Safrole Induces Apoptosis in Human Oral Cancer HSC-3 Cells

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Running title: Safrole-induced apoptosis in HSC-3 cells

KEY WORDS: Safrole; oral cancer HSC-3 cells; apoptosis; caspase cascades; xenograft model

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ABSTRACT

Phytochemicals have been used as potential chemopreventive or chemotherapeutic agents however, there are data suggesting a mutagenic effect of some phytochemicals. We hypothesized that safrole would have anticancer effects on human oral squamous cell carcinoma HSC-3 cells. Safrole decreased the percentage of viable HSC-3 cells which was *via* induction of apoptosis by increasing the level of cytosolic Ca^{2+}, reducing the mitochondrial membrane potential ($\Delta\Psi_m$). Changes in the membrane potential were associated with changes in the Bax, release of cytochrome *c* from mitochondria and activation of down-stream caspase-9 and -3, resulting in apoptotic cell death. *In vivo* studies also showed that safrole reduced the size and volume of an HSC-3 solid tumor on a xenograft nu/nu mouse model. Western blotting and flow cytometric analyses studies confirmed that safrole-mediated apoptotic cell death of HSC-3 cells is regulated by cytosolic Ca^{2+}, and mitochondria- and Fas-dependent pathways.

INTRODUCTION

Tobacco and alcohol consumption are major contributors to oral cancer (Franco *et al.*, 1989; Schlecht *et al.*, 1999). In addition, diets low in carotenoids and vitamin A, poor oral hygiene and indoor air pollution are also reported to be associated with oral cancer (Franceschi *et al.*, 1991; Pintos *et al.*, 1998; Velly *et al.*, 1998). In Taiwan, betel quid chewing is a significant factor associated with oral cancer. In Taiwan, 9.6 individuals per 100,000 died of oral cancer in 2008 and it is the fourth most frequent cause of cancer death among of males in Taiwan (Department of Health). Conventional treatments for patients with oral cancer are surgery, radiotherapy and chemotherapy (Ichimiya *et al.*, 2005), but the cure rates are not satisfactory.

In Taiwan, the composition of betel quid consists of areca nut, slaked lime and *Piper betle* inflorescence or leaf instead of tobacco (Chen *et al.*, 1999). It was reported that chewing betel quid with tobacco or combining it with cigarette smoking are recognized as a major risk factor for development of oral squamous cell carcinoma (OSCC) (Ko *et al.*, 1995). Chewing betel quid containing *piper betle* inflorescence can generate a high concentration of safrole (420 μM) in the saliva (Wang and Hwang, 1993). The International Agency for Research on Cancer (IARC) categorized safrole as a group 2B carcinogen (safrole is a documented rodent carcinogen). There are however, no adequate studies elucidating the relationship between exposure to safrole and human cancers (IARC monographs on the evaluation of the carcinogenic risk of chemicals to man: some naturally occurring substances, 1976). Carcinogenicity of safrole has typically been found to be caused by safrole–DNA adduct formation (Chen *et al.*, 1999; Daimon *et al.*, 1997; 1998; Liu *et al.*, 2004).

In human oral cancer cells, safrole induced a $[Ca^{2+}]i$ rise by causing release of stored Ca^{2+} from the endoplasmic reticulum in a phospholipase C- and protein kinase

C-independent fashion and by inducing Ca^{2+} influx *via* nifedipine-sensitive Ca^{2+} entry (Huang *et al.*, 2005). It was reported that safrole caused marked [Ca^{2+}]i elevation and decreased cell viability in human osteosarcoma cells (Lin *et al.*, 2006). Safrole can bind to DNA, resulting in safrole-DNA adduct formation (Daimon *et al.*, 1997; 1998; Lee *et al.*, 2005), but there is no information on whether safrole can reduce cell viability and induce apoptosis in human oral cancer cells. The HSC-3 cells, a human oral squamous cell carcinoma cell line, exists high metastatic and migrated potential (Kawahara *et al.*, 1999; Shigeta *et al.*, 2008). Therefore, in the present study we demonstrate for the first time that safrole induced apoptosis in HSC-3 cells through mitochondria-dependent pathway, and it inhibited tumor of HSC-3 cells in a mouse xenograft model.

MATERIALS & METHODS

Cell Culture

The human oral squamous cell carcinoma HSC-3 cell line was provided by Professor Pei-Jung Lu (National Cheng Kung University). Cells were plated in 75 cm^2 tissue culture flasks at 37°C under a humidified 5% CO_2 and 95% air atmosphere in DMEM/F-12 (1:1) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin as described elsewhere (Lin *et al.*, 2007).

In Vitro Studies

Morphological Changes and Cell Viability

Cells (2×10^5 per well) were placed in 12-well plates, and then safrole (Sigma Chemical Co., St. Louis, MO, USA) was added to each well at final concentrations of 10, 25, 50, 75 and 100 μM. DMSO, 0.2% in media served as a vehicle control. Cells were incubated for 24 and 48 h. Cell viability was determined by propidium iodide (PI, Sigma Chemical Co.) exclusion and flow cytometric procedure as previously described (Lin *et al.*, 2007). For morphological changes, cells in each well were examined for 24 and 48 h and photographed under a phase-contrast microscope (Chen *et al.*, 2009; Tsou *et al.*, 2009).

4',6'-Diamidino-2-Phenylindole Hydrochloride (DAPI) Staining and Comet Assay

Cells (2×10^5 per well) in 12-well plates were treated with 0 and 75 μM of safrole for 24 h. Cells were then individually stained by DAPI (Molecular Probes/Invitrogen Corp., Eugene, OR, USA) and photographed under fluorescence microscopy as described elsewhere (Chiu *et al.*, 2009; Yang *et al.*, 2009). The levels of DNA damage were determined using the Comet assay as previously described (Lu *et al.*, 2009).

Flow Cytometric Assays for Apoptotic Cells, Levels of $\Delta\Psi_m$ and Cytosolic Ca^{2+}

Cells (2×10^5 per well) were placed in 12-well plates, and then safrole was added to each well at final concentrations of 0, 25, 50, 75 and 100 μM for 24 h. The cells were stained with PI and were analyzed by flow cytometry. Formation of cells in the sub-G1 phase was indicative of apoptotic cells (Chen *et al.*, 2009). Cells were exposed to safrole at 75 μM for 0, 1, 3, 6, 12 or 24 h to determine the level of $\Delta\Psi_m$

and the cytosolic Ca^{2+}. Cells were harvested and suspended in 500 μl of $DiOC_6(3)$ (4 μmole/l) and Fluo-3/AM (2.5 μg/ml) (Molecular Probes) for the levels of $\Delta\Psi_m$ and cytosolic Ca^{2+}, respectively in a dark room for 30 min at 37°C, and then were analyzed by flow cytometry as previously described (Kuo *et al.*, 2009; Lin *et al.*, 2009b).

Assessment of Caspase-3, -8 and -9 Activity and Determination of Viability of HSC-3 Cells after Inhibitors of Caspase-3 and -8 Pre-treatment

Cells (2×10^5 per well) in 12-well plates were pre-treated with or without caspase inhibitors (Z-IETD-FMK for caspase-8 or Z-DEVD-FMK for caspase-3) and then were incubated with safrole at 75 μM for 0, 6, 12, 18 and 24 h to determine the caspase-3, -8 and -9 activity or viability of HSC-3 cells as described elsewhere (Ji *et al.*, 2009). The cells were in a 10 μM substrate solution (PhiPhiLux®-G_1D_2, CaspaLux®8-L_1D_2, CaspaLux®9-M_1D_2 kits, OncoImmunin, Inc. Gaithersburg, MD, USA) and incubated at 37°C for 60 min. The cells were washed again and then analyzed by flow cytometery (Lin *et al.*, 2009b).

Western Blotting for Protein Levels Analyses

Cells (1×10^6 per dish) were treated with 75 μM safrole and incubated for 0, 6, 12 and 24 h. Abundance was determined of selective proteins associated with apoptosis by Western blotting analysis. The levels of apoptotic relative proteins were determined in cell lysates using antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). To ensure equal protein loading, each membrane was stripped and reprobed with anti-β-actin antibody (Lin *et al.*, 2009a).

Assay for mRNA Levels of Caspase-3, -8, -9 and Endo G

Cells (1×10^6 per dish) were incubated with 75 μM safrole for 0 and 24 h mRNAs of caspase-3, -8 , -9 and Endo G were determined. Total RNA was extracted, RNA samples was reverse-transcribed and then quantitative PCR was performed with the forward and reverse primers (Appendix Table 1) as described elsewhere (Ho *et al.*, 2009a; Ji *et al.*, 2009).

In Vivo Studies

Mouse Xenograft Model

Twenty six-week-old male BALB/c athymic nude mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were housed a regular 12-hour light/12-hour dark cycle. Water and food were autoclaved and provided *ad libitum*. HSC-3 cells (1×10^7/100 μl) in PBS were subcutaneously (*s.c.*) injected into the flanks of mice. Animals bearing tumors were randomly assigned to a control or safrole treatment group (ten mice per group). Treatment was initiated when xenografts reached a volume of about 200 mm^3 and mice were then injected *i.p.* every four day (in the morning) with 30 μl of control vehicle (DMSO) or safrole (15 mg/kg). When mice started to exhibit tumors then were monitored, counted, and tumor sizes were measured initially after 2 weeks, with the final measurement taken 5 weeks after tumor cell inoculation. At the end of the study (5 weeks after cell inoculation), animals were sacrificed. For each animal, tumors were removed, measured and weighted (Ho *et al.*, 2009b; Yang *et al.*, 2008). All animal studies were conducted according to institutional guidelines (Affidavit of Approval of Animal Use Protocol)

approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan).

Statistical Analyses

Difference between treatment groups were calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. Data are presented as mean ± standard deviation (S.D.) and the results are representative of at least two or three independent experiments. A p value of <0.05 was considered significant.

RESULTS

Safrole Induces Morphological Changes, Viability, Apoptosis and DNA Damage

Safrole induced morphological changes (Fig. 1A) and decreased the percentage of viable cells in a dose-and time-dependent manner with an IC_{50} value of about 75 μM after 48 h-treatment (Fig. 1B). Compared with DMSO-treated controls, each safrole treatment resulted in the formation of sub-G1 in cells. The data showed that safrole induced apoptosis (sub-G1 phase) in a dose-dependent manner (Fig. 1E). DAPI staining assay confirmed chromatin condensation (an apoptotic characteristic) occurring in HSC-3 cells and the effects were dose-dependent (Fig. 1C and D). Comet assay also demonstrated that safrole induced DNA damage in HSC-3 cells (Fig. 1C and D).

Safrole Alters $\Delta\Psi_m$ and Cytosolic Ca^{2+} Levels

The results showed that safrole significantly decreased the levels of $\Delta\Psi_m$ in HSC-3 cells in a time-dependent manner (Fig. 2A). Safrole also significantly increased cytosolic Ca^{2+} levels (Fig. 2B).

Safrole Stimulates the Activity of Caspase-3, -8 and -9

Safrole significantly increased the activity of caspase-3, -8 and -9 (Fig. 2C, D and E). These effects were time-dependent with the exception of caspase-9 which after a 18 h incubation there was a reduction in activity compared with the 12 h-treatment (Fig. 2E). HSC-3 cells were pre-treated with inhibitors (Z-IETD-FMK for caspase-8 or Z-DEVD-FMK for caspase-3) and then exposed to safrole led to promote the percentage of viable cells when compared with safrole-treatment only (Fig. 2F).

Safrole Affects Abundance of Apoptotic Associated Proteins, Protein Translocation and mRNA Expression

Figure 3A shows that safrole increased levels of pro-apoptotic proteins such as Bax and Bid (Fig. 3B) as were the active forms of caspase-9 and -3 and PARP (Fig. 3B). The levels of Fas L, Fas and caspase-8 protein (Fig. 3A) were also up-regulated but earlier as compared with the aforementioned proteins. Expression levels of mRNA of caspase-3, -8 and -9 and Endo G were increased (Fig. 3C). The results from confocal laser microscopy shown in Appendix Figure 1 indicated that safrole promoted the release of cytochrome *c* and Endo G from the mitochondria to the cytosol and nuclei, respectively.

Safrole Inhibits Tumor Size of HSC-3 Cells in a Mouse Xenograft Model

Safrole significantly decreased the tumor weight compared to control (Fig. 4C) and the percentage of inhibition of tumors is shown in Figure 4D. Safrole induced a 40% inhibition of tumors compared with control treatment. Comparison of tumor volume between the control and safrole treatment groups showed that 15 mg/kg safrole significantly reduced the occurrence of tumors compared with control mice (Fig. 4E). Tumors in the treatment groups were significantly smaller than those in the control group. The final tumor size of mice treated with 15 mg/kg safrole were smaller than the tumor size of control mice after cell inoculation (treated for 20 days) with safrole.

DISCUSSION

Several studies have shown that safrole is a carcinogen and that it can induce carcinogenesis in animals (Chen *et al.*, 1999; Daimon *et al.*, 1997; 1998; Liu *et al.*, 2004). However, there is no report to show safrole induced apoptosis in any human cancer cell lines, therefore, the purpose of the present study was to examine effects of safrole in human oral cancer cells *in vitro* and its effects on solid tumor in a mouse xenograft model. We found that safrole can induce apoptosis and reduce viability in human oral squamous cell carcinoma HSC-3 cells and it also can inhibit the tumor size of oral cancer in a mouse xenograft model *in vivo*. Safrole also promoted the activity of caspase-3, -8 and -9. It is well-known that the activation of these caspases is a major mechanism which promotes apoptosis in response to death-inducing signals from cell surface receptors and mitochondrial stress (Eeva *et al.*, 2009). HSC-3 cells were pretreated with the inhibitors of caspase-8 and -3 (Z-IETD-FMK and

Z-DEVD-FMK, respectively) then were treated with safrole, the results showed that the activities of caspase-8 and -3 were inhibited and the percentage of viable HSC-3 cells were increased. Apparently, safrole-induced apoptosis is involved in a caspase-dependent pathway. We also observed that safrole promoted cytosolic Ca^{2+} release and decreased the levels of $\Delta\Psi_m$. The importance of mitochondrial changes induced by safrole is that mitochondria act as a nodal point for execution of apoptosis and the permeability transition pore opening and collapse of the $\Delta\Psi_m$ leads to a rapid release of cytochrome *c* into the cytoplasm and activation of caspase-3 *via* caspase-9, culminating in cell death (Robertson and Orrenius, 2000; Zou *et al.*, 1999). In order to examine whether or not safrole inhibited apoptotic associated protein levels are also affected gene expression of mRNA, cells after exposure to safrole were isolated total RNA and then were examined by real-time PCR and the results indicated safrole also activated mRNA expression of caspase-3, -8 and -9 and Endo G in examined HSC-3 cells.

There is not any information to show safrole inhibits HSC-3 tumor in mouse xenograft model. Therefore, for the *in vivo* effect of safrole on HSC-3 cells in a mouse xenograft model were undertook. Here, in the present study, we provide the first *in vivo* evidence to show safrole decrease tumor size and weight of HSC-3 tumor in mouse xenograft *in vivo*. A novel finding of the present study was that safrole inhibited tumor growth in a xenograft mice model. Tumors in mice that received safrole alone were about 40% smaller than these of the control group, and tumors that received safrole treatment continued to grow slowly. Importantly, the growth inhibitory effect of safrole on HSC-3 xenografts *in vivo* was consistent with the results obtained *in vitro*. The *in vitro* and *in vivo* findings in the present study provide initial support for further investigation in the use of safrole in treatment of oral cancer.

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Figure legends

Figure 1. Safrole affects on cell morphology, percentage of viable cells, apoptotic cells and DNA damage in HSC-3 cells. Cells were cultured in DMEM/F-12 (1:1) + 10% FBS with various concentrations of safrole for 24 and 48 h. The cells were examined and photographed by phase-contrast microscopy (100x) for morphological changes (A), percentage of viable cells (B), DAPI staining (a and c) and DNA damage (b and d) (C), quantification of fluorescence intensity (folds of difference between control and safrole treatment) and comet tail (% of difference between control and safrole treatment) (D) and apoptosis (E) were determined as described in Materials and Methods. a and b: control; c and d: 75 μM safrole. Each point is mean ± S.D. of three experiments. a, $p < 0.05$, significantly different compared with DMSO-treated control; b, c, d and e, $p < 0.05$, significantly different compared with 10, 25, 50 and 75 μM of safrole treatment, respectively by one-way ANOVA followed by Bonferroni's multiple comparison test. *$p < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA.

Figure 2. Safrole alters the levels of mitochondria membrane potential ($\Delta\Psi_m$), cytosolic Ca^{2+} and stimulates caspase cascade activity in HSC-3 cells. Cells were treated with 75 μM safrole for indicated time of intervals, and stained with $DiOC_6(3)$ and the level of $\Delta\Psi_m$ determined (A), stained by Fluo-3/AM and the level of cytosolic Ca^{2+} release were calculated (B). Cells were pre-treated with inhibitors (Z-IETD-FMK for caspase-8 or Z-DEVD-FMK for caspase-3), and then were treated with 75 μM safrole for 0, 6, 12, 18 and 24 h. The cells were determined for caspase-3 (C), -8 (D) and -9 (E) activity and the percentage of viability (F) as described in Materials and Methods. Columns, mean (n= 3); bars, S.D. a, $p < 0.05$, significantly different compared with DMSO-treated control; b, c and d, $p < 0.05$, significantly

different compared with 6, 12 and 24 h-treatment of 75 μM safrole, respectively by one-way ANOVA followed by Bonferroni's multiple comparison test.

Figure 3. Representative Western blotting and real-time PCR analyses show changes in the levels of associated proteins and genes in the apoptotic HSC-3 cells after exposure to safrole. Cells were treated with 75 μM safrole for 0, 6, 12 or 24 h before the total proteins and mRNA were prepared and determined, as described in Materials and Methods. The levels of apoptotic relative proteins (A: Fas L, Fas and caspase-8; B: Bax, Bid, caspase-9, caspase-3, PARP) were estimated by Western blotting analysis. For real-time PCR assay, cells after exposure to 75 μM safrole for 24 h were isolated total RNA, and then determined the gene expression of mRNA *caspase-3, -8* and *-9,* and *Endo G* (D) were performed as described in Materials and Methods. $*p < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA.

Figure 4. Representative tumor on the xenograft animal model and antitumor activity safrole against HSC-3 oral squamous cell carcinoma tumors. Twenty nude mice were *s.c.* implanted with HSC-3 cells (1×10^7 per mice) for 14 days, and then randomly divided into 2 groups. Group 1 was treated with DMSO only. Group 2 were treated with 15 mg/kg safrole, and then 34th day all animal were sacrificed. During the treatment, each animal will be measure tumor size and weight as described in Materials and methods. (A): representative animal with tumor; (B) representative solid tumor weight; (C) quantification of tumor weight; (D) representative tumor and rate of inhibition (%). The tumor size observed in DMSO-treated control and safrole groups (E) were compared as analyzed by one-way ANOVA. A p value of < 0.05 was considered statistically significant.

Figure 1

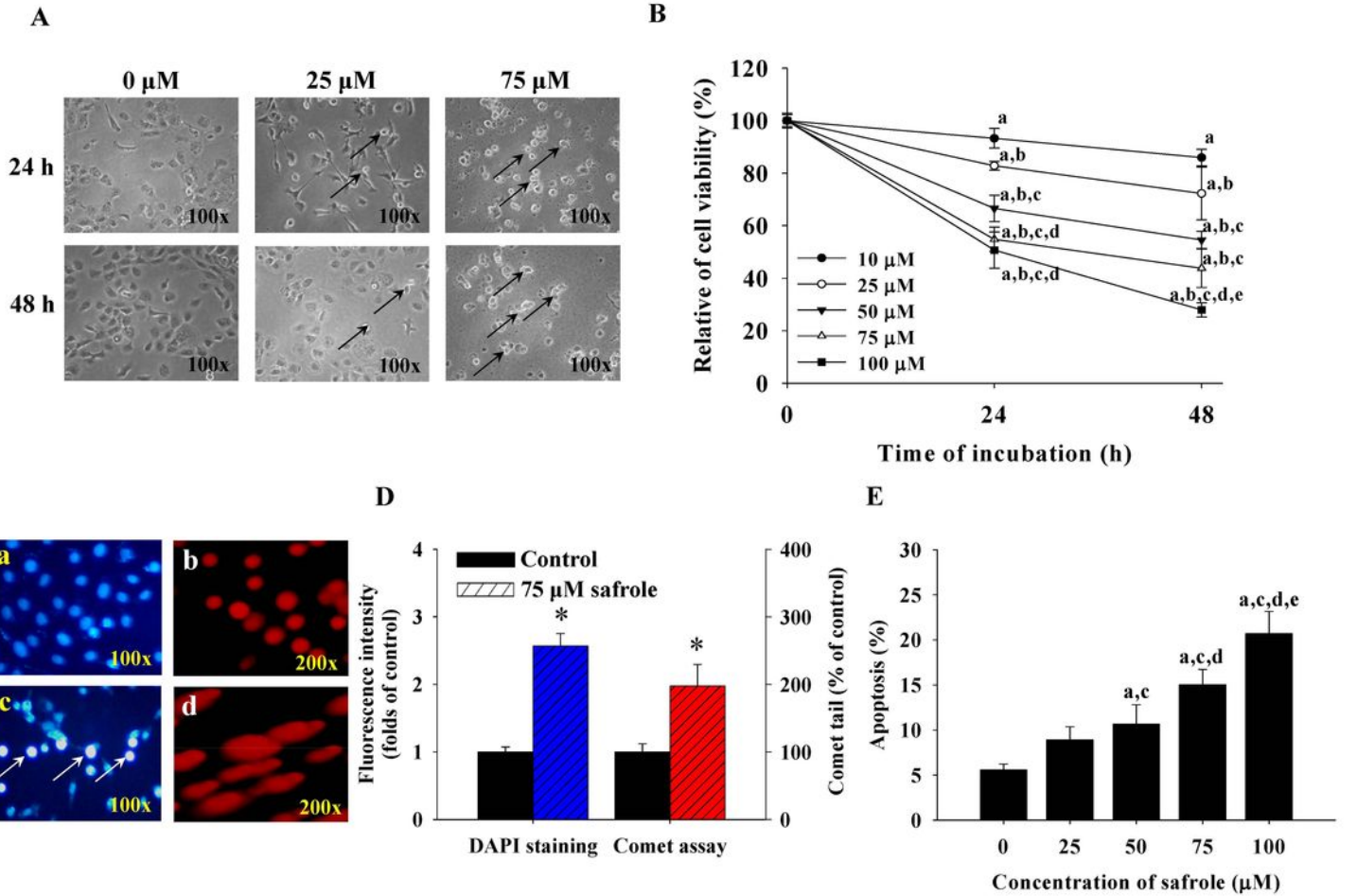

A
0 μM
25 μM
75 μM
24 h
48 h
100x
B
Relative of cell viability (%)
Time of incubation (h)
10 μM
25 μM
50 μM
75 μM
100 μM
a
a,b
a,b,c
a,b,c,d
a,b,c,d,e
C
a
b
c
d
100x
200x
D
Control
75 μM safrole
Fluorescence intensity (folds of control)
Comet tail (% of control)
DAPI staining
Comet assay
E
Apoptosis (%)
Concentration of safrole (μM)
a,c
a,c,d
a,c,d,e

Figure 2

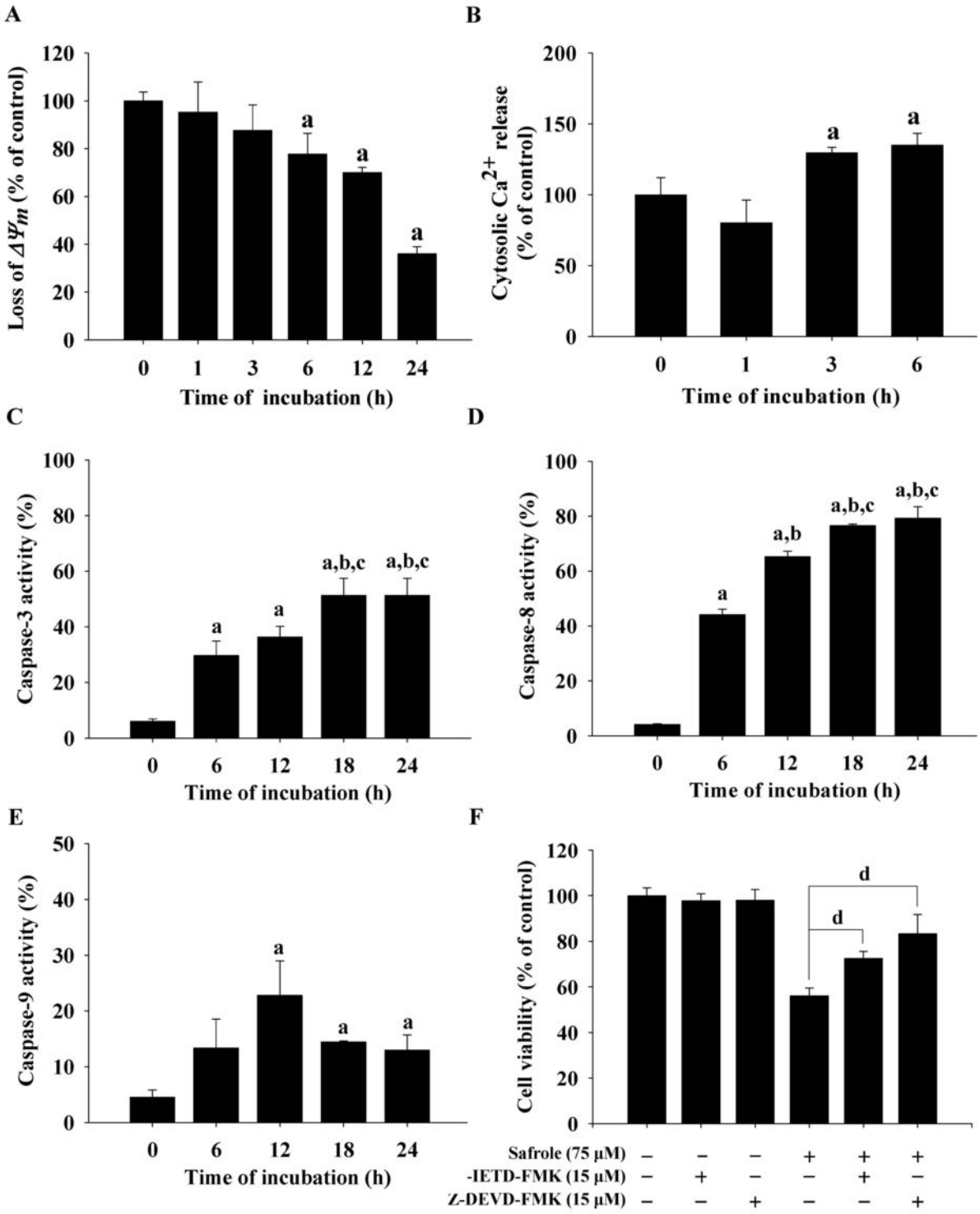

A
Loss of ΔΨm (% of control)
120
100
80
60
40
20
0
a
a
a
0
1
3
6
12
24
Time of incubation (h)
B
Cytosolic Ca2+ release (% of control)
200
150
100
50
0
a
a
0
1
3
6
Time of incubation (h)
C
Caspase-3 activity (%)
100
80
60
40
20
0
a
a
a,b,c
a,b,c
0
6
12
18
24
Time of incubation (h)
D
Caspase-8 activity (%)
100
80
60
40
20
0
a
a,b
a,b,c
a,b,c
0
6
12
18
24
Time of incubation (h)
E
Caspase-9 activity (%)
50
40
30
20
10
0
a
a
a
0
6
12
18
24
Time of incubation (h)
F
Cell viability (% of control)
120
100
80
60
40
20
0
d
d
Safrole (75 μM) – – – + + +
-IETD-FMK (15 μM) – + – – + –
Z-DEVD-FMK (15 μM) – – + – – +

Figure 3

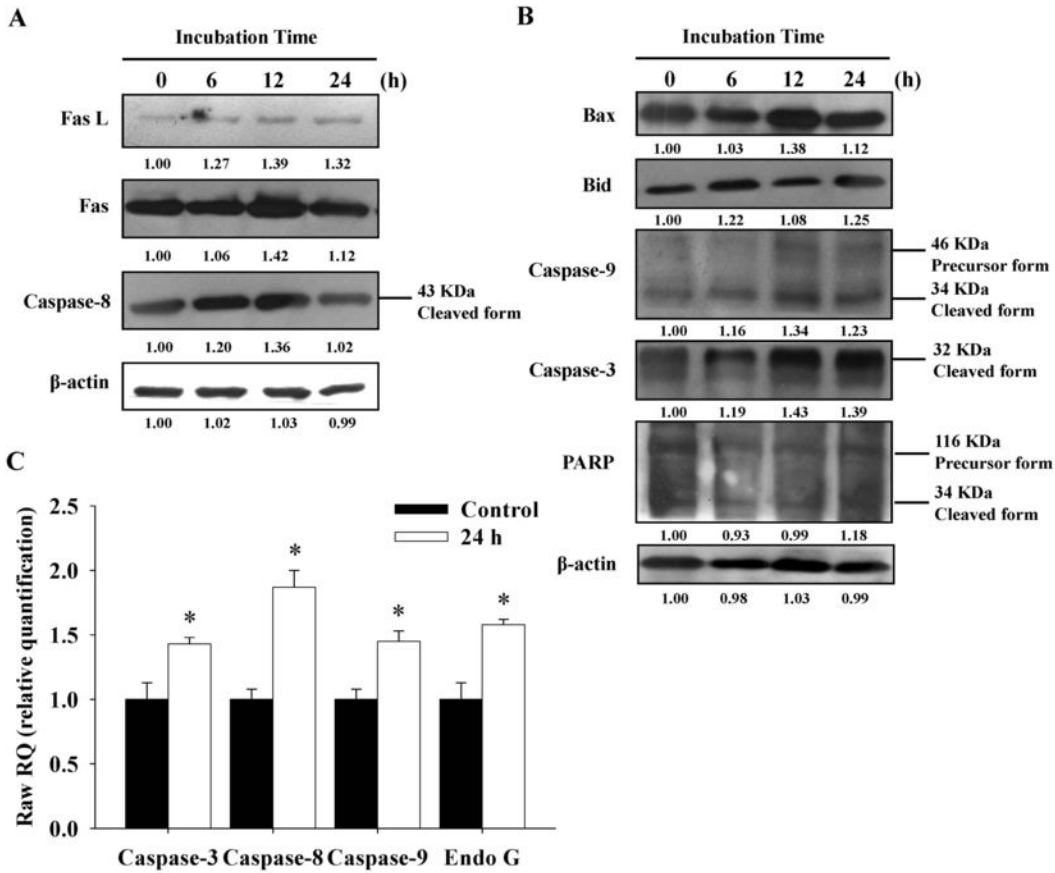
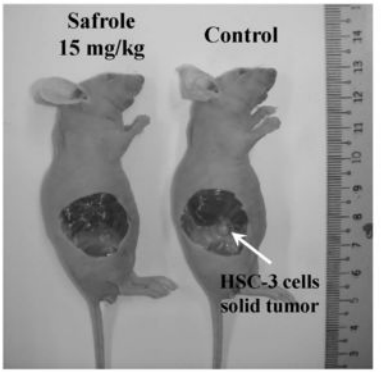
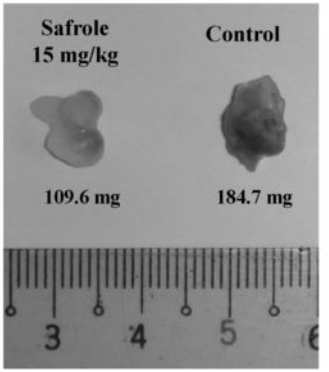

A
Incubation Time
0 6 12 24 (h)
Fas L
1.00 1.27 1.39 1.32
Fas
1.00 1.06 1.42 1.12
Caspase-8
43 KDa
Cleaved form
1.00 1.20 1.36 1.02
β-actin
1.00 1.02 1.03 0.99
B
Incubation Time
0 6 12 24 (h)
Bax
1.00 1.03 1.38 1.12
Bid
1.00 1.22 1.08 1.25
Caspase-9
46 KDa
Precursor form
34 KDa
Cleaved form
1.00 1.16 1.34 1.23
Caspase-3
32 KDa
Cleaved form
1.00 1.19 1.43 1.39
PARP
116 KDa
Precursor form
34 KDa
Cleaved form
1.00 0.93 0.99 1.18
β-actin
1.00 0.98 1.03 0.99
C
Control
24 h
Raw RQ (relative quantification)
2.5
2.0
1.5
1.0
0.5
0.0
Caspase-3 Caspase-8 Caspase-9 Endo G

Figure 4

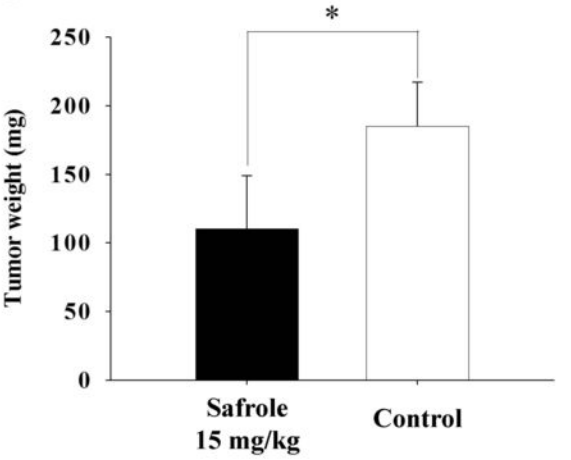
A



B



C



D

Treatment Dosage	Tumor weight (mg)	Rate of inhibition (%)
Control	185.5±32.2	---
Safrole 15 mg/kg	110.3±38.9 *	40.54

E