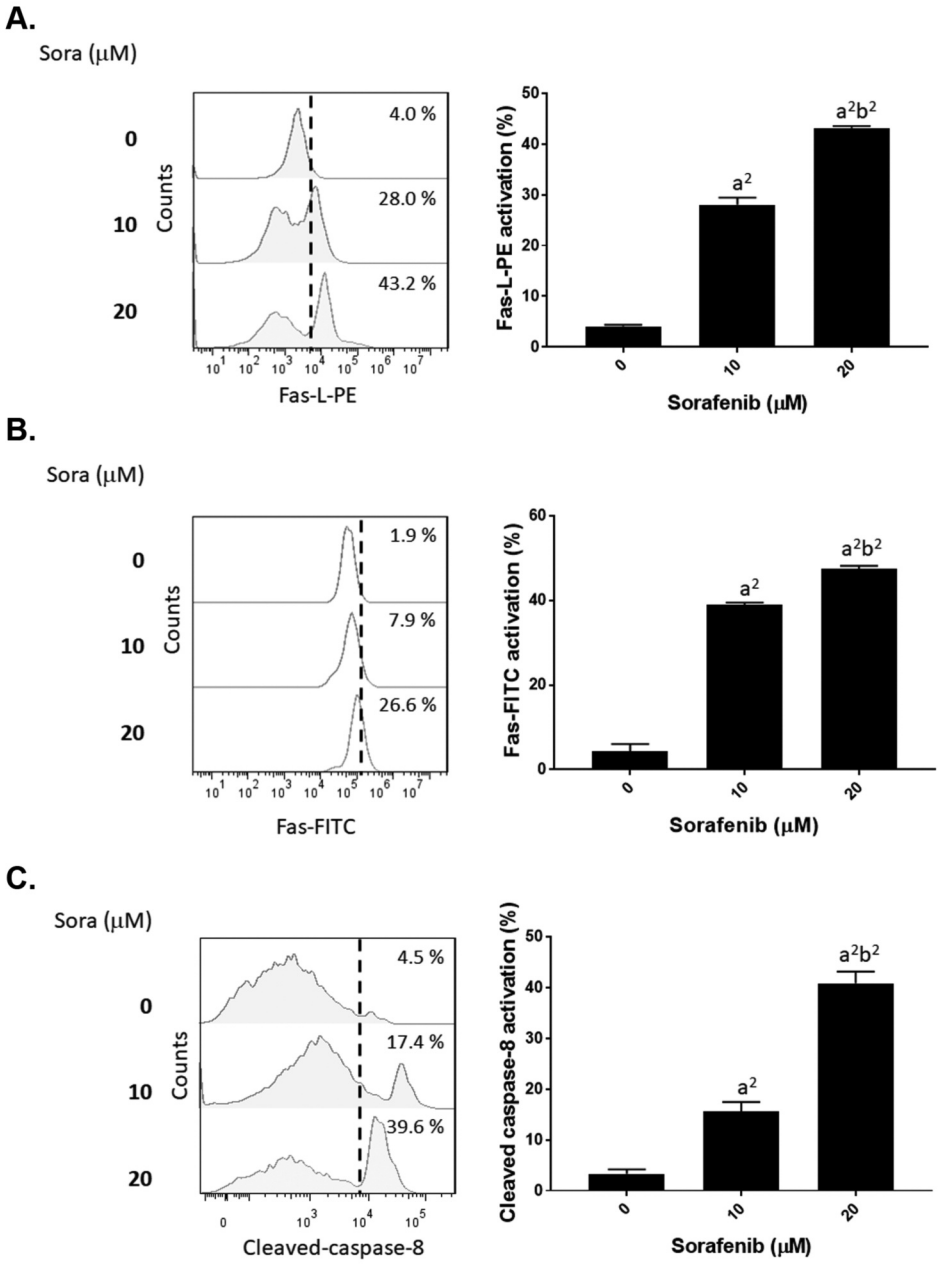

Figure 3. *Extrinsic apoptosis pathway induction by sorafenib in U-2 OS cells. U-2 OS cells were treated with 0, 10 or 20 μM sorafenib for 48 h and its effect on the extrinsic apoptotic pathway was tested by flow cytometry after staining for Fas-L, Fas, and cleaved caspase-8. Histogram patterns from each group and quantification results of (A) Fas-L, (B) Fas and (C) cleaved caspase-8 after sorafenib treatment are shown (a^2 $p<0.01$ vs. 0 μM sorafenib; b^2 $p<0.01$ vs. 10 μM sorafenib).*

According to our results, we suggest that sorafenib is an apoptosis inducer, which may not only reduce the expression of anti-apoptotic proteins but also induce apoptosis through extrinsic and intrinsic apoptotic pathways.

Patients with metastatic OS at diagnosis have worse survival rates (35). Metastasis-associated proteins MMP-2, -9, and VEGF, potentiate tumor invasion and metastasis through degradation of the extracellular matrix and new vessel formation (36, 37). Increased expression of MMP-2, -9, and VEGF has been associated with distant metastasis and poor outcome in patients with OS (38-40). Suppression of NF-κB signaling by QNZ (EVP4593), a NF-κB activator

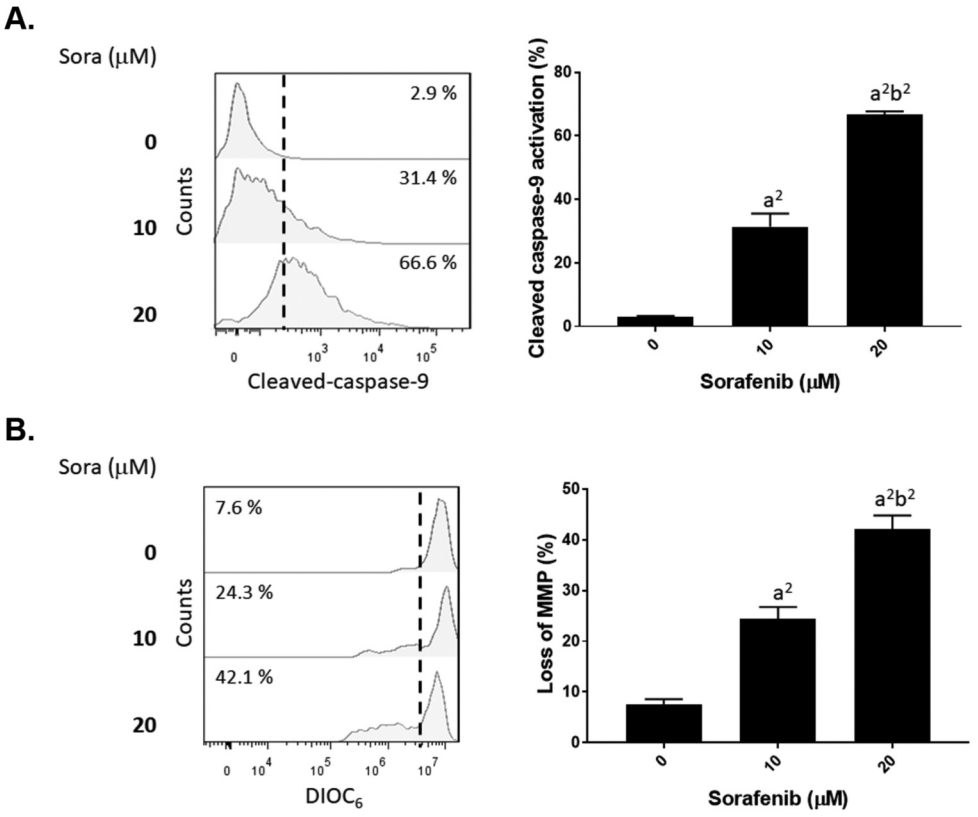

Figure 4. *Sorafenib induced the intrinsic apoptotic pathway in U-2 OS cells. U-2 OS cells were treated with 0, 10 or 20 μM sorafenib for 48 h and the intrinsic apoptosis effect was tested by cleaved caspase-9 and $DIOC_6$ staining. Histogram patterns from each group and quantification results of (A) cleaved caspase-9 and (B) mitochondria membrane potential (MMP) after sorafenib treatment, are shown (a^2 $p<0.01$ vs. 0 μM sorafenib; b^2 $p<0.01$ vs. 10 μM sorafenib).*

inhibitor, has been shown to reduce the expression of the above-mentioned metastasis-associated protein and attenuate cell invasion in OS (21). Our results showed that sorafenib diminished NF-κB activation, the protein levels of MMP-9, -2, and VEGF, and the invasion/migration ability of U-2 OS cells (Figure 5D).

In conclusion, sorafenib stimulated the extrinsic/intrinsic apoptotic pathways and suppressed NF-κB signaling and the metastatic potential of OS cells.

Conflicts of Interest

The Authors declare that they no conflicts of interest in relation to this article.

Authors' Contributions

Data curation, CH Wu, KH Lin, BS Fu, FT Hsu and JJ Tsai; funding acquisition, CH Wu and BS Fu; writing – original draft, CH Wu, FT Hsu, JJ Tsai and PJ Pan; writing – review, PJ Pan. All Authors have read and agreed to the published version of the manuscript.

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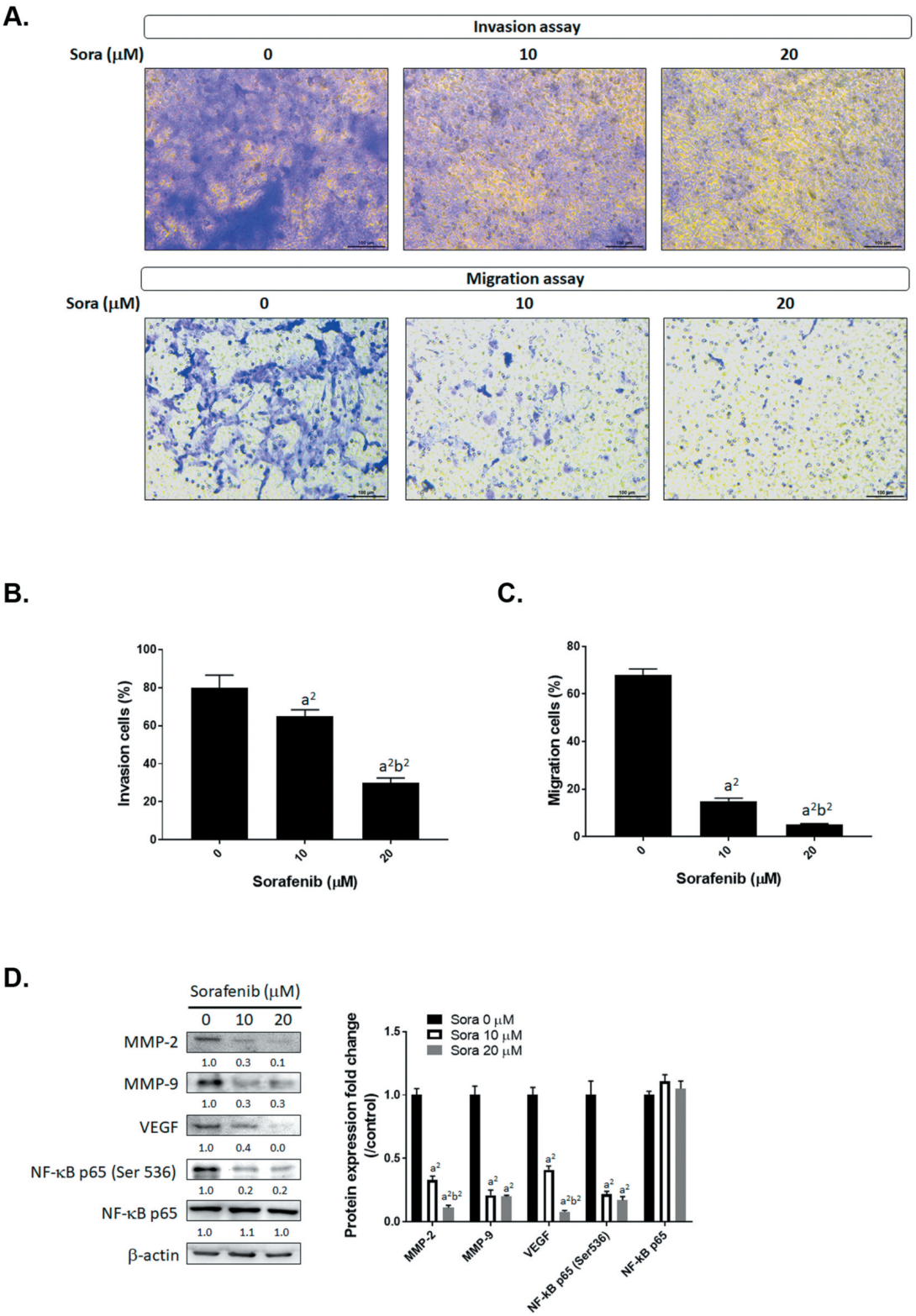

Figure 5. *Sorafenib suppresses the invasion and migration ability of U-2 OS cells. U-2 OS cells are treated with 0, 10 or 20 μM sorafenib for 48 h and the invasion/migration potential is tested by trans-well and western blotting assays. (A) Invasion and migration as measured by trans-well assay and (B-C) quantification of the results of each group are displayed. (D) Protein expression pattern and quantification results of MMP-2, MMP-9, VEGF, NF-κB p65 (ser 536) and NF-κB p65 are shown (a^2 $p<0.01$ vs. 0 μM sorafenib; b^2 $p<0.01$ vs. 10 μM sorafenib).*

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