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Reinforcement of Sorafenib Anti-osteosarcoma Effect by Amentoflavone Is Associated With the Induction of Apoptosis and Inactivation of ERK/NF-κB

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Abstract. *Background/Aim: Sorafenib has been reported to show anti-osteosarcoma (anti-OS) efficacy by inhibiting metastasis; however, a phase II trial suggested that further combination with other agents could be necessary to achieve permanent remission. Herein, we aimed to identify whether amentoflavone, an abundant natural bioflavonoid found in many medicinal plants, can improve the treatment efficacy of sorafenib in OS. Materials and Methods: Cell viability, metastasis, apoptosis, and nuclear translocation of NF-κB after amentoflavone combined with sorafenib were assayed by MTT, transwell migration/invasion, western blotting, flow cytometry, and immunofluorescence staining, respectively. Results: The sorafenib-induced cytotoxicity and apoptosis of U-2 OS was enhanced by combining treatment with QNZ (NF-κB inhibitor) or amentoflavone. NF-κB nuclear translocation, NF-κB phosphorylation, and metastasis capacity of U-2 OS cells were inhibited by amentoflavone combined with sorafenib. Conclusion: Amentoflavone may sensitize OS to sorafenib treatment by inducing intrinsic and extrinsic apoptosis and inhibiting ERK/NF-κB signaling transduction.*

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Osteosarcoma (OS) is the most common type of bone malignancy, which affects children, adolescents and adults older than 50 years of age (1, 2). The efficacy of conventional anti-OS treatments, such as surgery, chemotherapy, and radiotherapy, is attenuated by the exuberant invasion capacity and chemoradioresistance, which are causes leading to treatment failure (3, 4). Several tyrosine kinase inhibitors (TKIs) have been shown to provide survival benefits in patients with relapsed OS (5).

Sorafenib is a multi-tyrosine kinase inhibitor that elicits tumor regression through inactivating angiogenic and oncogenic kinases, including platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptors (VEGFRs), Ret, c-Kit, and Raf (6, 7). Sorafenib presented encouraging clinical activity and a manageable safety profile in patients with relapsed OS after failure of standard treatment (8).

Preclinical and clinical studies have indicated that the effect of sorafenib against cancers can be enhanced by combinations with critical molecular inhibitors (9, 10). For instance, the nuclear factor-kappa B (NF-κB) inhibitor and mammalian target of rapamycin (mTOR) inhibitor have been reported to sensitize hepatocellular carcinoma (HCC) cells to sorafenib (9) and to improve the therapeutic efficacy of sorafenib in patients with unresectable OS (10), respectively.

Demonstrated by cell and animal models, bioactive compounds isolated from natural plants, such as curcumin, berberine, genistein, quercetin, tanshinone, resveratrol, silibinin, galangin, and betulin exert anti-OS activities *via* mediating

apoptosis, cell cycle arrest, autophagy, and suppression of oncogenic signaling transduction pathways (11, 12).

Amentoflavone, a bioactive polyphenolic compound extracted from many natural plants, was shown to inhibit progression of OS by suppressing extracellular signal-regulated kinase (ERK)/NF-κB signaling (2, 13). Previous studies have shown that amentoflavone sensitized HCC cells to sorafenib by triggering apoptosis (14, 15). However, whether amentoflavone enhances the anti-OS effect of sorafenib is ambiguous. The major goal of the present study was to evaluate the inhibitory efficacy and possible underlying mechanism of amentoflavone combined with sorafenib in OS *in vitro*.

Table I. *Combination index of amentoflavone combined with various doses of sorafenib.*

Concentration of drugs		U2-OS cells	
Amentoflavone (μM)	Sorafenib (μM)	Fa	CI
100	5	0.16	1.63
100	10	0.30	1.18
100	15	0.38	1.14
100	20	0.56	0.76*
100	30	0.80	0.39*

Fa: Fraction affected % inhibition; CI: Combination index; CI=1, additivity; CI>1, antagonism; CI<1, synergy. Values marked with * indicate increased synergistic effect.

Materials and Methods

Reagents and antibodies. Sorafenib was purchased from LC Laboratories (Woburn, MA, USA), Amentoflavone was purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, Hubei, PR China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). McCoy's 5A medium, fetal bovine serum (FBS), and penicillin-streptomycin (PS) were purchased from GIBCO®/Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies X-linked inhibitor of apoptosis protein (XIAP, #2042, 1:1,000, Cell Signaling Technology (CST), Danvers, MA, USA), Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (C-FLIP, #8510, 1:1,000, CST), matrix metallopeptidase 9 (MMP9, PA5-13199, 1:1,000, Invitrogen), vascular endothelial growth factor (VEGF, ab46154, 1:1,000, Abcam, Cambridge, UK), extracellular signal-regulated kinase (ERK) Thr202/Try204 (#9101, 1:1,000, CST), ERK (#4695, 1:1,000, CST), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) Ser536 (93H1) (#3033, 1:1,000, CST), NF-κB (D14E12) XP® (#8242, 1:1,000, CST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (D16H11) XP® (#5174, 1:1,000, CST), β-actin (sc-47778, 1:1,000, Santa Cruz Biotechnology, Dallas, TX, USA) and GAPDH (Elabscience, Houston, TX, USA) were purchased from the indicated companies. Secondary antibodies for western blotting, including peroxidase affiniPure Goat Anti-Mouse IgG and Goat Anti-Rabbit IgG were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA).

Cell culture. Human bone osteosarcoma epithelial cell, U-2 OS cells, purchased from Bioresource Collection and Research Center (Hsinchu City, Taiwan, ROC), and maintained in McCoy's 5A medium containing 10% FBS and 1% PS in 5% CO_2 and 37˚C.

MTT assay and combination index (CI). U-2 OS cells were seeded into 96-well plates with 5×10^3 cells per well and incubated at 37˚C overnight. After incubation, cells were treated with sorafenib (0-30 μM), QNZ (0-4 μM), amentoflavone (0-200 μM), sorafenib plus QNZ or sorafenib plus amentoflavone for 24 h. Then, the supernatant was discarded, replaced with MTT solution (0.5 mg/ml MTT in PBS), and incubated at 37˚C for 2 h. After the formazan formation, the MTT solution was removed from the 96-well plate, and 100 μl of DMSO was added into each well. The SpectraMax iD3 microplate reader (Molecular Device, San Jose, CA, USA) was the used for signal detection at the absorbance wavelength of 570 nm. The blank value was defined as zero (+/–0.1). The CI was calculated and analyzed by CompuSyn software (PD Science, LLC, Paramus, NJ, USA)

Migration/invasion transwell assay. U-2 OS cells were seeded into a 6-cm petri dish with 5×10^5 cells and incubated at 37˚C overnight. After incubation, U-2 OS cells were pre-treated with 20 μM of sorafenib, 100 μM amentoflavone or sorafenib plus amentoflavone for 24 h. The drug pre-treated U-2 OS cells were collected by trypsinization, and diluted to 1×10^6/ml in serum-free medium. Then, the 8 μm diameter pore size transwell inserts (Merck, Kenilworth, NJ, USA) were placed into 24-well plates. Seven hundred 10% completed medium was then added in the bottom layer, and 100 μl of drug pre-treated cells was added in the upper layer of the transwell inserts for another 24 h. For the invasion assay, 8 μm diameter pore size transwell inserts were pre-coated with 50 μl matrigel one day before the experiment. The transwell insert was fixed with fixation buffer (methanol: acetic acid=3:1) for 15 min at room temperature. Transwell insert membranes were then stained with 0.1% crystal violet solution for 5 min, and observed by using light microscopy. The percentage of migration/invasion was analyzed by using ImageJ software 1.50 (National Institutes of Health, Bethesda, MD, USA) (16).

Flow cytometry. U-2 OS cells were seeded into 6-well plates with 1×10^5 cells per well and incubated at 37˚C overnight. After incubation, U-2 OS cells were treated with 20 μM of sorafenib, 100 μM amentoflavone or sorafenib plus amentoflavone for 24 h. U-2 OS cells were then collected by trypsinization, and washed twice with ice-cold PBS. Next, U-2 OS cells were conjugated with different antibodies or reagents, including FITC-DEVD-FMK (cleaved-caspase-3) (Abcam, Waltham, MA, USA), Red-IETD-FMK (cleaved-caspase-8) (Abcam), FITC-VAD-FMK (cleaved-caspase-9) (Abcam), Fas-L-PE (NOK-1, BioLegend, San Diego, CA, USA), Fas-FITC (DX2, BioLegend), and $DIOC_6$ (mitochondria membrane potential, $\Delta\Psi m$) for apoptosis detection and analyzed by flow cytometry (NovoCyte, Agilent Technologies, Santa Clara, CA, USA) (17).

Western blotting assay. U-2 OS cells were seeded into a 6-cm petri dish with 5×10^5 cells and incubated at 37˚C overnight. After incubation, U-2 OS cells were treated with 20 μM of sorafenib, 100 μM amentoflavone or sorafenib plus amentoflavone for 24 h. U-2 OS cells

were washed twice with ice-cold PBS and lysed with RIPA buffer containing proteinase and phosphatase inhibitors for 1 h on ice. The protein from the whole cell lysate was separated by 8-12% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were further blocked with 5% non-fat milk for 1 h at room temperature and incubated with primary and secondary antibodies for protein detection. For protein band detection, PVDF membranes were incubated and reacted with Immobilon Western Chemiluminescent HRP Substrate (Pierce, Rockford, IL, USA). Finally, the intensities from protein bands were detected and quantified by using VisionWorks (Analytik Jena, Jena, Germany). Quantification data were all normalized by control GAPDH and β-actin expression.

NF-κB translocation assay. U-2 OS cells were seeded into a 6-well containing 2 mm × 2 mm coverslips at 1×10^5 cells per well and incubated at 37˚C overnight. U-2 OS cells were treated with 20 μM of sorafenib, 100 μM amentoflavone or sorafenib plus amentoflavone for 24 h. Coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X 100 both for 15 min. Then, U-2 OS cells on coverslips were blocked with 3% bovine serum albumen (BSA) and stained with NF-κB primary antibody (1:300) at 4˚C overnight. The Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG (H+L) secondary antibody (1:200) and 1 μg/ml DAPI (in PBS) were administered to cells and incubated at room temperature for 1 h and 2 min, respectively. The stained coverslips were mounted onto glass slides with fluorescence protection mounting buffer and stored at 4˚C in the dark. Fluorescence signal from U-2 OS cells was detected by using DAPI and FITC channels with fluorescence microscopy (Revolve, ECHO, San Diego, CA, USA) and quantified by ImageJ software 1.50 (18).

Statistical analysis. One-way ANOVA with Tukey's test was used in this study for comparing different concentrations of single drug treatments and combined treatments by using Microsoft Excel 2016. *p*-Values less than 0.05 were defined as significant differences. Each value in this study is displayed as mean±standard error.

Results

Amentoflavone enhanced sorafenib-induced cytotoxicity and inhibited the migration/invasion capacity of OS cells. We used the MTT assay to investigate the cytotoxicity of sorafenib and amentoflavone as monotherapy and in combination on U-2 OS cells. As illustrated in Figure 1A and B, cell viability was decreased in a dose-dependent manner in response to sorafenib and amentoflavone. Furthermore, sorafenib-induced cytotoxicity was enhanced by the combination treatment with amentoflavone in U-2 OS cells (Figure 1C). In Figure 1D, the synergistic effect of amentoflavone combined with sorafenib was found at 100 μM amentoflavone plus 20 μM sorafenib (Combination index=0.76, Table I); these concentrations were used to perform further experiments. Moreover, the sorafenib-induced inhibition of migration and invasion was validated by transwell migration and invasion assay. The positive staining of migration and invasion cells was found to be markedly decreased by the combination of sorafenib and amentoflavone compared to single treatments (Figure 1E and F). Then, we identified whether the migration- and invasion-associated genes were reduced by sorafenib combined with amentoflavone. As indicated in Figure 1G, the expression of MMP-9 and VEGF was effectively decreased by the combination of sorafenib and amentoflavone (over 95% reduction). Taken together, amentoflavone may sensitize U-2 OS cells to sorafenib treatment.

Amentoflavone enhanced the sorafenib-induced death receptor-dependent and mitochondria-dependent apoptosis pathways in OS cells. We then examined whether the apoptosis induced by sorafenib may also be enhanced by the combination treatment with amentoflavone by using flow cytometry. As illustrated in Figure 2A, cleaved caspase 3 was significantly activated by the combination treatment. The activity of cleaved caspase-3 increased to 80% in the combination group (Figure 2B). Caspase 8, the most proximal caspase in death receptor signaling, was also activated by treatment with sorafenib and amentoflavone as compared to single therapy (Figure 2A and C). Next, we found that the cell death surface receptor Fas and its ligand (Fas-L) were induced by the combination treatment (Figure 2D-F). We also validated whether the mitochondria-dependent apoptosis pathway may be activated by sorafenib combined with amentoflavone. In Figure 2G-H, cleaved caspase-9, involved in the mitochondria-mediated apoptosis, was increased by the combination treatment. Subsequently, sorafenib combined with amentoflavone decreased the mitochondrial membrane potential ($\Delta\Psi m$) of U-2 OS cells (Figure 2G and I). Sorafenib in combination with amentoflavone not only induced apoptosis but also increased the suppression effect on anti-apoptosis related proteins, such as XIAP and c-FLIP (Figure 2J). In summary, amentoflavone sensitized U-2 OS cells to sorafenib-induced extrinsic (death receptor-mediated apoptosis) and intrinsic (mitochondria-mediated apoptosis) apoptosis signaling.

The amentoflavone enhancement of the sorafenib-induced toxicity in OS cells was associated with the inactivation of the ERK/NF-κB signaling pathway. To identify the role of NF-κB after treatment with the combination of amentoflavone and sorafenib, we replaced amentoflavone with a NF-κB inhibitor (QNZ). As indicated in the MTT assay results, QNZ suppressed the viability of U-2 OS cells (Figure 3A). The cell viability reduction was markedly increased after treatment with the combination of QNZ and sorafenib as compared to treatment with sorafenib alone (Figure 3B). The synergistic combination effect of QNZ combined with sorafenib was also confirmed by CI (Figure 3C and Table II). Thus, the inhibition of NF-κB may sensitize U-2 OS cells to sorafenib treatment. Next, we further investigated whether amentoflavone suppressed nuclear translocation of NF-κB by using immunofluorescence staining. As shown in Figure 3D, nuclear translocation of NF-κB was significantly abolished by amentoflavone alone and

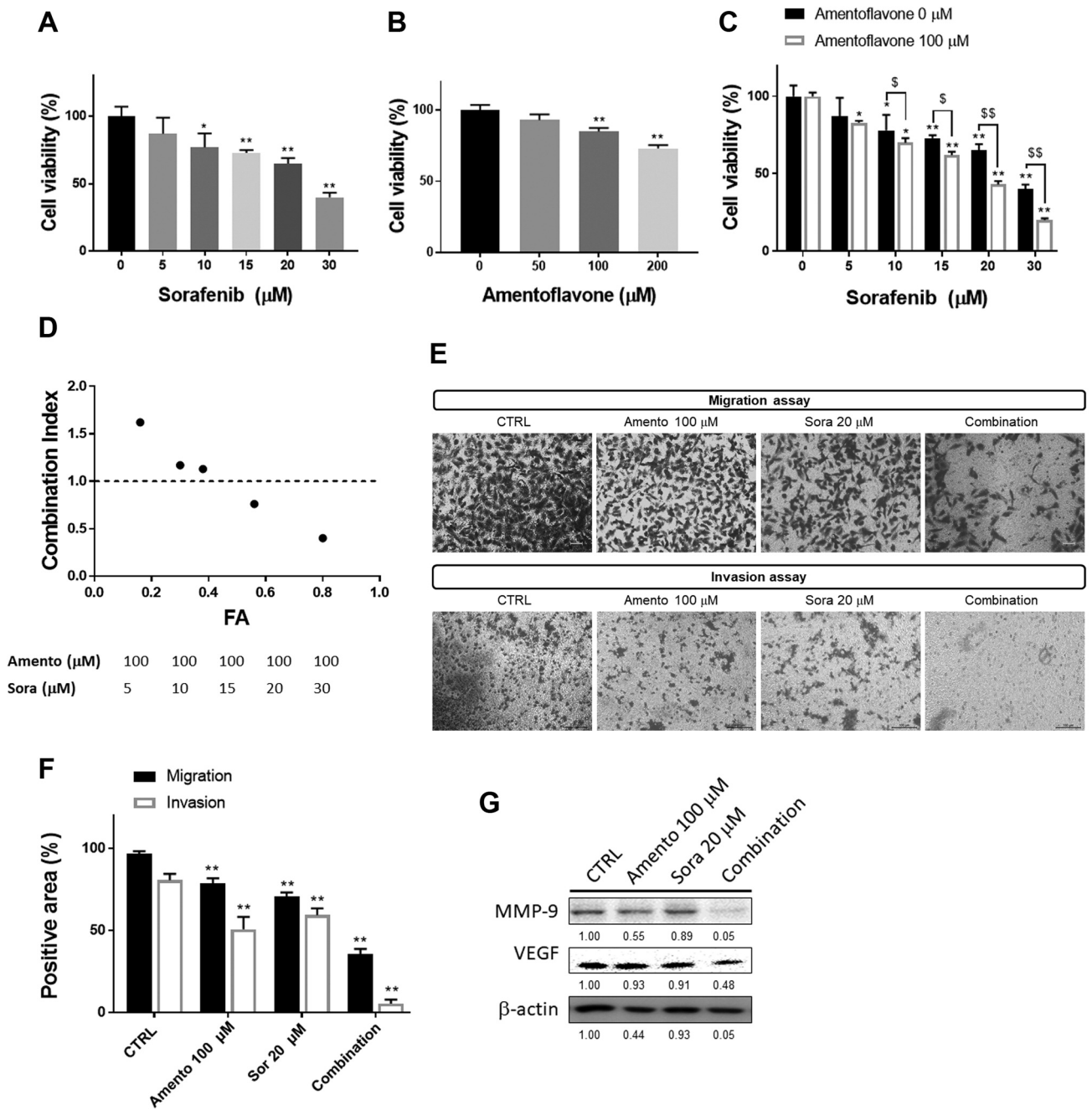

Figure 1. *The induction of sorafenib-induced toxicity and sorafenib-suppressed migration and invasion by amentoflavone in U-2 OS cells. U-2 OS cells were treated with (A) sorafenib (0-30 μM), (B) amentoflavone (0-200 μM) or (C) the combination of both for 24 h, and then assayed by MTT. (D) The CI index of the combination treatment is displayed. The (E) stained transwell images of migration and invasion and (F) the quantification of data are presented. (G) The western blotting results of MMP-9 and VEGF after treatment are presented. (*p<0.05, **p<0.01 vs. 0 μM of sorafenib or 0 μM of amentoflavone; $p<0.05, $$p<0.01 vs. 0 μM of amentoflavone).*

amentoflavone combined with sorafenib. In Figure 3E, the expression of NF-κB in the nucleus was reduced even further after the combination treatment. Moreover, the upstream regulator of NF-κB, ERK, was also found to be decreased by amentoflavone combined with sorafenib. The phosphorylation of ERK and NF-κB was effectively decreased after the combination treatment with amentoflavone and sorafenib (Figure 3F). Altogether, amentoflavone may aggravate the

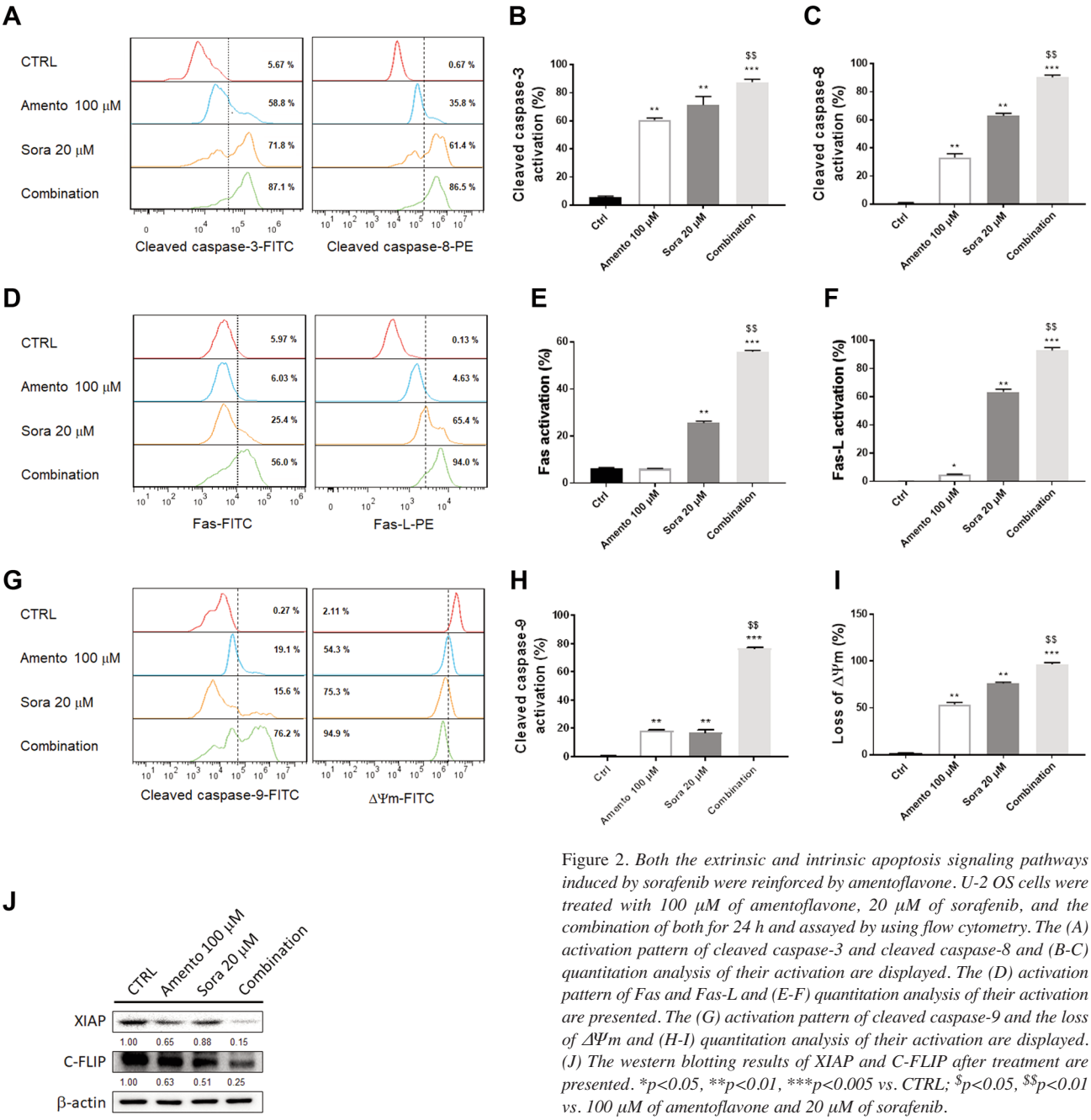

Figure 2. *Both the extrinsic and intrinsic apoptosis signaling pathways induced by sorafenib were reinforced by amentoflavone. U-2 OS cells were treated with 100 μM of amentoflavone, 20 μM of sorafenib, and the combination of both for 24 h and assayed by using flow cytometry. The (A) activation pattern of cleaved caspase-3 and cleaved caspase-8 and (B-C) quantitation analysis of their activation are displayed. The (D) activation pattern of Fas and Fas-L and (E-F) quantitation analysis of their activation are presented. The (G) activation pattern of cleaved caspase-9 and the loss of ΔΨm and (H-I) quantitation analysis of their activation are displayed. (J) The western blotting results of XIAP and C-FLIP after treatment are presented. *p<0.05, **p<0.01, ***p<0.005 vs. CTRL; $p<0.05, $$p<0.01 vs. 100 μM of amentoflavone and 20 μM of sorafenib.*

sorafenib-induced toxicity in U-2 OS cells *via* down-regulation of the ERK/NF-κB signaling.

Discussion

Invasion-associated proteins VEGF and MMP-9 are essential for tumor growth, angiogenesis, and metastasis. Positive expression of both VEGF and MMP-9 is associated with metastasis and poor prognosis in patients with OS (19, 20). Inhibition of MMP-9 expression and VEGF signaling inhibit the growth and invasion of OS cells (21, 22). Sorafenib was reported to reduce the protein levels of MMP-9 and VEGF as well as to suppress the migration and invasion of OS cells (23). Our data show that the sorafenib-induced inhibition of the invasion ability of OS cells was promoted by treatment with amentoflavone (Figure 1E and F).

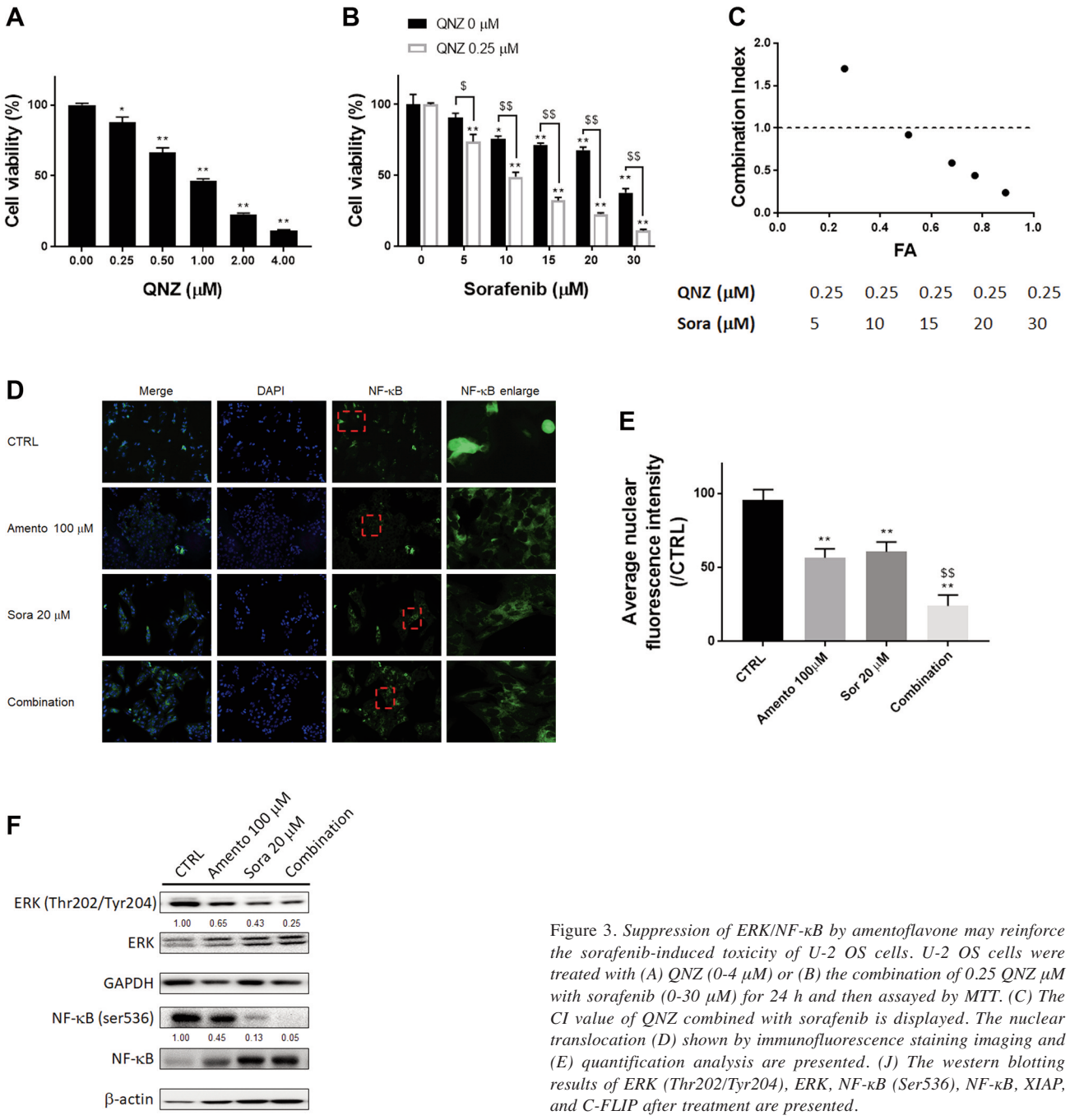

Figure 3. *Suppression of ERK/NF-κB by amentoflavone may reinforce the sorafenib-induced toxicity of U-2 OS cells. U-2 OS cells were treated with (A) QNZ (0-4 μM) or (B) the combination of 0.25 QNZ μM with sorafenib (0-30 μM) for 24 h and then assayed by MTT. (C) The CI value of QNZ combined with sorafenib is displayed. The nuclear translocation (D) shown by immunofluorescence staining imaging and (E) quantification analysis are presented. (J) The western blotting results of ERK (Thr202/Tyr204), ERK, NF-κB (Ser536), NF-κB, XIAP, and C-FLIP after treatment are presented.*

Anti-apoptotic pathway mediate drug resistance of OS cells by blocking apoptotic signaling (24, 25). The anti-apoptotic proteins XIAP and C-FLIP abrogate the caspase-3 and caspase-8 activation, leading to inhibition of apoptosis (26). Inhibition of XIAP and C-FLIP sensitized OS cells to therapeutic agents, such as cisplatin and TNF-related apoptosis inducing ligand (TRAIL) (27, 28). In this study, we examined the effect of amentoflavone on sorafenib treatment of OS cells. Amentoflavone significantly enhanced the sorafenib-induced extrinsic and intrinsic apoptosis (Figure 2A-I). We also found that the combination treatment reduced the expression of XIAP and C-FLIP even more compared to treatment with amentoflavone or sorafenib alone (Figure 2J).

Table II. *Combination index of QNZ combined with various doses of sorafenib.*

Concentration of drugs		U2-OS cells	
QNZ (μM)	Sorafenib (μM)	Fa	CI
0.25	5	0.26	1.71
0.25	10	0.50	0.92*
0.25	15	0.68	0.59*
0.25	20	0.77	0.45*
0.25	30	0.89	0.24*

Fa: Fraction affected % inhibition; CI: Combination index; CI=1, additivity; CI>1, antagonism; CI<1, synergy. Values marked with *indicate increased synergistic effect.

Constitutive NF-κB activation promotes cancer survival and invasion *via* the upregulation of anti-apoptotic and invasion-associated proteins, which are encoded by NF-κB-related genes (29, 30). The increase in NF-κB nuclear translocation is correlated with poor survival of patients with OS (31). NF-κB inactivation suppresses growth, survival, and invasion of OS cells (32). NF-κB inhibition also enhanced the anti-HCC efficacy of sorafenib (9). Therefore, we verified the effect of NF-κB inhibition on sorafenib-induced cytotoxicity in OS cells. We found that both QNZ (NF-κB inhibitor) and amentoflavone sensitized U-2OS cells to sorafenib (Figure 1C-D and Figure 3B-C). Sorafenib-inhibited NF-κB phosphorylation and nuclear translocation were potentiated by treatment with amentoflavone (Figure 3D-F). Phospho-ERK has been shown to be required for the constitutive NF-κB activation in U-2OS cells (2). Lower levels of ERK (Thr202/Tyr204) were observed after the combination treatment compared to those in cells treated with amentoflavone or sorafenib alone (Figure 3F).

In conclusion, induction of apoptosis and suppression of NF-κB signaling are associated with amentoflavone-sensitized OS cells to sorafenib. We suggest that the addition of amentoflavone as complimentary agent may increase the therapeutic benefits of sorafenib in patients with OS.

Conflicts of Interest

The Authors declare no competing financial interests regarding this study.

Authors' Contributions

CMS, CHL, MCH, PFY and FTH performed the experiments. CMS, CHL, MCH, PFY and JGC prepared the initial version of the paper. FTH, RFL and LCH designed the study, performed the literature review, and prepared the final versions of the paper.

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