Amentoflavone enhances sorafenib-induced apoptosis through extrinsic and intrinsic pathways in sorafenib-resistant hepatocellular carcinoma SK-Hep1 cells in vitro

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Abstract. The present study aimed to evaluate the effects of amentoflavone on sorafenib-induced apoptosis in sorafenib-resistant hepatocellular carcinoma (HCC) cells. The sorafenib-resistant SK-Hep1 (SK-Hep1R) cell line was established for the present study. Initially, the differences in sorafenib-induced cytotoxicity and apoptosis between wild-type SK-Hep1 and SK-Hep1R cells were verified using the MTT assay and flow cytometry. The effects of amentoflavone on sorafenib-induced cytotoxicity and apoptosis were then investigated using MTT, flow cytometry, DNA gel electrophoresis and western blot analysis. The results demonstrated that cell viability of SK-Hep1R cells was increased compared with that of SK-Hep1 cells following treatment with different concentrations of sorafenib for 24 h. Apoptosis of SK-Hep1R cells was lower than that of SK-Hep1 cells following treatment with 20 µM sorafenib for 24 h. Amentoflavone alone did not inhibit cell viability but significantly triggered sorafenib-induced cytotoxicity and apoptosis in SK-Hep1R cells. Amentoflavone not only reversed sorafenib-induced anti-apoptotic protein levels but also enhanced sorafenib-induced pro-apoptotic protein expression in SK-Hep1R cells. In conclusion, amentoflavone may be used as a sorafenib sensitizer to enhance sorafenib-induced cytotoxicity and trigger sorafenib-induced apoptosis through extrinsic and intrinsic pathways in SK-Hep1R cells.

Introduction

Sorafenib, a multi-kinase inhibitor, has been approved by the US Food and Drug Administration to improve overall survival and time to progression of patients with advanced hepatocellular carcinoma (HCC) (1). Sorafenib induces apoptosis and inhibits angiogenesis in HCC through blockade of the rapidly accelerated fibrosarcoma/mitogen-activated protein kinase/extracellular signal-regulated kinase cascade, vascular endothelial growth factor and platelet-derived growth factor receptor tyrosine kinase signaling (2,3). Sorafenib has also been demonstrated to enhance the therapeutic efficacy of anticancer agents and radiotherapy via inhibition of nuclear factor-κB (NF-κB) or signal transducer and activator of transcription 3 (STAT3)-modulated resistance to anticancer treatments in HCC models in vitro and in vivo (4,5). However, long-term exposure to sorafenib for HCC cells induces sorafenib resistance and results in tumor progression (6,7). Therefore, development of sorafenib sensitizers, which reverse sorafenib resistance and results in sorafenib-inhibited tumor progression in sorafenib-resistant HCC cells, is important.
Previous studies have identified the molecular mechanism of sorafenib resistance and have identified different types of sorafenib sensitizers. For example, Chen et al (8) reported that activation of phosphatidylinositol 3-kinase/protein kinase B (Akt) signaling modulates acquired resistance to sorafenib in HCC cells. Akt inhibitors may enhance sorafenib-induced apoptosis in HCC cells with sorafenib resistance. Tai et al (9) reported that dovitinib, a novel Src homology region 2 domain-containing phosphatase-1 (SHP-1) activator, induces apoptosis and overcomes sorafenib resistance through SHP-1-inhibited STAT3 activation in HCC cells. Cell cycle and anti-apoptosis associated proteins are overexpressed by sorafenib treatment in sorafenib-resistant HCC cells. In addition, Hsu et al (10) proposed that Cyclin-E1 and myeloid cell leukemia-1 (Mcl-1) overexpression inhibits sorafenib-induced apoptosis, whereas suppression of Cyclin-E1 and Mcl-1 enhances induction of apoptosis. Based on these previous studies, it was hypothesized that restoration of sorafenib-induced apoptosis by sorafenib sensitizers is a critical mechanism in overcoming sorafenib resistance in HCC cells.

Amentoflavone, a polyphenolic compound isolated from Selaginella tamariscina, has been demonstrated to possess anticancer effects through the inhibition of molecules that are associated with tumor progression and modulation of apoptosis (11-13). Amentoflavone, as a NF-kB signal inhibitor, induces anti-angiogenic and anti-metastatic effects via suppression of NF-kB activation in breast cancer and melanoma cells in vitro and in vivo (11,12). Amentoflavone has also been suggested to induce apoptosis and inhibit Akt phosphorylation in cervical and breast cancer cells (14,15). However, whether amentoflavone, as a sorafenib sensitizer, triggers sorafenib-induced apoptosis in sorafenib-resistant HCC cells remains ambiguous. The present study aimed to investigate the effects of amentoflavone on sorafenib-induced apoptosis in sorafenib-resistant HCC cells. In the present study, sorafenib-resistant SK-Hep1 (SK-Hep1R) cells were established, and were selected following long-term sorafenib exposure. Effects of sorafenib on cell viability and apoptosis were evaluated in wild-type SK-Hep1 and SK-Hep1R cells by MTT assay and flow cytometry. Effects of sorafenib, amentoflavone and a combination of the two on cell viability, apoptosis and expression of anti-apoptotic and pro-apoptotic proteins were also investigated in SK-Hep1R cells, using MTT, flow cytometry, DNA gel electrophoresis and western blot analysis.

Materials and methods

Chemicals. Sorafenib (Nexavar) was provided by Bayer HealthCare Pharmaceuticals, Inc. (Whippany, NJ, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin were bought from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Propidium iodide (PI) and 3,3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>) were purchased from BioVision, Inc. (Milpitas, CA, USA) and Enzo Life Sciences, Inc. (Farmingdale, NY, USA), respectively. MTT and RNase were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and Fermentas; Thermo Fisher Scientific, Inc., respectively.

Primary antibodies for cleaved Caspase-3 (dilution, 1:500; catalog no. P42574; anti-rabbit) and cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (C-FLIP) (dilution, 1:500; catalog no. O15519; anti-rabbit) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies of cleaved Caspase-8 (dilution, 1:500; catalog no. MA5-15054; anti-rabbit) and X-linked inhibitor of apoptosis protein (XIAP) (dilution, 1:500; catalog no. PA1-84846; anti-rabbit) were purchased from Thermo Fisher Scientific, Inc. Primary antibodies of Mcl-1 (dilution, 1:500; catalog no. 3035-100; anti-rabbit) and cytochrome c (dilution, 1:500; catalog no. sc-13156; anti-mouse) were obtained from BioVision, Inc. and Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), respectively. Horseradish peroxidase-conjugated secondary antibodies were bought from Jackson ImmunoResearch Laboratories, Inc. (catalog nos. 31430 and 31460; dilution, 1:5000; West Grove, PA, USA). Nuclear and Cytoplasmic Extraction and Genomic DNA miniprep kits were purchased from Chemicon; EMD Millipore (Billerica, MA, USA) and Axygen; Corning Incorporated (Corning, NY, USA), respectively.

Cell culture. SK-Hep1 cells were provided by Professor Jing-Gung Chung (Department of Biological Science and Technology, China Medical University, Taichung, Taiwan). Cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, and maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ (16).

Establishment of sorafenib-resistant SK-Hep1 cells. The sorafenib-resistant SK-Hep1 (SK-Hep1R) cells were selected from SK-Hep1 cells that survived slowly escalating concentrations of sorafenib treatment (2.5 µM increase per month) till reached 10 µM was reached, as previously described by Zhai et al (17). Finally, after 3-4 month, SK-Hep1R cells were cultured in medium containing 10 µM sorafenib for use in the present study.

MTT assay. SK-Hep1 or SK-Hep1R cells were seeded onto 96-well plates at a density of 3x10<sup>3</sup> cells/well and incubated overnight. SK-Hep1 and SK-Hep1R cells were treated with 0, 10, 15, 20 and 25 µM sorafenib in 0.1% dimethyl for 24 h. In addition, SK-Hep1R cells were treated with 0-25 µM sorafenib alone or combined with 75 µM amentoflavone for 24 h. Cell viability was evaluated by MTT assay, as described previously (4).

Detection of mitochondrial membrane potential (MMP). SK-Hep1 or SK-Hep1R cells were seeded onto 12-well plates at a density of 2x10<sup>4</sup> cells/well and incubated overnight. SK-Hep1 and SK-Hep1R cells were treated with 0 µM or 20 µM sorafenib in 0.1% dimethyl for 24 h. For combination treatment, SK-Hep1R cells were treated with 20 µM sorafenib, 75 µM amentoflavone or a combination of these for 24 h. Cells from different groups were harvested by centrifugation, washed twice with PBS, resuspended in 500 µl PBS with 4 µM DiOC<sub>6</sub> and incubated for 30 min at 37°C. The changes of MMP were measured by flow cytometry (FACSCalibur FACSV101; BD Biosciences, Franklin Lakes, NJ, USA) as previously
described (18). All data were analyzed by FlowJo 7.6.1 software (Tree Star, Inc., Ashland, OR, USA).

Analysis of the subG<sub>1</sub> population. SK‑Hep1 or SK‑Hep1R cells were seeded onto 12‑well plates at a density of 2x10<sup>5</sup> cells/well and incubated overnight. SK‑Hep1 and SK‑Hep1R cells were treated with 0 µM or 20 µM sorafenib in 0.1% dimethyl for 24 h. For combination treatment, SK‑Hep1R cells were treated with 20 µM sorafenib, 75 µM amentoflavone or a combination of these for 24 h. Cells were collected, fixed with 70% ethanol and incubated overnight at −20°C. Cells were washed with PBS and then resuspended in 500 µl PI buffer (40 µg/ml PI, 100 µg/ml RNase and 1% Triton X‑100 in PBS) (catalog no. P1304MP; Thermo Fisher Scientific, Inc.) for 1 h in the dark at room temperature. Detection of the subG<sub>1</sub> population was evaluated by flow cytometry (FACSCalibur FACS101; BD Biosciences) as described by Huang et al (19). All data were analyzed by FlowJo 7.6.1 software (Tree Star, Inc.).

Detection of DNA fragmentation. SK‑Hep1R cells were seeded onto 6‑well plates at a density of 1x10<sup>6</sup> cells/well and incubated overnight. Cells were then treated with 20 µM sorafenib, 75 µM amentoflavone and their combination for 24 h. The genomic DNA miniprep kit (Chemicon; EMD Millipore) was used to purify genomic DNA from cells, following the protocol provided by the manufacturer. Detection of DNA fragmentation was analyzed using 1.5% agarose gel electrophoresis with SYBRsafe stain (4).

Western blot analysis. A total of 3x10<sup>6</sup> SK‑Hep1 or SK‑Hep1R cells were seeded in 10 cm diameter dishes and incubated overnight. SK‑Hep1 cells were treated with 20 µM sorafenib for 24 h. In addition, SK‑Hep1R cells were treated with 20 µM sorafenib, 75 µM amentoflavone or a combination of these for 24 h. Total proteins from cells were extracted with lysis buffer (50 mM Tris‑HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40 and 1 mM phenylmethylene sulfonyl fluoride). A cytosol extraction kit (catalog no. 2118936; EMD Millipore) was used to extract cytosolic cytochrome c from cells, following the protocol provided by the manufacturer. Expression levels of XIAP, Mcl‑1, C‑FLIP, Caspase‑3, Caspase‑8 and cytochrome c were determined by western blot analysis, as described by Ting et al (20). The levels of protein bands were quantified with ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as the mean ± standard error. Student’s t‑test was analyzed for comparison between the control and each treatment group by SigmaPlot version 10 (Systat Software, Inc., San Jose, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Differences in sorafenib‑induced cytotoxicity and apoptosis between SK‑Hep1 and sorafenib‑resistant SK‑Hep1 cells. Differences in sorafenib‑induced cytotoxicity were examined between SK‑Hep1 and SK‑Hep1R cells using the MTT assay. The viability of SK‑Hep1R cells was significantly increased compared with viability of wild‑type SK‑Hep1 cells following treatment with 10‑25 µM sorafenib for 24 h (Fig. 1A). Sorafenib treatment (10‑25 µM) significantly reduced cell viability by
15-46% compared with the control SK-Hep1 cells. Notably, no evident cytotoxicity was observed when SK-Hep1R cells were treated with 10 µM sorafenib for 24 h. Sorafenib treatment (15-25 µM) significantly reduced cell viability by 7-14% compared with that of the control in SK-Hep1R cells. Differences in sorafenib-induced apoptosis between SK-Hep1 and SK-Hep1R cells were investigated by detection of subG<sub>1</sub> and MMP with flow cytometry. The subG<sub>1</sub> population of SK-Hep1R cells was significantly decreased compared with wild-type SK-Hep1 cells following treatment with 20 µM sorafenib for 24 h. Sorafenib significantly increased the subG<sub>1</sub> population by 35% compared with the control SK-Hep1 cells, and only increased subG<sub>1</sub> population by 8% compared with the control SK-Hep1R cells (Fig. 1B). SK-Hep1R cells were also demonstrated to present resistance to sorafenib-induced loss of MMP. Sorafenib treatment (20 µM) significantly reduced MMP by 50% compared with the control SK-Hep1 cells (Fig. 1C). In contrast, the MMP of SK-Hep1R cells was not affected under similar experimental conditions.

Amentoflavone restores sorafenib-induced apoptosis in extrinsic and intrinsic pathways in sorafenib-resistant SK-Hep1 cells. The levels of anti-apoptotic proteins (XIAP, Mcl-1 and C-FLIP) were reduced by 0.7-0.8 fold in SK-Hep1 cells compared with SK-Hep1R cells following treatment with 20 µM sorafenib for 24 h, but anti-apoptotic protein levels of SK-Hep1R cells were not inhibited under similar experimental conditions (Fig. 3A and B). Amentoflavone not only inhibited sorafenib-induced anti-apoptotic protein levels (XIAP, Mcl-1 and C-FLIP) but also triggered sorafenib-induced pro-apoptotic protein expression (cleaved-Caspase-3, -8 and cytochrome c) in SK-Hep1R cells (Fig. 3B).

Discussion

Sorafenib is the only FDA approved drug for advanced HCC, but acquired resistance limits the therapeutic efficacy of sorafenib. Therefore, development of sorafenib sensitizers may benefit patients with HCC. Based on selected published studies, it was hypothesized that restoration of sorafenib-induced apoptosis by sensitizers is critical in overcoming acquired sorafenib resistance in HCC cells. Amentoflavone has been demonstrated to inhibit tumor growth through induction of apoptosis in breast and cervical cancer cells (14,15). However, whether amentoflavone is able to act as a sorafenib sensitizer, which restores sorafenib-induced apoptosis in sorafenib-resistant HCC cells, has not been elucidated. The present study aimed to evaluate the effect of amentoflavone on sorafenib-induced apoptosis in sorafenib-resistant HCC cells. A sorafenib-resistant SK-Hep1 cell line was established and used in the present study.
Apoptosis is the process of programmed cell death, which may be triggered by extrinsic and intrinsic signal pathways. Apoptosis results in morphological change and DNA fragmentation, resulting in cell death (21). Various anticancer agents inhibit tumor growth through induction of apoptosis (22). Multiple anti-apoptotic proteins, including C-FLIP, XIAP and Mcl-1, are induced and overexpressed by anticancer agents and subsequently block apoptotic pathways (23). Caspase-8 is a critical mediator of the extrinsic apoptotic pathway. C-FLIP disrupts initiation of extrinsic apoptotic pathway through inhibition of Caspase-8 activation (21). The intrinsic apoptosis pathway is characterized by loss of mitochondrial membrane potential and release of cytochrome c. Mcl-1 inhibits the intrinsic apoptosis pathway by preventing loss of mitochondrial membrane potential and the release of cytochrome c (24,25). A previous study indicated that sorafenib enhances vorinostat-induced extrinsic and intrinsic apoptotic pathways via inhibiting expression of NF-kB-modulated anti-apoptotic proteins in HCC HuH7 cells in vitro and in vivo (4). The present study also revealed that sorafenib induced accumulation of the subG₀ population and loss of MMP, and inhibited protein levels of XIAP, Mcl-1 and C-FLIP in wild-type SK-Hepl cells (Figs. 1B, C and 3A).

Apoptosis is inhibited and anti-apoptotic proteins are overexpressed in HCC cells with acquired resistance to sorafenib (8-10). Tai et al (9) reported that protein levels of activated Cyclin D1, Mcl-1 and STAT-3 in sorafenib-resistant HCC cells were increased compared with those in wild-type cells. Cytotoxicity, subG₀ population and loss of MMP were increased in SK-Hepl cells compared with in SK-HeplR cells following treatment with 20 µM sorafenib for 24 h (Fig. 2B and C). Protein levels of XIAP, Mcl-1 and C-FLIP were not decreased by sorafenib treatment in SK-HeplR cells (Fig. 3B). Hsu et al (10) suggested that Mcl-1 suppression is critical to restore sorafenib-induced apoptosis in sorafenib-resistant HCC cells. The present results revealed that amentoflavone not only decreased sorafenib-induced anti-apoptotic protein levels (XIAP, Mcl-1 and C-FLIP) but also triggered sorafenib-induced pro-apoptotic protein expression (cleaved-Caspase-3, -8 and cytochrome c) in SK-HeplR cells (Fig. 3B). Notably, amentoflavone alone did not induce apoptosis but enhanced sorafenib-induced increases in the subG₀ population, loss of MMP and DNA fragmentation. Inhibition of sorafenib-induced protein levels of XIAP, Mcl-1 and C-FLIP by amentoflavone was associated with enhancement of sorafenib-induced apoptosis in SK-HeplR cells. Application of amentoflavone as a sorafenib sensitizer may help to enhance the therapeutic efficacy of sorafenib in patients with HCC.

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