MSFTZ, a Flavanone Derivative, Induces Human Hepatoma Cell Apoptosis via a Reactive Oxygen Species- and Caspase-Dependent Mitochondrial Pathway

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most common malignancy of the liver. It is unfortunate that HCCs are highly refractory to conventional chemotherapy, radiation therapy, and even immunotherapy. Thus, novel therapeutic targets need to be sought for the successful treatment of HCCs. We now report that 5-(and-6)-carboxy-2SR,4SR)-2-(2-chloro-4-methylsulfonylphenyl)-4SR-chloro-3SR,4SR-diethoxy-flavane[4,3-d]-D1,9b-1,2,3-thiadiazoline (MSFTZ), a synthesized flavanone derivative, induced growth arrest and apoptosis of HCCs both in vitro and in vivo. MSFTZ induced a time- and dose-dependent increase in HCC apoptosis through caspase-3 activation and poly(ADP-ribose) polymerase-1 cleavage. Activation of caspase-9 induced by MSFTZ suggested that MSFTZ-induced signaling was mediated through a mitochondrial death pathway. In addition, we observed an elevation of reactive oxygen species (ROS) and a consequent loss of mitochondrial membrane potential, further suggesting that MSFTZ-induced death signaling was mediated through a mitochondrial oxygen stress pathway. These events were associated with a decrease and increase in Bcl-2 and Bax expression, respectively, as well as phosphorylation of mitogen-activated protein kinase (MAPK) and activation of p53-DM2 pathway. However, the antioxidant N-acetylcysteine opposed MSFTZ-mediated mitochondrial dys-function, caspase activation, Bcl-2/Bax modulation, and apoptosis, supporting the role of ROS in the apoptotic process. We were surprised that we failed to observe the protective effect of N-acetylcyisteine against MSFTZ-induced MAPK activation. Furthermore, MSFTZ had an antitumor effect in vivo by 34.8 to 78.7% reduction of tumor size in SMMC-7721-xenografted nude mice. We conclude that MSFTZ induces HCC cell apoptosis both in vivo and in vitro via caspase- and ROS-dependent mitochondrial pathway. In addition, MSFTZ has potential as a novel therapeutic agent for the treatment of HCC.

Hepatocellular carcinoma (HCC) is currently the fifth most common solid tumor worldwide and the fourth leading cause of cancer-related death, and its incidence is rising in many countries (El-Serag, 2004; Thomas and Zhu, 2005). Thus, novel therapeutic strategies are needed to combat this group of cancers that are resistant to systemic chemotherapies, radiation therapy, and even immunotherapy (Wirth et al., 2005). Thus, novel therapeutic strategies are needed to improve the efficacy in treating HCC.

Natural products derived from plants have recently received much attention as potential chemopreventive and chemotherapeutic agents. Flavanones (Fig. 1A), a subclass of flavonoids, are plant polyphenols found in vegetables, fruit, and beverages of plant origin that is well known for their physiological antipyretic, analgesic, and anti-inflammatory activities. The antitumor activity of flavonoids has recently been identified as an early stage. However, approximately 70% of patients are inoperable because of advanced tumor growth or liver cirrhosis, or, alternatively, the hepatoma belongs to the group of cancers that are resistant to systemic chemotherapies, radiation therapy, and even immunotherapy (Wirth et al., 2005). Thus, novel therapeutic strategies are needed to improve the efficacy in treating HCC.

ABBREVIATIONS: HCC, hepatocellular carcinoma; MSFTZ, (±)-3aRS,4SR)-2-(2-chloro-4-methylsulfonylphenyl)-4SR-chloro-3SR,4SR-diethoxy-flavane-[4,3-d]-D1,9b-1,2,3-thiadiazoline; ROS, reactive oxygen species; Δψm, mitochondrial membrane potential; MAPK, mitogen-activated protein kinase; DMSO, dimethyl sulfoxide; NAC, N-acetylcysteine; JC-1, 5,5’6,6’-tetrachloro-1,1’3,3’-tetraethylbenzimidazol-carbocyanine iodide; carboxy-DCFDA, 5-(and-6)-carboxy-2’7’-dichlorofluorescin diacetate; XIAP, X-linked inhibitor of apoptosis protein; PARP, poly(ADP-ribose) polymerase; ERK, extracellular signal-regulated kinase; p-, phosphorylated; T/C%, 100 x (mean RTV of treated group)/(mean RTV of control group); RTV, relative tumor volume; PI, propidium iodide; PBS, phosphate-buffered saline; AO, acridine orange; EB, ethidium bromide.
attracted much attention. Previous reports have demonstrated that flavanone possessed antiproliferative effects (Sonoda et al., 2004; Tokalov et al., 2004) and that it could induce apoptosis in hepatocellular carcinoma cells (Lee et al., 2002). One of the flavanone analogs, silibinin, has been shown to be highly effective and nontoxic in the prevention and intervention of various cancers in animals and humans (Varghese et al., 2005). 1,2,3-Thiadiazoline (Fig. 1B) is a pharmacophore, and some of its derivatives have antiproliferative activity relative to the promotion of apoptosis (Zhang et al., 2003). We hypothesized that introduction of 1,2,3-thiadiazoline might improve the flavanone-induced apoptotic effects, and we synthesized the compound (±)-(3aRS,4SR)-2-(2-chloro-4-methylsulfonylphenyl)-4'-chloro-3a,4-dithioxy-flavane[4,3-d]-D1,9b-1,2,3-thiadiazoline (MSFTZ) (Fig. 1C).

In our previous report (He et al., 2006), we have shown that MSFTZ inhibited leukemia cell proliferation and that it induced apoptosis in HL-60 leukemia cells in vitro. In the present study, MSFTZ exhibits antiproliferative activity in all three human hepatocellular carcinoma cell lines (SMMC-7721, Bel-7402, and HepG2). Therefore, we investigated both in vitro and in vivo anticancer activities and the mechanism of action of MSFTZ in SMMC-7721 cells, including mitochondrial dysfunction.

It is obvious that the mitochondrial dysfunction plays an important role in apoptosis (Desagher and Martinou, 2000; Kroemer and Reed, 2000; Wang, 2001). An increase of reactive oxygen species (ROS) (Kamata and Hirata, 1999; Ruffels et al., 2004) and a consequent loss of mitochondrial membrane potential (Δψm) (Rotem et al., 2005) were reported as typical phenomena in the process of apoptosis related to mitochondria (Woo et al., 2003). It is known that mitochondrial-mediated apoptosis is regulated by two major pathways. The death receptors on the cell surface can affect the mitochondria indirectly by regulation of MAPKs (Chang and Karin, 2001), and the convergence of the signaling at the mitochondria can influence the mitochondria directly by regulation of Bcl-2 protein family (Li et al., 2004). Thus, we have looked at the possibility that MSFTZ may play a role in mitochondrial dysfunction in HCCs, as well as downstream regulation at the postmitochondrial level.

Our results showed that MSFTZ is a potent agent against human hepatoma cells both in vitro and in vivo, and a caspase- and ROS-dependent mitochondrial pathway might be involved in signaling MSFTZ-induced apoptosis. Our data provide the molecular theoretical basis for clinical application of flavanone derivative in patients with HCC.

Materials and Methods

Drugs and Chemicals. MSFTZ was synthesized according to a previous report (He et al., 2006), and then it was dissolved in DMSO (20.0 μM stock solution) and stored at −20°C. The thiol antioxidant N-acetylcysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO). NAC was freshly dissolved in medium at a stock concentration of 40 mM. The pH adjusted to 7.4, and then the NAC was sterilized by 0.22-μm filtration and diluted to different concentrations. All the NAC treatments mentioned in this article are pretreatments. The mitochondrial fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carboxycyanine iodide (JC-1) and the 5-(and-6)-diborboxy-2′-7′-dichlorofluorescin diacetate (carboxy-DCFDA) were purchased from Invitrogen (Carlsbad, CA). Stock solutions of JC-1 (2.0 mg/ml) and carboxy-DCFDA (15.0 mM) were dissolved in DMSO, and then they were stored at −20°C. The primary antibodies to caspase-9, caspase-3, X-linked inhibitor of apoptosis protein (XIAP), PARP, ERK1/2, p38, p-ERK1/2, p-p38, Bel-2, Bax, p53, Mdm2, α-tubulin, and β-actin, and horseradish peroxidase-labeled secondary anti-goat, anti-mouse, and anti-rabbit antibodies, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence, a Western blot detection reagent, was purchased from Pierce Chemical (Rockford, IL).

Cell Lines. Bel-7402 and SMMC-7721 cells were maintained in RPMI 1640 medium (2 g/l glucose; Invitrogen), and HepG2 cells were maintained in HG-Dulbecco’s modified Eagle’s medium (4.5 g/l glucose). Invitrogen; both media were supplemented with 10% fetal calf serum (newborn calf serum; Invitrogen) plus 2 mM glutamine and 50 U/ml penicillin. All of the cell lines were purchased from the Institute of Cell Biology (Shanghai, People’s Republic of China), and they were grown at 37°C in a 5% CO2 atmosphere.

Cytotoxicity Assay. The cytotoxic activity of MSFTZ in three hepatoma cell lines was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Cell lines were seeded in 96-well microtiter plates (4000 cells/well). After treatment (1.25–40.0 μM MSFTZ for 72 h) in 96-well plates, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution (5.0 mg/ml in RPM 1640 medium; Sigma-Aldrich) was added (10.0 μl/well), and plates were incubated for a further 4 h at 37°C. The purple formazan crystals were dissolved in 100.0 μl of DMSO. After 5 min, the plates were read on an automated microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 570 nm. Assays were performed in triplicate on three independent experiments. The concentration of drug inhibiting 50% of cells (IC50) was calculated using the software of dose-effect analysis with microcomputers.

Measurement of in Vivo Activity. Tumors were established by injection of SMMC-7721 cells (5 × 106 cells/animal, s.c. into the armpit of the athymic mice) to 4- to 5-week-old BALB/c female athymic mice (National Rodent Laboratory Animal Resource, Shanghai Branch, People’s Republic of China). Treatments were initiated when tumors reached a mean group size of approximately 130 mm3. The tumor growth inhibition rate was calculated as (W2/mean RTV of control group)/(mean RTV of treated group)/(mean RTV of control group)/(mean RTV of treated group). The tumor growth inhibition rate was calculated by using the formula inhibition rate (%) = (1 - TW/TWc) × 100, where TWc and TWc were the mean tumor weight of control and control groups, respectively. Athymic mice was administrated i.p. with MSFTZ (10.0, 20.0, and 40.0 mg/kg) dissolved in ethanol/0.9% sterile sodium chloride solution (1:9, vol) once every 2 days. Mouse weight and tumor volume were recorded every 3 days until animals were sacrificed. Animal care was in accordance with institutional guidelines.

Flow Cytometric Analysis for Apoptosis. Annexin V-fluorescein isothiocyanate/PI is sensitive in detecting very early stages of apoptotic process, and it allows precise quantification of apoptotic cells through flow cytometric analysis. Exponentially growing SMMC-7721 cells were

Fig. 1. Chemical structures of flavanone (A), 1,2,3-thiadiazoline (B), and MSFTZ (C).
seeded at $4 \times 10^5$ cells/flask in 75-cm² flasks containing 10 ml of complete medium. The cells were cultured at 37°C in a 5% CO₂ atmosphere for 24 h before they were exposed to MSFTZ (0.0, 5.0, 10.0, and 20.0 μM) for 48 h. Cells were harvested, washed, and resuspended with phosphate-buffered saline (PBS). Fluorescein isothiocyanate-conjugated Annexin V and PI (both from BD Biosciences Pharmingen, San Diego, CA) were added at manufacturer's recommended concentrations to 0.1-ml aliquots containing 10⁶ cells. Cells were incubated for 15 min at 18°C in the dark. Cell suspensions were diluted with 0.4 ml of binding buffer, and then they were analyzed by flow cytometry within 1 h. Flow cytometry was performed on FACSScan (BD Biosciences, San Jose, CA), with collection and analysis of data performed using CellQuest software (BD Biosciences).

Acridine Orange/Ethidium Bromide Staining. AO/EB staining provides a reliable method to measure cells in different compartments (or pathways) of cell death, and it detects the morphological change of apoptotic cells directly. SMMC-7721 cells were plated in 96-well plates at a concentration of $5 \times 10^5$ cells/well in a final volume of 100 μl of complete medium. After treatments were completed, cell culture medium was removed, cells were washed with PBS twice, and 100 μl of the same buffer was added. Then, 4 μl of AO/EB solution (1 part 100 μg/ml AO in PBS and 1 part 100 μg/ml EB in PBS) was added. Samples were examined in a fluorescence microscope, and they were photographed using a digital color camera DFC 300 FX (Leica, Wetzlar, Germany).

JC-1 Stain for Δψm. The harvested cells ($2 \times 10^5$) were resuspended in 0.5 ml of complete medium containing 10.0 μg/ml JC-1 for 10 min at 37°C. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green ($525 \pm 10$ nm) to red ($610 \pm 10$ nm). Samples ($1 \times 10^4$ cells/sample) were analyzed by fluorescent microscope (Leica) and FACSCalibur using an argon laser (488 nm). Mitochondrial depolarization is specifically indicated by a decrease in the red-to-green fluorescence intensity ratio (Yang and Reynolds, 2005).

Detection of Intracellular ROS. The production of intracellular ROS was measured in the SMMC-7721 cell line using the oxidation-sensitive fluorescent dye carboxy-DCFDA. An increase in green fluorescence intensity is used to quantify the generation of intracellular ROS. After adding carboxy-DCFDA at a final concentration of 15.0 μM to the culture medium, the cells were incubated at 37°C for an additional 30 min, and then they were harvested, washed with PBS, and measured immediately by FACSCalibur using an argon laser at 488 nm and a 525-nm band pass filter (Ling et al., 2003).

Western Analysis. Proteins of SMMC-7721 cells were extracted in immunoprecipitation assay buffer [150 mM NaCl, 50 mM Tris, 2 mM ethylene glycol-bis(β-aminoethyl ether), 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.2% Triton X-100, 0.3% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride], and 40.0 to 80.0 μg of total protein was loaded per lane. Proteins were fractionated on 10 to 15% Tris-glycine precast gels, and then they were transferred to nitrocellulose membrane (Pierce Chemical) and probed with primary antibodies followed by horseradish peroxidase-labeled secondary antibodies. Proteins were visualized using enhanced chemiluminescence (Pierce Chemical).

Statistical Analysis. For all parameters measured, the values for all samples in different experimental conditions were averaged, and the SD of the mean was calculated. The significance of differences between the values of the groups was determined with unpaired Student's t test. Significance levels were set at $p < 0.05$.

Results

MSFTZ Caused Cytotoxicity and Apoptosis in Human Hepatoma Cells in Vitro. The antiproliferative activity of MSFTZ was determined in three human hepatoma cell lines (SMMC-7721, Bel-7402, and HepG2), and the dose-response curves are shown in Fig. 2A. All three cell lines exhibited dose-dependent sensitivity to MSFTZ (0–40.0 μM) at 72 h, with IC₅₀ values ranging from 4.3 to 7.7 μM. The results suggested that MSFTZ had promising antitumor activity.

The time and dose dependence for MSFTZ activation of apoptosis was measured by flow cytometry and by staining with the fluorescent probe AO/EB. The percentage of apoptotic cells (Annexin V⁻/PI⁻) in the control group was 4.7% (Fig. 2B). After treatment with 5.0, 10.0, and 20.0 μM MSFTZ for 48 h, the percentages of apoptotic cells were 4.5, 12.7, and 92.9%, respectively (Fig. 2B). The results of AO/EB staining also indicated MSFTZ-induced SMMC-7721 cells apoptosis in both dose-dependent and time-dependent manner, and features of typical apoptosis—cell shrinkage, blebbing, and chromatin condensation—were clearly observed (Fig. 2C). These results indicated that MSFTZ induced apoptosis, rather than necrosis, in SMMC-7721 cells.

Caspase Activation and PARP Cleavage in MSFTZ-Induced Apoptosis. Caspases, known as crucial mediators of apoptosis, depend on proteolytic activation of the procaspase forms to enzymatically active forms. When SMMC-
7721 cells were treated with 5.0, 10.0, and 20.0 μM MSFTZ for 24 h, significant proteolytic cleavage of procaspase-3 and procaspase-9 was detected using Western blotting (Fig. 3A). PARP is a 116-kDa protein, and it is cleaved by caspases during apoptosis to generate 89- and 23-kDa fragments. We analyzed PARP protein using an antibody able to detect the full-length 116 kDa and the characteristic apoptosis-related 85-kDa fragment. Treatment of the SMMC-7721 cells with 20.0 μM MSFTZ for 24 h induced cleaved PARP protein expression (Fig. 3B). XIAP is able to inhibit activation of caspase-3 to protect cells from apoptosis. As shown in Fig. 3B, MSFTZ decreased XIAP protein levels in a dose-dependent manner. These results suggest that activation of caspase-3, caspase-9, and cleavage of PARP are ultimately responsible for MSFTZ-induced apoptotic process in hepatoma cells.

**MSFTZ Induces Loss of Δψ_m and Increase of ROS.** To assess the role of mitochondria in MSFTZ-induced cell death, we tested whether MSFTZ caused a loss of Δψ_m using the JC-1 staining. A loss of Δψ_m is specifically indicated by a decrease in the ratio of red-to-green fluorescence intensity. Compared with the corresponding control, MSFTZ caused an obvious decrease of Δψ_m in SMMC-7721 cells in a time-dependent manner (Fig. 4A), and 89.6% cells presented low Δψ_m at 24-h incubation, whereas the value was 14.6% in control group (Fig. 4B).

It has also been reported that production of ROS contributes to mitochondrial damage that may facilitate the further release of ROS into the cytoplasm (Simon et al., 2000). To further address the possibility that the MSFTZ-induced apoptosis could be related to contributions from the mitochondrial pathway, SMMC-7721 cells were treated with 5.0, 10.0, and 20.0 μM MSFTZ for the indicated times, and the change in ROS production was examined using the specific fluorescence probe carboxy-DCFDA. A significant production of ROS for all concentrations was observed after 2-, 4-, and 6-h exposure period compared with untreated cells (Fig. 4C).

**Involvement of MAPK, Bcl-2 Family, and p53 Proteins in MSFTZ-Induced Apoptosis.** Because increased ROS production has been associated with activation of ROS levels in the control group were arbitrarily assigned a fluorescence value of 1. All data are mean ± S.D. (n = 3—5). *, p < 0.05 versus nontreated control.

**MAPK (Kulisz et al., 2002),** we next examined the effect of MSFTZ in MAPK. As shown in Fig. 5C, the phosphorylation of ERK1/2 and p38 was reduced and increased, respectively, after the MSFTZ treatment, but expression level of ERK1/2 and p38 did not change.

The proto-oncoprotein Bcl-2 is a powerful antagonist of the mitochondrial pathway of apoptosis initiated by a variety of extra- and intracellular stresses. The properties of Bcl-2 also extend to its ability to function as an antioxidant, exerting a particular buffering effect on mitochondrial ROS production (Agostinis, 2003). Therefore, we examined whether MSFTZ...
could alter the balance between proapoptotic Bax and antiapoptotic Bcl-2 proteins at the mitochondrial membrane. Exposure to MSFTZ (24 h; 5.0–20.0 μM) resulted in decrease of Bcl-2 and increase of Bax (Fig. 5, A and B), with an increase in Bax/Bcl-2 ratio (Fig. 5B).

It is reported that p53 results in apoptosis through a multistep process, including the generation of ROS (Ye et al., 1999), so we examined whether wild-type p53 protein is involved in MSFTZ-induced apoptosis. SMMC-7721 cells express wild-type p53 (Huang et al., 2000; Gong et al., 2007). After an exposure to MSFTZ, the wild-type p53 protein level was elevated in a dose-dependent manner. An MSFTZ-induced increase in wild-type p53 was not noticeable at 5.0 μM during the period of MSFTZ treatment (Fig. 5D). However, an increase in the wild-type p53 level was observed at 10.0 and 20.0 μM after a 24-h incubation of the cells with MSFTZ (Fig. 5D). Moreover, MSFTZ resulted in a rapid down-regulation of Mdm2 (Fig. 5D). Taken together, these results imply that MAPK, Bcl-2 family, and p53 proteins take part in MSFTZ-induced apoptotic process in hepatoma cells.

Effect of NAC on Cytotoxicity and Apoptosis Caused by MSFTZ. To further demonstrate whether ROS participates in MSFTZ-induced apoptosis, the antioxidant NAC was added into SMMC-7721 cells growing in the presence of MSFTZ. A concentration of 5.0 mM NAC showed a significant protective effect against cytotoxicity of MSFTZ (Fig. 6A). All concentration of NAC (5.0, 10.0, and 20.0 mM) were active in SMMC-7721 cells treated with MSFTZ concentration larger than 20.0 μM (Fig. 6A). However, no protective effect was observed with the concentration of NAC lower than 5.0 mM (data not showed). In addition, the protective effect was only partial because MSFTZ cytotoxicity was not completely prevented.

The treatment of the cells with 20.0 μM MSFTZ alone or with different concentrations of NAC for 72 h induced morphological alterations. The cells then seemed smaller, retracted, and rounded in MSFTZ alone group (Fig. 6C), whereas SMMC-7721 cells exposed to 20 μM MSFTZ in the presence of 5.0 or 10.0 mM NAC were similar in appearance to untreated cells (Fig. 6C). As showed in Fig. 6C, morphology of 20.0 mM NAC group was also altered, probably because the highest concentration of NAC induces cytotoxicity in SMMC-7721 cells (Fig. 6B).

Because NAC protected SMMC-7721 cells against cytotoxicity of MSFTZ, we investigated whether NAC inhibited MSFTZ-induced apoptosis. After a 48-h exposure to 20.0 μM MSFTZ and 5.0 and 10.0 mM NAC, the percentage of apoptotic cells was inhibited by 2.7 to 30.6% (Fig. 6B). NAC (5.0 mM) cotreatments significantly decreased the percentage of apoptotic cells from 90.3 to 59.7%. Taken together, these results suggest that NAC is an effective compound to inhibit MSFTZ-induced apoptosis and that ROS generation takes part in the MSFTZ-induced apoptotic process in hepatoma cells.

Effect of NAC on Mitochondrial Pathway and Caspase Activation Induced by MSFTZ. Because NAC could inhibit MSFTZ-induced apoptosis and the mitochondrial pathway take part in the MSFTZ-induced apoptotic process, we next examined whether NAC could inhibit the effect of MSFTZ on the proteins related to mitochondrial pathway. We pretreated SMMC-7721 cells with or without 5.0 mM NAC, and then we added 20.0 μM MSFTZ 8 h after administration of NAC. NAC blocked MSFTZ-induced decrease of Bcl-2 (Fig. 7B) and increase of Bax (Fig. 7C), and the Bax/Bcl-2 ratio was decreased from 3.0 to 0.8 (Fig. 7D). However, NAC had no effect on the MAPK pathway activated by MSFTZ (data not shown).

We next explored the protective effect of NAC against MAFZ-induced caspase activation. NAC could significantly inhibit the activation of caspase-3 and caspase-9, as well as cleavage of PARP (Fig. 7E). Indeed, NAC inhibited the decrease of XIAP induced by MSFTZ (Fig. 7E). These findings showed that NAC inhibited MSFTZ-induced apoptosis by maintaining the Bcl-2 level and reducing the Bax level, and at the postmitochondrial level by blocking caspase activation.

Antitumor Activity of MSFTZ in Nude Mice. The in vivo antiproliferation activity of MSFTZ was evaluated using human tumor models xenografted in athymic mice as described under Materials and Methods. MSFTZ inhibited tumor growth in a dose-dependent manner (Table 1). Compared with the control group, MSFTZ showed significant effect on tumor weight, and the inhibition rates caused by 10.0, 20.0, and 40.0 mg/kg MSFTZ was 34.8, 51.2, and 78.7%, respectively. MSFTZ also showed moderate-to-good therapeutic activity, and the T/C% was 47 and 21% in the groups treated with MSFTZ (20.0 and 40.0 mg/kg, respectively). Furthermore, there was no significant change on athymic mice body weight during the experiment (Table 1), which could be considered as the antineoplastic activity of MSFTZ taking precedence over the toxicity on athymic mice.

![Fig. 6. Effect of NAC on cytotoxicity and apoptosis caused by MSFTZ.](image-url)
that the antihepatoma effect on SMMC-7721 xenografted weight. On the basis of the previous analysis, it was possible to select one hepatoma cell line SMMC-7721 and evaluated the effects of MSFTZ in inhibiting tumor growth in a dose-dependent manner, without any significant influences on mouse body weight. The results indicated the potent antihepatoma activity of MSFTZ in inhibiting tumor growth in a dose-dependent manner, without any significant influences on mouse body weight. On the basis of the previous analysis, it was possible to consider the antihepatoma effect on SMMC-7721 xenografted athymic mice was due to MSFTZ-mediated apoptosis, and the results of the apoptosis assay were consistent with our hypothesis. Moreover, the results suggested that the MSFTZ treatment induces apoptosis via mitochondrial dysfunction.

Mitochondria play a pivotal role in the regulation of apoptosis induced by diverse death stimuli (Desagher and Martinou, 2000; Kroemer and Reed, 2000; Wang, 2001), and the changes of mitochondria in SMMC-7721 cells treated with MSFTZ were examined. The results showed an MSFTZ-mediated loss of Δψm, Δψm loss can induce the opening of permeability transition pores in mitochondria, and several apoptogenic factors are released from mitochondria to cytosol by apoptosis-inducing stimuli. For example, cytochrome c, once released, forms an apoptosome with apoptotic protease activating factor 1 and procaspase-9 in the presence of deoxyadenosine triphosphate, resulting in activation of caspase-9 (Liu et al., 1996; van Loo et al., 2002). The active subunit of caspase-9 further activates downstream caspase-3. Therefore, the observation of MSFTZ-mediated activation of procaspase-9, procaspase-3, and subsequent cleavage of the substrate of caspase-3, PARP (Naumovsk et al., 2005), suggested that mitochondrial-mediated caspase cascade pathway played a very important role in MSFTZ-induced apoptosis. Furthermore, the MSFTZ-caused down-regulation of XIAP, one of the inhibitors of apoptosis proteins family (Amantana et al., 2004; Devi, 2004; Naumovsk et al., 2005) was detected in SMMC-7721 cells. Because the XIAP is able to inhibit activation of caspase-3 to protect cells from apoptosis, the down-regulation of XIAP provided an additional documentation that MSFTZ-induced antitumor was related to activate caspase cascade.

ROS including H2O2 activates a variety of intracellular signaling cascades closely associated with both cell death and cell survival pathways (Kamata and Hirata, 1999; Ruffels et al., 2004). It has been proposed that ROS plays a role as a mediator of apoptosis (Woo et al., 2003). ROS was recently shown to induce apoptosis by regulating the phosphorylation and ubiquitination of Bcl-2 family proteins, resulting in increased proapoptotic protein levels and decreased antiapoptotic protein expression (Li et al., 2004). MAPKs, a protein family, related to death receptors on the cell surface, can indirectly affect mitochondrial function by interaction with Bcl-2 family and other factors. It was suggested that the increased ROS production in choline deficiency-induced apoptosis of rat hepatocytes might be related to dysfunction of the mitochondrial respiratory chain (Guo et al., 2004). A few natural products such as curcumin (Woo et al., 2003) and phycocyanin (Pardhasaradhi et al., 2003) have already been shown to generate ROS mediating their proapoptotic effects preceded by down-regulation of the antiapoptotic Bcl-2. As shown in Fig. 4C, ROS greatly increased in SMMC-7721 cells treated with 20.0 μM MSFTZ. Bax/Bcl-2 ratio in SMMC-7721 cells treated with MSFTZ was increased, indicating that the regulation of Bcl-2 protein family expression played an important role in MSFTZ-induced apoptosis. In addition, our results also exhibited that MSFTZ obviously up-regulated p-p38 and down-regulated p-ERK. In contrast, there was little change in the expressions of p38 and ERK1/2. All the results suggested that MSFTZ-induced apoptosis in hepatoma cells was related to ROS-dependent mitochondrial pathway.

NAC is a precursor of l-cysteine and the glutathione path-

Fig. 7. Effect of NAC on mitochondrial pathway and caspase activation induced by MSFTZ. SMMC-7721 cells, with or without pretreatment of 5.0 mM NAC, were incubated with 20.0 μM MSFTZ for 24 h, and whole-cell lysates were collected and immunoblotted with Bcl-2 and Bax (A) and caspase-9, caspase-3, PARP, and XIAP antibodies (E). Densitometric analyses of expression of Bcl-2 (B), Bax (C), and Bax/Bcl-2 ratio (D) are relative to the untreated control. Data are mean ± S.D. (n = 3). * p < 0.05 versus nontreated control.

Discussion

The lack of effective therapy for the treatment of HCC has recently led to the investigation and development of various immunological treatment strategies (Wirth et al., 2005). However, despite this overall response rates of 0 to 25% remain inadequate (Thomas and Zhu, 2005). These poor clinical responses, together with the toxicity associated with such treatment, have meant that identification of other less toxic biological molecules is imperative. It is unfortunate that few antineoplastic compounds have been identified in a recent specific search for agents active in this disease. In this respect, more and more researchers focused on the effects of flavonoids in cancer treatment. In the present study, MSFTZ, which was previously found to induce apoptosis in human leukemia cells in vitro, was clearly demonstrated to inhibit cell proliferation in all three human hepatoma cell lines, including SMMC-7721, Bel-7402, and HepG2. We further selected one hepatoma cell line SMMC-7721 and evaluated the effects of MSFTZ on SMMC-7721 xenografted athymic mice. The results indicated the potent antitumor activity of MSFTZ in inhibiting tumor growth in a dose-dependent manner, without any significant influences on mouse body weight. On the basis of the previous analysis, it was possible to consider the antihepatoma effect on SMMC-7721 xenografted athymic mice. On the basis of the previous analysis, it was possible to consider the antihepatoma effect on SMMC-7721 xenografted athymic mice. On the basis of the previous analysis, it was possible to consider the antihepatoma effect on SMMC-7721 xenografted athymic mice.
way and a scavenger of free radicals because it interacts with ROS (Zafarullah et al., 2003). The reactive thiol group on NAC may directly bind to and inactivate platinum agents, as has been shown with other thiols (Muldoon et al., 2001). Data suggesting that a direct effect of NAC on MSFTZ binding includes the data showing that NAC protected against MSFTZ-induced apoptosis only if NAC was present in the culture medium, and loss of antiapoptotic effect of NAC was observed if NAC was washed off the cells. NAC reduced dissipation of the mitochondrial membrane potential, caspase-9 activation, and apoptosis, indicating a role for hepatocarcinogen-induced ROS in human HepG2 cells (Panaretakis et al., 2001). Our results show that NAC can block MSFTZ-induced apoptosis at both the mitochondrial and postmitochondrial levels. Caspase-9 activation, which is considered an important indicator for activation of the mitochondrial pathway of apoptosis, was totally prevented by NAC. NAC inhibited MSFTZ-induced activation of caspase-3, PARP cleavage, and chromatin condensation (Supplemental Fig. 1), which are downstream events from both caspase-9 and -8. Total inhibition of caspase-9 activation suggests that the mitochondrial pathway is primarily involved in protective mechanisms involving NAC. Caspase-8 activation mediates apoptotic signaling via death receptor pathways. At the mitochondrial level, NAC maintained antiapoptotic Bcl-2 protein and reduced proapoptotic Bax protein at the mitochondrial membrane. Therefore, NAC changed the balance between proapoptotic Bax and antiapoptotic Bcl-2 proteins at the mitochondrial membrane in favor of an antiapoptotic state, as confirmed by protection against the MSFTZ-induced decrease in MMP.

Surprisingly, NAC did not protect cell against MSFTZ-induced MAPK pathway activation. However, it was reported that NAC inhibits activation of JNK, p38 mitogen-activated protein kinase, and nuclear factor-κB transcription factor activities regulating expression of numerous genes (De Flora et al., 2001; Hashimoto et al., 2001). NAC can also prevent apoptosis and promote cell survival by activating ERK pathways (Wung et al., 1999; Li et al., 2000). Our results suggested that the protective role of NAC against MSFTZ-induced cytotoxicity and apoptosis may be a subsequent event after MAPK activation. However, it was also reported that NAC inhibited p38 MAPK activation in HASMC, whereas there was no effect of NAC on the activation of JNK or ERK (Wuyts et al., 2003). Therefore, further work is required to characterize the mechanisms involved in the mitochondrial regulation of apoptosis.

### References

- Guo WX, Pye QN, Williamson KS, Stewart CA, Hensley KL, and Kastake Y (2004) Anticancer drug. A proposed mechanism of apoptosis induced by MSFTZ in hepatoma cells was shown in Fig. 8.

Our experiments demonstrated that MSFTZ is a potent compound against human hepatoma cells both in vitro and in vivo, and the caspase- and ROS-dependent mitochondrial pathway, mediated by Bcl-2 protein family and MAPKs, might be involved in signaling MSFTZ-induced apoptosis. The results indicated that MSFTZ could be a promising novel anticancer drug. A proposed mechanism of apoptosis induced by MSFTZ in hepatoma cells was shown in Fig. 8.

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Body Weight Start</th>
<th>Tumor Size Start</th>
<th>Tumor Size End</th>
<th>Tumor Weight Inhibition Rate T/C (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>14</td>
<td>16.75 ± 1.20</td>
<td>73.1 ± 22.0</td>
<td>0.66 ± 0.24</td>
</tr>
<tr>
<td>MSFTZ 10 mg/kg</td>
<td>7</td>
<td>7</td>
<td>16.60 ± 0.85</td>
<td>85.2 ± 34.4</td>
<td>0.43 ± 0.18*</td>
</tr>
<tr>
<td>MSFTZ 20 mg/kg</td>
<td>7</td>
<td>7</td>
<td>16.44 ± 1.30</td>
<td>64.6 ± 22.9</td>
<td>0.32 ± 0.09**</td>
</tr>
<tr>
<td>MSFTZ 40 mg/kg</td>
<td>7</td>
<td>7</td>
<td>15.71 ± 1.01</td>
<td>72.8 ± 29.8</td>
<td>0.14 ± 0.05***</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01
*** p < 0.001 vs. control.


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