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Amentoflavone Induces Apoptosis and Reduces Expression of Anti-apoptotic and Metastasis-associated Proteins in Bladder Cancer

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Abstract. *Background/Aim: Amentoflavone has been shown to be effective against a variety of cancer cells, but its role in bladder cancer remains unclear. Thus, the aim of this study is to evaluate whether amentoflavone may induce toxicity effect of bladder cancer. Materials and Methods: Herein, we evaluated amentoflavone effects in a human bladder cancer cell line TSGH8301 in vitro. Results: Amentoflavone caused significant cytotoxicity in TSGH8301 cells at a concentration as low as 200 μM. FAS/FASL-dependent extrinsic apoptosis and mitochondria-dependent intrinsic apoptosis were observed in amentoflavone-treated cells in a dose-dependent manner. Levels of several proapoptotic proteins, such as FAS, FAS-ligand and BAX (B-cell lymphoma 2 associated X) were increased following amentoflavone treatment. Meanwhile, anti-apoptotic MCL-1 (myeloid cell leukemia sequence 1) and cellular FLICE-inhibitory protein (C-FLIP) protein levels were reduced. Additionally, angiogenesis and proliferation-related proteins, including matrix metalloproteinase (MMP)-2, -9, vascular endothelial growth factor (VEGF), urokinase-type plasminogen actvator (uPA) and cyclin D1 were diminished by amentoflavone. Conclusion: Amentoflavone induced toxicity of bladder cancer by inhibiting tumor progression and inducing apoptosis signaling transduction.*

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Many carcinogens lead to bladder cancer through genetic alterations (1). The molecules that cause genetic alterations promote the overexpression of oncogenes and the silencing of tumor suppressor genes, leading to apoptosis dysregulation and metastasis in bladder cancer (2-4). Overexpression of anti-apoptotic and metastasis-associated proteins often observed in high-grade and invasive bladder tumors inhibit the therapeutic efficacy of current treatment approaches for bladder cancer, including radical cystectomy, chemotherapy, and radiotherapy (5-7). Anti-apoptotic and metastasis-associated proteins are recognized as potential targets for the treatment of bladder cancer (8, 9).

Herbal medicine, including compounds and formulas derived from natural plants, are applied to treat and prevent urinary tract diseases, such as urinary stone disease and urinary tract infections (10, 11). In addition, herbal medicines have been demonstrated to possess anticancer properties in bladder cancer. Lee *et al*., presented their work on herbal medicines and have shown that they can effectively reduce the number and size of multiple metastatic nodules in both lungs in a 74-year-old Korean man (12). Flavonoids, bioactive compounds extracted from plants, such as curcumin and apigenin, are indicated to inhibit cell invasion and induce apoptosis in bladder cancer (13, 14) Zamora-Ros *et al*., have found that dietary intake of

flavonoid subclasses (flavonol) and lignans are associated with a reduction in bladder cancer risk (15).

Amentoflavone, a bioflavonoid isolated from *Selaginella tamariscina*, has been indicated to modulate the activity of several cancer types. Amentoflavone inhibits metastatic activity by suppressing the activation of nuclear factor-kappaB (NF-κB) in melanoma, osteosarcoma, and breast cancer (16-18). Amentoflavone can also induce apoptosis and reduce the expression of anti-apoptotic proteins in glioblastoma and breast cancer (19, 20). In a previous study, we found amentoflavone enhances the therapeutic efficacy of sorafenib, the oral multikinase inhibitor, by inducing apoptosis in HCC (21). However, whether amentoflavone promotes apoptosis and abolishes the expression of anti-apoptotic and metastasis-associated proteins in bladder cancer is ambiguous. The aim of the present study was to investigate the anticancer properties of amentoflavone in bladder cancer *in vitro*.

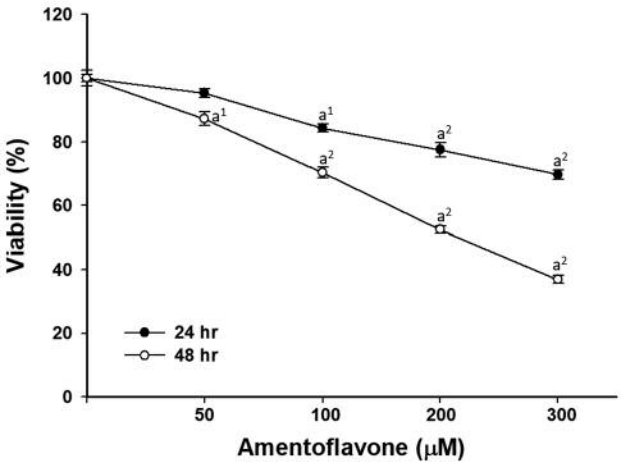

Figure 1. *Cell viability was markedly decreased by amentoflavone. MTT assay was used to validate the viability of TSGH 8301 cells following amentoflavone treatment. (a^1 $p<0.05$ and a^2 $p<0.01$ vs. 0 μM amentoflavone).*

Materials and Methods

Chemical reagents and antibodies. Amentoflavone, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Primary antibodies used in Western blots were acquired from different companies, such as XIAP (Thermo Fisher Scientific, Fremont, California, USA), C-FLIP (Cell signaling Technology, Danvers, Massachusetts, USA), MCL-1 (BioVision, Milpitas, California, USA), BAX (Proteintech Inc., Rosemont, Illinois, USA), FAS (Elabscience Biotechnology Inc., Houston, Texas, USA), FASL (Elabscience Biotechnology Inc.), VEGF (EMD Millipore, Billerica, MA, USA), MMP-2 (Proteintech Inc.), MMP-9 (EMD Millipore), uPA (abcam, Cambridge, MA, USA), Cyclin D1 (Thermo Fisher Scientific), β-actin (Santa Cruz, California, USA). All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, Pennsylvania, USA).

TSGH8301 cells culture. Human bladder carcinoma cell line (TSGH 8301) was kindly provided from Professor Jing-Gung Chung's lab, China Medical University (22, 23). TSGH 8301 cells were cultured in 10 cm culture plates and were preserved in flasks in a humidified atmosphere of 5% CO_2 at 37˚C. The culture medium of TSGH 8301 contained RPMI-1640 medium, 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cell culture reagents were all obtained from GIBCO®/Invitrogen Life Technologies (Carlsbad, California, USA).

Cell viability analysis. TSGH 8301 cells were seeded into 96-well plates with a density of 2×10^4 cells/well overnight. Cells were then treated with 0-300 μM amentoflavone for 24 or 48 h, respectively. Cell viability was performed using the MTT assay, as previous described (1). The absorbance of MTT was detected using SpectraMax iD3 at 570 nm wavelength (Molecular Devices, San Jose, California, USA).

Annexin-V/PI flow cytometry analysis. TSGH 8301 cells were seeded into 6-well plates with a density of 5×10^5 cells/well overnight. Cells were then treated with 0, 150, 200 μM amentoflavone for 48 h. Cells were finally collected and double stained using Annexin V/PI in the dark for 15 min (24). Detection and quantification of the positive Annexin V-FITC/PI-PE population was performed using FACSCalibur flow cytometry (Becton-Dickinson, Franklin Lakes, New Jersey, USA) and FlowJo software, version 7.6.1 (FlowJo LLC, Ashland, Oregon, USA), respectively. Annexin V-FITC apoptosis detection kit was purchased from Vazyme Biotech Co. Lt (Nanjing City, PR China).

Cleaved caspase-3 and caspase-8 flow cytometry analysis. TSGH 8301 cells were seeded into 6-well plates with a density of 5×10^5 cells/well overnight. Cells were then treated with 0, 150, 200 μM amentoflavone for 48 h. Cells were finally collected and stained using the CaspGlow fluorescein active Caspase-3 and -8 staining kit (BioVision). The detailed staining procedure has been described in our previous study (24). The percentages of cleaved caspase-3 and caspase-8 were detected and quantified using FACSCalibur flow cytometry and FlowJo software, respectively.

Cell-cycle flow cytometry analysis. TSGH 8301 cells were seeded into 6-well plates with a density of 5×10^5 cells/well overnight. Cells were then treated with 0, 150, 200 μM amentoflavone for 48 h. For cell cycle phase analysis, cells were harvested, fixed in 70% ethanol and stored at –20˚C overnight. The second day, cells were centrifuged and stained with propidium iodide (PI: 40 μg/ml) at 25˚C for 60 min (24) (Sigma Chemical Co., St. Louis, MO, USA). Detection and quantification of the sub-G1 cell population was performed using FACSCalibur flow cytometry and FlowJo software, respectively. PI and RNase were purchased from purchased from Sigma-Aldrich and Fermentas (St. Leon-Rot, Baden-Württemberg, Germany), respectively (25).

FAS/FASL flow cytometry analysis. TSGH 8301 cells (5×10^5 cells/well) were seeded into 6-well plates overnight before treatment. Cells were then treated with 0, 150, 200 μM amentoflavone. After a 48-hour treatment, cells were harvested and double stained usingut anti-FAS-FITC and anti-FASL-PE for 15 min on ice, purchased from Thermo Fisher Scientific (1, 24). Detection and quantification of the FAS and FASL percentage was assayed by

FACSCalibur flow cytometry and FlowJo software, respectively.

Mitochondria membrane potential flow cytometry analysis. Half a million of TSGH 8301 cells were seeded into 6-well plates overnight. Cells were then treated with 0, 150, 200 μM amentoflavone. Following a 48-hour treatment, cells were collected and stained by 4 μM $DiOC_6$ in 500 μl PBS for 30 minutes at 37˚C. The loss of mitochondria membrane potential was assayed using FACSCalibur flow cytometry and was quantified by FlowJo software. $DiOC_6$ was purchased from Enzo Life Sciences (Farmingdale, New York, NY, USA) (1, 24, 25).

Western blot analysis. Three million TSGH 8301 cells were incubated in 10 cm culture plates overnight and then treated with i) 0, ii) 150, iii) or 200 μM amentoflavone. Total cells were collected and re-suspended in lysis buffer [50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.5% Nonide P-40] for sonication. Following centrifugation at 13,000 rpm, total proteins from supernatant were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific) with bovine serum albumin (BSA) as the standard. Proteins were then separated by 8-12% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene difluoride (PVDF) membrane (EMD Millipore). Membranes were then processed with blocking (5% low-fat milk), followed by primary and secondary antibody staining, as described previously (1). Finally, membranes were visualized using enhanced chemiluminescence solution (EMD Millipore) and the chemiluminescent image system (ChemiDoc-It 515, UVP, Upland, CA, USA). VisionWorks LS Analysis Software (UVP) was used to quantify changes in protein expression by densitometry analysis and using β-actin as the loading control.

Statistical analysis. Statistical significance was measured by Student's *t*-test analysis. *p*-Values<0.05 and <0.01 were both considered statistically significant. The statistical analysis was conducted using the Excel 2017 software (Microsoft, Redmond, WA, USA). All experiments were repeated independently three times.

Results

Amentoflavone induced cytotoxicity of TSGH 8301 bladder cancer cells. Following incubation with various concentrations of amentoflavone (0, 50, 100, 200 and 300 μM) for 24 and 48 h, MTT assay was performed in TSGH 8301 cells to measure their viability. Figure 1 results indicated that cell viability decreased in a time- and dose-dependent manner compared to the untreated control. The half maximal inhibitory concentration (IC_{50}) of amentoflavone on TSGH 8301 was defined as 200 μM treatment for 48 h.

Amentoflavone induced apoptosis and suppressed anti-apoptosis effect of TSGH 8301 bladder cancer cells. TSGH 8301 cells treated with 150 and 200 μM amentoflavone for 48 h contained a significantly higher percentage of AnnexinV/PI positive late apoptotic cells compared to the untreated control group (Figure 2A). In Figure 2B, the cleaved form of caspase-3 was markedly increased about 30-50% by amentoflavone as compared to the untreated control group. Moreover, cells in the sub-G1 phase are also recognized as apoptotic; therefore, treatment with amentoflavone induced apoptosis in TSGH 8301 cells (Figure 2C). Additionally, the expression of XIAP, an inhibitor of caspase-3, was markedly decreased by amentoflavone (Figure 2D). In the amentoflavone-treated group, further mitochondria-related anti-apoptosis molecules, such as C-FLIP and MCL-1 were also decreased by 50-80% as compared to the control group.

Amentoflavone boost apoptosis effect through the induction of extrinsic apoptosis mechanism. TSGH 8301 cells with distinct doses of amentoflavone were evaluated for an extrinsic apoptosis effect. As showed in Figures 3A and B, death receptor FAS and death receptor ligand FASL was effectively increased by amentoflavone treatment. Furthermore, the downstream apoptosis mechanism of FAS/FASL contained caspase-8 molecule. As shown in Figure 3C, amentoflavone significantly boosted the activation of cleaved caspase-8. The protein expression of BAX, FAS and FASL following amentoflavone treatment showed obviously enhancement as compared to untreated control (Figures 3D and E). Taken together, amentoflavone can successfully trigger FAS/FASL-dependent extrinsic apoptosis signaling in bladder cancer cells.

Amentoflavone triggered intrinsic apoptosis mechanism through the modulation of mitochondria membrane potential ($\Delta\Psi m$). TSGH 8301 cells were treated with 150 and 200 μM amentoflavone for 48 h. The level of $\Delta\Psi m$ was analyzed and quantified using flow cytometry. Levels of $\Delta\Psi m$ loss were significantly increased in TSGH 8301 cells treated with amentoflavone (Figure 4) in a dose-dependent manner. These results indicate that amentoflavone can induce mitochondria-dependent intrinsic apoptosis signaling.

Amentoflavone diminished angiogenesis, metastasis and proliferation effect in TSGH 8301 bladder cancer cells. For further investigating whether amentoflavone suppressed cell angiogenesis, metastasis and proliferation we assessed the expression of proteins related to tumor progression in TSGH 8301 cells. Following incubation with amentoflavone (0, 150 and 200 μM) for 48 h, cells were harvested for Western blotting (Figures 5A and B). The results revealed several key angiogenesis-related and metastasis-related proteins, such as VEGF, MMP-2, MMP-9, uPA, which all underwent a significant reduction following 48-hour treatment with amentoflavone. Additionally, protein levels of the proliferation-related molecule, cyclin D1, were also reduced about 50-80% with amentoflavone. In sum, amentoflavone successfully suppressed the expression of tumor progression related proteins in TSGH 8301 bladder cancer cells, with a profound anti-cancer effect.

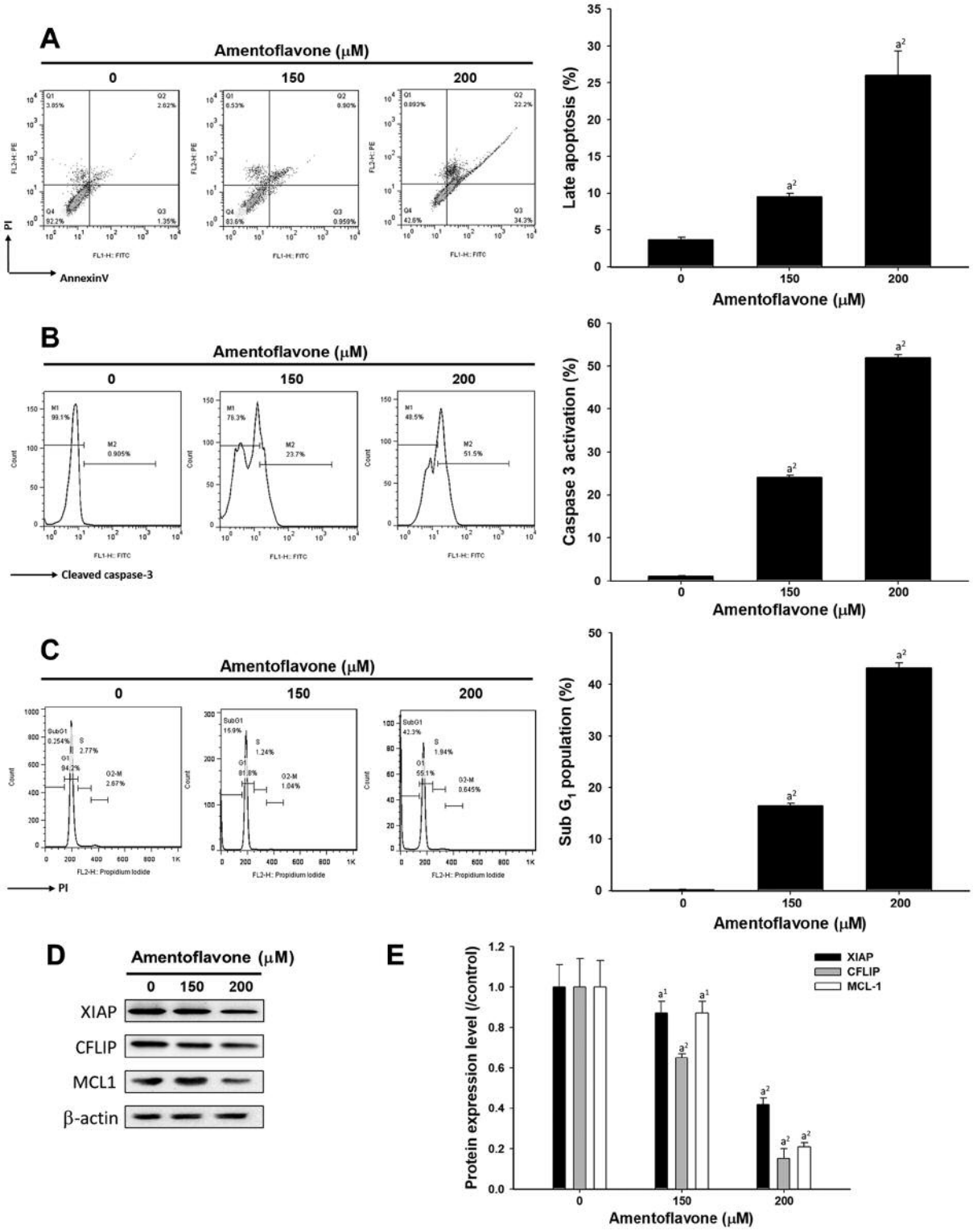

Figure 2. *Apoptosis effect was enhanced and anti-apoptosis-related protein expression was reduced by amentoflavone. TSGH 8301 cells were treated with 150 and 200 μM amentoflavone for 48 h and were stained with (A) Annexin-V/PI (B) cleaved caspase-3 (C) cell cycle-PI and were analyzed by flow cytometry. Histogram images of each group and quantification results of three repeated experiments are displayed (left and right panels, respectively). (D) Representative chemiluminescence images of XIAP, C-FLIP and MCL-1. (E) Quantification results of the protein expression of XIAP, C-FLIP and MCL-1. (a^1 $p<0.05$ and a^2 $p<0.01$ vs. 0 μM amentoflavone).*

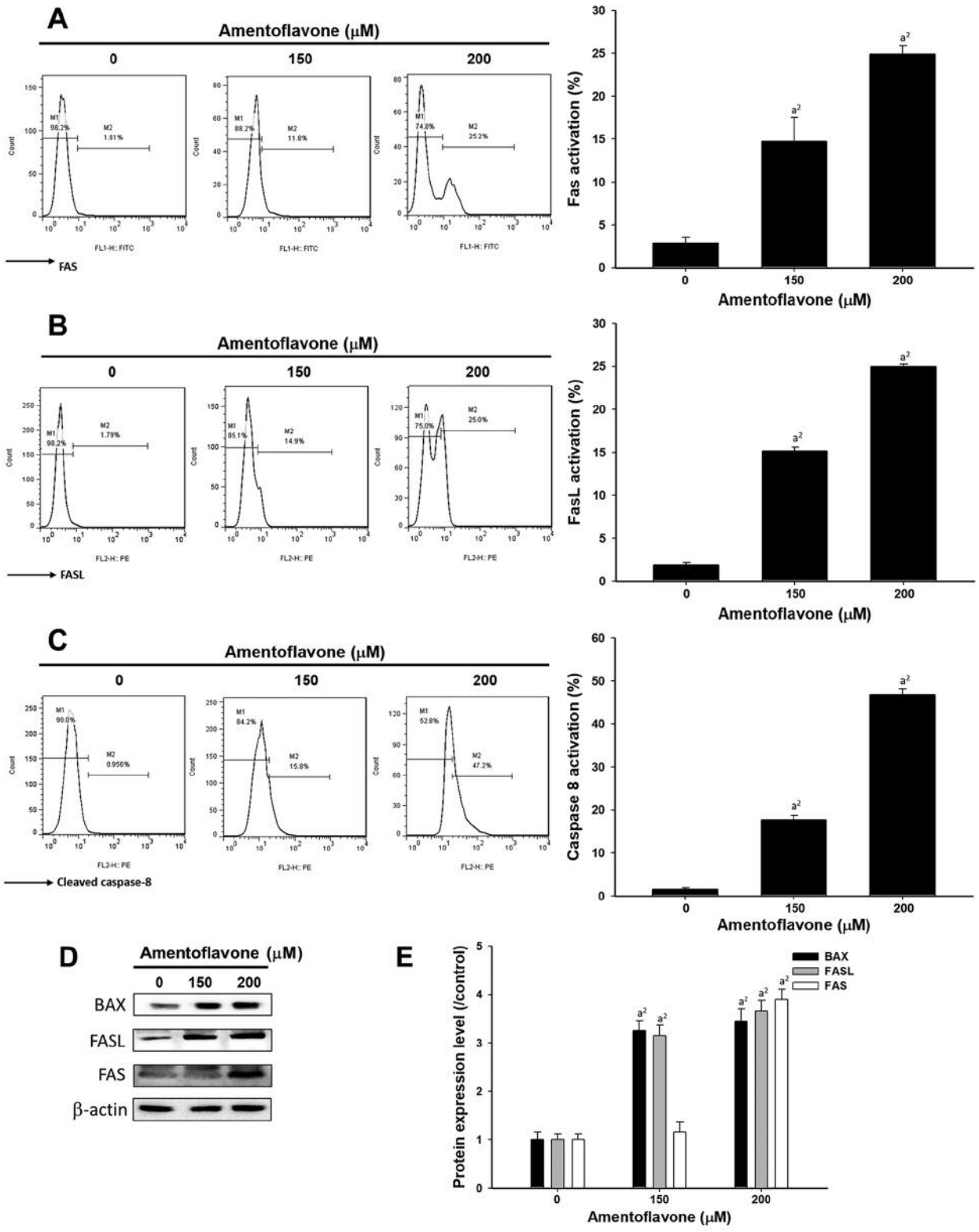

Figure 3. *Extrinsic apoptosis signaling was induced by amentoflavone. TSGH 8301 cells were treated with 150 and 200 μM amentoflavone for 48 h. Cells were then harvested and stained with (A) FAS (B) FASL (C) cleaved caspase-8 and analyzed by flow cytometry. Histogram images of each group and quantification results of three repeated experiment are displayed. (D) Representative chemiluminescence images of BAX, FAS and FASL. (E) Quantification results of the protein expression of BAX, FAS and FASL. (a^2 $p<0.01$ vs. 0 μM amentoflavone).*

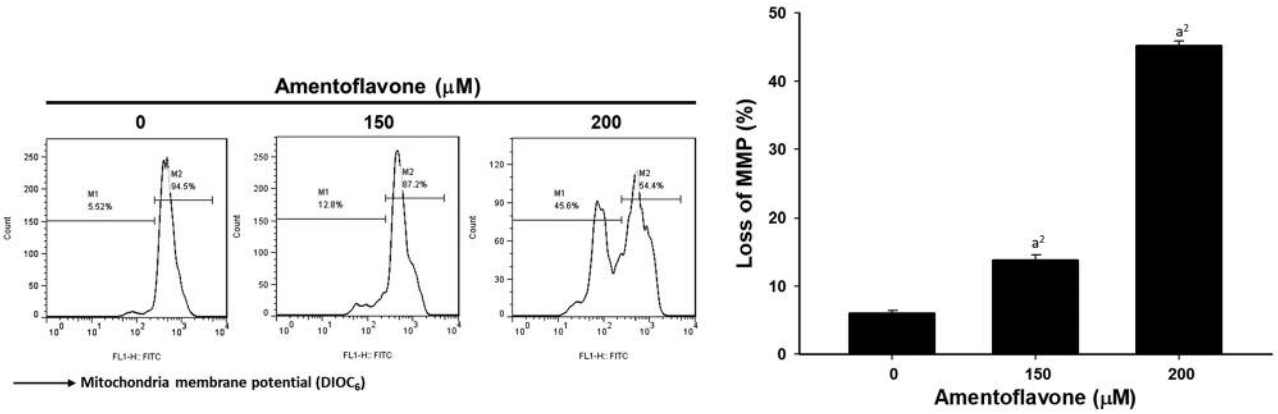

Figure 4. *Intrinsic apoptosis signaling was induced by amentoflavone. TSGH 8301 cells were treated with 150 and 200 μM amentoflavone for 48 h and were assesed for ΔΨm by flow cytometry. Histogram images of each group and quantification results of three repeated experiment were displayed. (a^2 p<0.01 vs. 0 μM amentoflavone).*

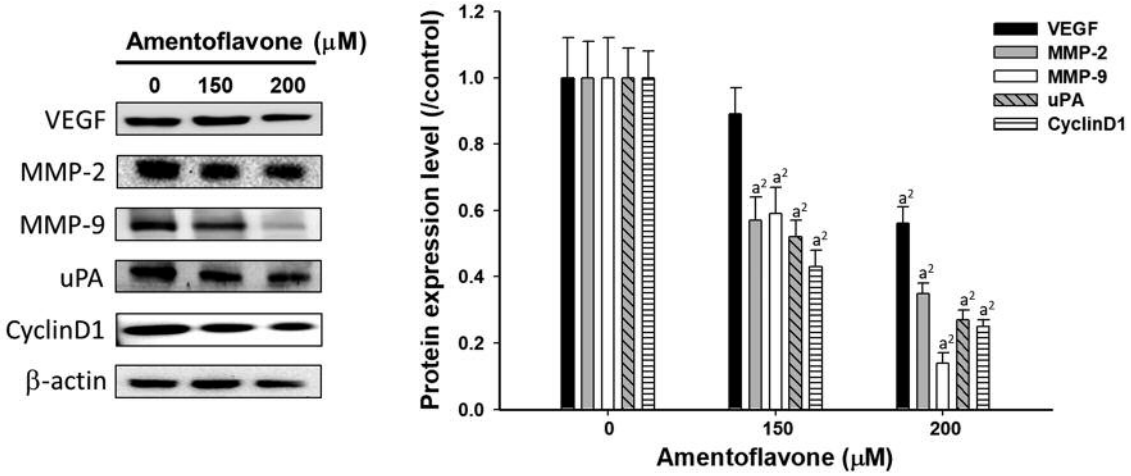

Figure 5. *Proliferation and angiogenesis-related protein expression were both decreased by amentoflavone. TSGH 8301 cells were treated with 150 and 200 μM amentoflavone for 48 h and were collected for western blotting analysis. (A) Representative chemiluminescence images of various proteins. (B) Protein level quantification results of three independent experiments. (a^2 p<0.01 vs. 0 μM amentoflavone).*

Discussion

Dysregulation of apoptosis is associated with absence or inactivation of pro-apoptotic proteins and overexpression of anti-apoptotic proteins. Evasion of apoptosis is required for disease progression and development of treatment resistance in cancer (26). The function of several apoptotic proteins is modulated or diminished in bladder cancer (6). The death receptor FAS/FAS ligand (FAS-L) interaction is recognized as the initiator for the induction of the apoptosis extrinsic pathway (27). Bax, the pro-apoptotic member of the B-cell lymphoma 2 (Bcl-2) family, promotes the apoptosis intrinsic pathway by inducing loss of mitochondrial membrane potential (ΔΨm) (28). Both extrinsic and intrinsic apoptotic signaling pathways activate caspase-3, which is essential for apoptotic DNA fragmentation (29). Lack of FAS, Bax, or caspase-3 expression has been shown to correlate with poor prognosis in patients with bladder cancer (30-32).

Several anti-apoptotic proteins have been shown to mediate resistance to apoptosis by blocking the extrinsic and intrinsic apoptotic pathways (33). Overexpression of XIAP (the inhibitor of caspase-3 activation), C-FLIP (the inhibitor of caspase-8 activation), and MCL-1 (the inhibitor of BAX function at mitochondria) have all been correlated with poor prognosis in patients with bladder cancer (34-36). Inhibition of MCL-1, C-FLIP, or XIAP expression can downregulate cell survival and enhance the antitumoral efficacy of

chemotherapeutic agents in bladder cancer (36-38). Our results here showed that amentoflavone significantly induces cytotoxicity and apoptosis in bladder cancer cells. Amentoflavone increased the expression of apoptotic proteins, FAS, FAS-L, and BAX, and diminished the expression of XIAP, MCL-1, and C-FLIP. Furthermore, extrinsic and intrinsic apoptotic signaling pathways, including FAS, FAS-L, caspase-8 activation, and loss of $\Delta\Psi m$ were significantly increased by amentoflavone treatment in TSGH-8301.

Overexpression of metastasis-associated proteins contributes to tumor metastasis, which is the leading cause of death in cancer patients (17,19). Both MMP-2 and MMP-9 have been shown to mediate cancer invasion and metastasis through the disruption of extracellular matrix (ECM) (39). Tumor cells attract blood vessels through the secretion of VEGF and the formation of new blood vessels that supports tumor growth and metastasis (40). The serine protease, uPA, promotes tumor invasion and metastasis by inducing the conversion of plasminogen to plasmin that also participates in ECM degradation (41). Overexpression of uPA, MMP-2, MMP-9, and VEGF has been associated with a poor outcome in patients with bladder cancer (41-43). Our results also demonstrated that protein levels of VEGF, MMP-2, MMP-9, and uPA were significantly inhibited when TSGH-8301 cells were treated with amentoflavone.

In conclusion, amentoflavone not only induces apoptosis through extrinsic and intrinsic pathways, but also inhibits the expression of anti-apoptotic and metastasis-associated proteins in TSGH-8301 cells. We suggest that amentoflavone may be used as a potential adjuvant to provide therapeutic efficacy for the treatment of bladder cancer.

Conflicts of Interest

The Authors declare that they have no conflicts of interest for this article.

Authors' Contribution

CC, IC and FH designed, performed the experiment and analyzed the data, CY and IC supervised the study, JC CC, IC and FH wrote the original manuscript and reviewed and edited the final version.

Acknowledgements

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