Antileukemia activity of MSFTZ–a novel flavanone analog

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A newly synthesized flavanone derivative, (±)-(3a*RS*,4*SR*)-2-(2-chloro-4-methyl- sulfonylphenyl)-4′-chloro-3a, 4-diethoxy-flavane[4,3-d]-$\Delta^{1,9b}$-1,2,3-thiadiazoline (MSFTZ), was investigated for its antileukemia activity and molecular mechanisms. Cytotoxicity assay confirmed the high antiproliferative efficiency of MSFTZ in six leukemia cell lines (including two drug-resistant cell lines), with 50% inhibition of cell viability values ranging from 1.0 to 9.2 μmol/l. The results of flow cytometry assay showed that the percentage of apoptotic HL-60 cells was 68.3% after 48 h treatment with MSFTZ, suggesting that the activation of the apoptosis pathway was an anticancer property of MSFTZ. Furthermore, the protein changes related to apoptosis were investigated. Exposure of HL-60 cells to MSFTZ induced pro-caspase-9 and pro-caspase-3 cleavage, X-linked inhibitor of apoptosis protein and Bcl-X_L downregulation, and poly(ADP-ribose) polymerase degradation. Treatment of cells with MSFTZ resulted in a time-dependent reduction in phosphorylation (activation) of extracellular signal-regulated kinase 1/2 and an increase in phosphorylation (activation) of Jun N-terminal kinase. Taken together, our results demonstrated that activation of mitogen-activated protein kinase and apoptotic cascade is involved in MSFTZ-induced antileukemia activity. All data suggest that MSFTZ is a promising chemotherapy drug. ***Anti-Cancer Drugs*** **17:641–647 © 2006 Lippincott Williams & Wilkins.**

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Introduction

For most cancer patients, currently available therapies have been only temporarily successful because of resistance or unacceptable levels of toxicity. As for treatment of leukemia, although the administration of all-*trans*-retinoic acid for acute promyelocytic leukemia and Gleevec for chronic myeloid leukemia has greatly improved the clinical outcome, resistance and relapse still occur [1,2]. Therefore, it is important to develop alternative therapeutic agents with improved efficacy and tolerability, particularly ones that exploit the apoptotic pathway.

Flavanone (Fig. 1a) of flavonoids exists in plants. One of the flavanone analogs, silibinin, has been shown to be highly effective and non-toxic in the prevention and intervention of various cancers in animals and humans [3,4]. Previous reports have demonstrated that flavanone possessed of antiproliferative effects [5] and could induce apoptosis [6] in leukemia cell lines. 1,2,3-Thiadiazoline (Fig. 1b) is a pharmacophore and some of its derivatives have antiproliferative activity relative to the promotion of apoptosis [7]. We hypothesized that introduction of 1,2,3-thiadiazoline might improve the flavanone-induced apoptotic effects. Here, we synthesized a novel compound (±)-(3a*RS*,4*SR*)-2-(2-chloro-4-methylsulfonylphenyl)-4′-chloro-3a,4-diethoxy-flavane[4,3-d]-$\Delta^{1,9b}$-1,2,3-thiadiazoline (MSFTZ, Fig. 1c) and examined the cytotoxic effects and its mechanism on human promyelocytic leukemia cells HL-60, serving as a useful model for testing antileukemic or general antitumoral compounds [8]. Our results suggest that MSFTZ is able to activate mitogen-activated protein kinase (MAPK) and the caspase cascade, resulting in DNA fragmentation to exhibit its antileukemic activity.

Materials and methods

Reagents and antibodies

The primary antibodies to caspase-3, caspase-9, Bcl-2, Bax, Bcl-X_L, p-ERK1/2, p-JNK, p-p38, X-linked inhibitor of apoptosis protein (XIAP), poly(ADP-ribose) polymerase (PARP), α-tubulin and β-actin, and horseradish peroxidase-labeled secondary anti-goat, anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). ECL, a Western blot detection reagent, was purchased from Pierce Biotechnology (Rockford, Illinois, USA).

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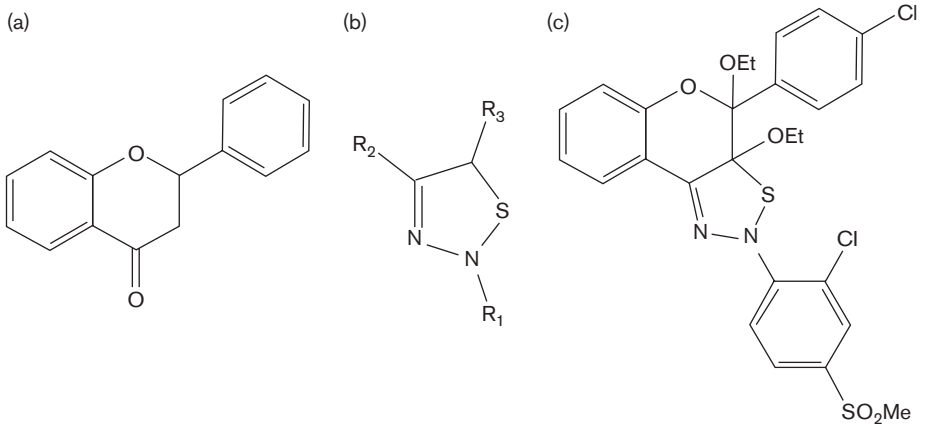
Fig. 1



The chemical structures of flavanone (a), 1,2,3-thiadiazoline (b) and MSFTZ (c).

Cell culture

The human leukemia cell lines (K562, HL-60 and Molt-4) were obtained from the American Type Culture Collection, and two drug-resistant cell lines (K562R and HL-60R) [9] and an acute promyelocytic leukemia line (NB4) were endowed by Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences). The K562R displayed 76.1-fold resistance to vincristine and HL-60R displayed 26.9-fold resistance to daunorubicin compared with their corresponding parental cells. The major mechanism of resistance of K562R is overexpression of mdr1 and P-gp [9]. We also found the overexpression of mdr1 and P-gp in HL-60R (data not shown). All cell lines were maintained in RPMI 1640 medium (Gibco, Grand Island, New York, USA) with 10% heat-inactivated fetal bovine serum (Gibco) at 37°C in 20% O_2.

Viability assay

The cytotoxic activity of MSFTZ on six leukemia cell lines was measured using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tertrazolium bromide assay. 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tertrazolium bromide solution (10.0 µl/well) in RPMI 1640 (Sigma, St Louis, Missouri, USA) was added after cells were treated with 0.5–16.0 µmol/l MSFTZ for 72 h and cells were incubated for a further 4 h at 37°C. The purple formazan crystals were dissolved in 100.0 µl dimethylsulfoxide. After 5 min, the plates were read on an automated microplate spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA) at 570 nm. Assays were performed in triplicate on three independent experiments. The concentration of MSFTZ required for 50% inhibition of cell viability (IC_{50}) was calculated using the software 'Dose–Effect Analysis with Microcomputers' (Elsevier–Biosoft, Cambridge, UK).

DNA fragmentation

HL-60 cells at a density of 2×10^5 cells/ml were treated with various concentrations of MSFTZ (0.8–3.2 µmol/l) for 24 h and then collected by centrifugation. The cell pellets were lysed in 200.0 µl lysis buffer [10.0 mmol/l ethylenediaminetetraacetic acid (EDTA); 50.0 mmol/l Tris–HCl, pH 8.0; 0.5% sodium lauryl sulfate; 100.0 µg/ml proteinase K] at 37°C for 12 h, then incubated with RNase (50.0 µg/ml) at 37°C for an additional 1 h. After incubation, DNA in the lysate was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), then with chloroform. DNA was precipitated with two volumes of ethanol in the presence of 0.3 mol/l sodium acetate. After centrifugation at 12 000 *g* for 15 min, the DNA pellets were washed with 70% ethanol, air-dried, and resuspended in 20.0 µl TE (10.0 mmol/l Tris–HCl and 1.0 mmol/l EDTA, pH 8.0). DNA was separated on 1.5% agarose gels containing 0.5 µg/ml ethidium bromide and photographed by Bio-Rad GD2000 (Bio-Rad, Hercules, California, USA) [10].

Flow cytometric analysis of apoptosis

For flow cytometry analysis of DNA content, HL-60 cells in exponential growth were treated with graded concentrations of MSFTZ (0.8–3.2 µmol/l) for 48 h. Cells were washed twice with phosphate-buffered saline and fixed in 70% ethanol at −20°C. The cell pellet was resuspended in 100.0 µl of phosphate-buffered saline containing 50.0 µg/ml RNase (Amersco, Solon, Ohio, USA) and then incubated at 37°C for 1 h. After incubation, the cells were stained with 200.0 µg/ml propidium iodide (Sigma) at 4°C for 30 min. The fluorescence of 10^4 cells was measured with FACSCalibur (Becton Dickinson, Lincoln Park, New Jersey, USA) [11].

Western blot analysis

HL-60 cells in exponential growth were treated with 3.2 μmol/l MSFTZ for 0, 6, 12 and 24 h. Protein extracted from HL-60 cells treated with 0.2 μmol/l podophyllotoxin for 24 h was used as positive control in the detection of MAPK proteins. Cells were lysed at a density of 1×10^6 per 30 μl in protein lysis buffer containing 50 mmol/l Tris–HCl, 150 mmol/l NaCl, 2 mmol/l EDTA, 2 mmol/l ethylene glycol-bis(*b*-aminoethyl ether), 25 mmol/l NaF, 25 mmol/l β-glycerophosphate, 0.1 mmol/l Na Vanadate, 5 μg/ml leupeptin, 0.1 mmol/l phenylmethylsulfonyl fluoride, 0.2% Triton-100 and 0.3% NP-40. The lysed solution was centrifuged at 10 000 *g* for 30 min at 4°C. Supernatants were then loaded onto a 10 or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. After electrophoresis, proteins were transferred to polyvinylidine diflouride membrane (Millipore, Bedford, Massachusetts, USA) followed by immunoblotting. Signals were detected using ECL.

Statistical analysis

Assays were set up in triplicates and the results were expressed as the mean ± SD. Statistical analysis and *P*-value determinations were performed by two-tailed paired *t*-test. $P < 0.05$ was considered to be significant.

Results

Antiproliferative effect of MSFTZ

The antileukemia activity of MSFTZ was determined on six human leukemia cell lines, including HL-60, K562, Molt-4 and NB4 cell lines, and two drug-resistant cell lines, HL-60R and K562R. As shown in Table 1, MSFTZ has antileukemia activity on all six cell lines and the IC_{50} value of MSFTZ for all six leukemia cells is below 10.0 μmol/l in a dose-dependent manner. The results suggest that MSFTZ has promising antileukemia activity.

Apoptosis of HL-60 cells induced by MSFTZ

To confirm whether cell death was caused by apoptotic processes, we assessed the DNA fragmentation in the cells exposed to MSFTZ (0.8, 1.6 and 3.2 μmol/l) for 24 h. A DNA ladder induced by MSFTZ (1.6 and 3.2 μmol/l) on an agarose gel was observed (Fig. 2). By flow cytometry, the percentages of apoptotic cells were 3.9, 25.3, and 68.3% after 0.8, 1.6 and 3.2 μmol/l MSFTZ treatment for 48 h, respectively (Fig. 3). The induction of the DNA ladder and the increased sub-G_0/G_1 contents indicate that MSFTZ is able to induce apoptosis in leukemia cells.

Activation of the caspase cascade during MSFTZ-induced apoptosis

To characterize the molecular events involved in MSFTZ-induced apoptosis, we first examined whether this apoptosis involved the activation of a caspase cascade. As shown in Fig. 4(a), the degradation of procaspase-3 was observed at 12 and 24 h, whereas an increase of cleaved caspase-9 was observed as early as 6 h after treatment with 3.2 μmol/l MSFTZ. Consistently, MSFTZ cleaved the 116-kDa PARP, with the accumulation of the 89-kDa fragment in HL-60 cells. To determine whether the activity of caspase-3 was associated with the expression levels of caspase inhibitors in MSFTZ-induced apoptosis, the expression of XIAP protein in HL-60 cells exposed to MSFTZ was measured. MSFTZ treatment for 12 h caused a decrease in the level of XIAP expression.

Modulation of Bcl-2 protein families in MSFTZ-induced apoptosis

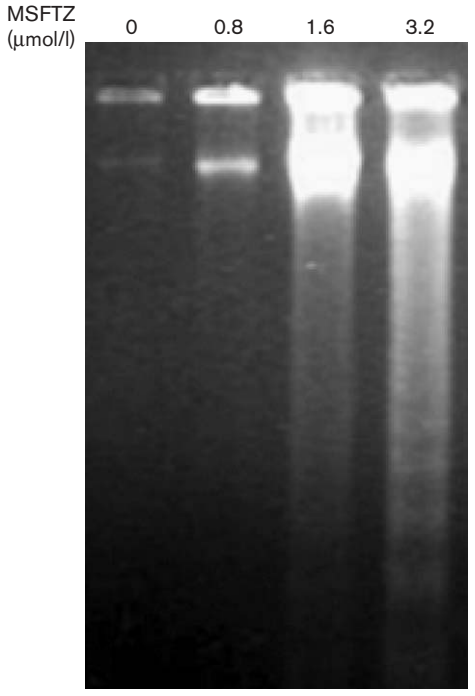
We also examined whether MSFTZ induced cell death by modulating the expression of Bcl-2 family members.

Table 1 Cytotoxicity of MSFTZ on human leukemia cell lines

Leukemia origin	Cell line	IC_{50} (μmol/l)
Acute promyelocytic leukemia	NB4	1.58 ± 0.23
Acute lymphoblastic leukemia	Molt-4	1.00 ± 0.02
Chronic myelocytic leukemia	K562	2.95 ± 1.02
Chronic myelocytic leukemia (76.1-fold resistance to vincristine)	K562R	9.27 ± 3.49
Acute promyelocytic leukemia	HL-60	1.61 ± 0.45
Acute promyelocytic leukemia (26.9-fold resistance to daunorubicin)	HL-60R	2.72 ± 0.51

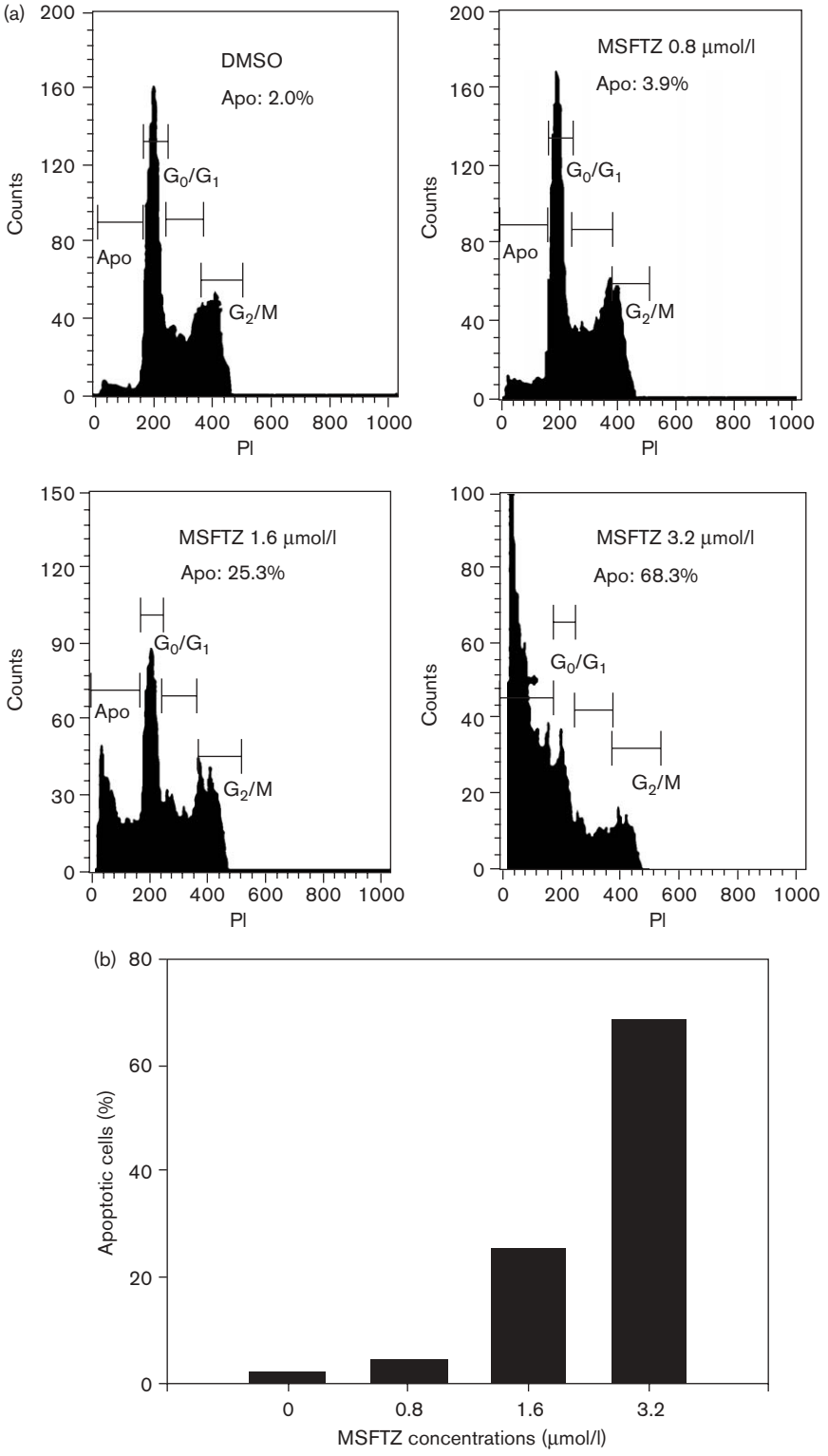
IC_{50}, 50% inhibition of cell viability.

Fig. 2



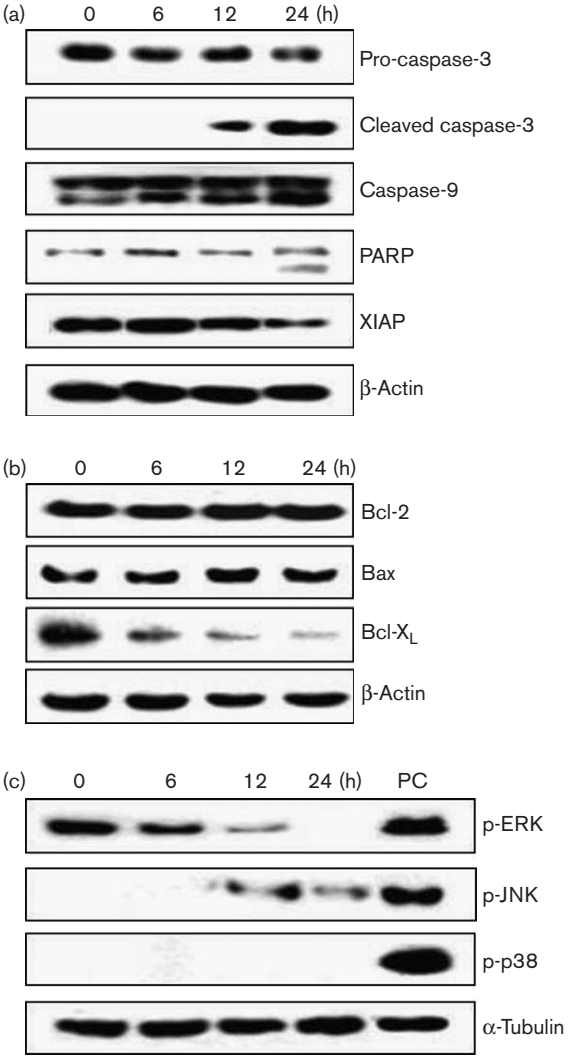
DNA fragmentation induced by MSFTZ in HL-60 cells. Cells (1×10^6/ml) were treated with indicated concentration of MSFTZ for 24 h. Equal amounts of genomic DNA (10 mg) were analyzed by 1.5% agarose gel electrophoresis and DNA laddering was visualized by staining the gel with ethidium bromide.

Fig. 3



Apoptosis induced by MSFTZ in HL-60 cells. (a) HL-60 cells were treated with MSFTZ (0.8, 1.6 and 3.2 μmol/l) for 48 h, and the DNA content analyzed by flow cytometry. (b) Analysis of the population of apoptotic cells in HL-60 cells by flow cytometry. PI, propidium iodide; DMSO, dimethylsulfoxide.

Fig. 4



Protein expression in HL-60 cells. HL-60 cells were incubated with 3.2 μmol/l MSFTZ. Protein extracts were prepared at 0, 6, 12 and 24 h, and analyzed on Western blots probed with specific antibodies to ascertain protein expression. (a) Activation of the caspase cascade. The cleavage of pro-caspase-9, and pro-caspase-3 and PARP was analyzed. In addition, the level of XIAP protein was determined. (b) Modulation of Bcl-2 protein families in MSFTZ-induced apoptosis. (c) Activation of mitogen-activated protein kinase by MSFTZ treatment. Protein extracted from HL-60 cells (treated with 0.2 μmol/l podophyllotoxin for 24 h) was used as positive control (PC). PARP, poly(ADP-ribose) polymerase; JNK, Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; XIAP, X-linked inhibitor of apoptosis protein.

MSFTZ obviously decreased the protein expression of Bcl-X_L, but not Bcl-2 or Bax in HL-60 cells (Fig. 4b).

Involvement of mitogen-activated protein kinases activation in MSFTZ-induced apoptosis

Recent studies have suggested that apoptotic stimuli are transmitted to caspases through the activation of MAPKs [12–14]. Therefore, we tested whether MAPK activation is involved in MSFTZ-induced apoptosis. As phosphorylation was the activated form of MAPK [15,16], we only determined the levels of phosphorylation of MAPK. In the present experiments, podophyllotoxin (0.2 μmol/l for 24 h) was used as a positive control [17]. Treatment of MSFTZ in HL-60 cells resulted in a decrease in the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in a time-dependent manner (Fig. 4c). In contrast, phosphorylation of Jun N-terminal kinase (JNK) was increased, which was easily detectable at 12 and 24 h. Detectable activation of P-p38 was not observed in HL-60 cells treated with MSFTZ, but was seen in HL-60 cells exposed to podophyllotoxin.

Discussion

In the present study, the cytotoxicity analysis *in vitro* indicates that MSFTZ is able to inhibit cell proliferation on all six leukemia cell lines in a time-dependent and dose-dependent manner. The most important finding is that, unlike vincristine and daunorubicin, there is no significant difference in antiproliferative effect of MSFTZ between drug-resistant cell lines and their parental cell lines.

MSFTZ was found to inhibit the growth of HL-60 cells by apoptosis, including an increase in the sub-G_1 population, caspase activation, DNA fragmentation and PARP cleavage. Apoptosis is an important response to most chemotherapeutic agents in leukemia cells [18,19]. It is a highly regulated process that involves the activation of a series of molecular events leading to cell death that is characterized by chromatin condensation, hypodiploid cells and apoptotic bodies that are associated with DNA cleavage into ladders [20]. Apoptosis involves two main death pathways: the receptor-mediated and mitochondria-mediated pathways [21]. The two pathways converge at caspase-3 activation. After caspase-3 activation, some specific substrates for caspase-3 such as PARP and D4-GDI proteins are cleaved, and these are important for the occurrence of apoptosis [20]. XIAP, the most potent member of the IAP gene family [22], can bind to and inhibit the processed caspase-9, caspase-7 and caspase-3, and it can also promote caspase degradation [23]. Therefore, the findings of MSFTZ-mediated activation of pro-caspase-3 and subsequent cleavage of its substrate PARP suggested that MSFTZ induced apoptosis via activation of the caspase cascade, which was also confirmed by the downregulation of XIAP and upregulation of cleaved caspase-9.

Bcl-2 family proteins play a pivotal role in controlling cell life and death. Three subfamilies of Bcl-2 protein have been identified in the apoptotic response. The Bcl-2 subfamily (e.g. Bcl-2 and Bcl-X_L) functions to inhibit apoptosis, whereas the Bax subfamily (e.g. Bax, Bak and Bcl-Xs) and the BH3-only subfamily (e.g. Bid and Bad)

promote apoptosis [24]. Bcl-X_L has been shown to be a stronger protector against apoptosis than Bcl-2 under certain circumstances [25,26]. Low levels of Bcl-X_L expression correlated with a greater tendency to undergo apoptosis [27]. The obvious downregulation of Bcl-X_L and no change of Bcl-2 and Bax expression in the present study indicate that the regulation of Bcl-X_L protein expression plays a key role in MSFTZ-induced apoptosis.

In addition to a continuously expanding family of proapoptotic and antiapoptotic proteins, apoptosis is also regulated by multiple signal transduction cascades, of which the MAPK pathways have received considerable attention. The MAPK family consists of a superfamily of three parallel signal transduction modules converging on the serine/threonine kinases JNK, ERK and p38 [28]. Although exceptions exist, the bulk of evidence suggests that p44/42 promotes cell survival, whereas p38 and JNK facilitate cell death [29]. Increased ERK activity has been observed in a variety of leukemias, including acute myeloid leukemia and chronic myeloid leukemia [30,31]. Inhibition of this pathway enhances apoptosis induction by a variety of agents [32–36]. Several reports have suggested that the JNK pathway is activated by various chemotherapeutic agents and its function may also be required for the induction of apoptosis by certain agents used in the treatment of acute myeloid leukemia [37,38]. Our data showed that MSFTZ resulted in a downregulation of ERK1/2 activation and an upregulation of JNK activation in a time-dependent manner, indicating that MSFTZ may induce apoptosis via inhibition of ERK1/2 and activation of JNK. The role of p38 in apoptosis is a matter of debate. Accumulating evidence suggests that the involvement of p38 MAPK in apoptosis is both cell-type and stimulus dependent [39]. Although p38 MAPK was phosphorylated by podophyllotoxin and has been reported to be activated by resveratrol [40], MSFTZ did not activate p38 MAPK in HL-60 cells in our study. This result implies that p38 MAPK is not involved in MSFTZ-induced apoptosis.

In conclusion, our data demonstrate that MSFTZ, a newly synthesized flavanone derivative, inhibits leukemia cells proliferation and induces apoptosis in HL-60 leukemia cells. The results suggest that MSFTZ may be used as a promising chemotherapy agent for leukemia and that the MAPK pathways have critical roles in MSFTZ-induced apoptosis in HL-60 cells.

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