Swainsonine promotes apoptosis in human oesophageal squamous cell carcinoma cells *in vitro* and *in vivo* through activation of mitochondrial pathway

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Swainsonine, a natural indolizidine alkaloid, has been reported to have antitumour effects, and can induce apoptosis in human gastric and lung cancer cells. In the present study, we evaluated the antitumour effects of swainsonine on several oesophageal squamous cell carcinoma cells and investigated relative molecular mechanisms. Swainsonine treatment inhibited the growth of Eca-109, TE-1 and TE-10 cells in a concentration-dependent manner as measured by MTT assay. Morphological observation, DNA laddering detection and flow cytometry analysis demonstrated that swainsonine treatment induced Eca-109 cell apoptosis *in vitro*. Further results showed that swainsonine treatment up-regulated Bax, down-regulated Bcl-2 expression, triggered Bax translocation to mitochondria, destructed mitochondria integrity and activated mitochondria-mediated apoptotic pathway, followed by the release of cytochrome c, which in turn activated caspase-9 and caspase-3, promoted the cleavage of PARP, resulting in Eca-109 cell apoptosis. Moreover, swainsonine treatment inhibited Bcl-2 expression, promoted Bax translocation, cytochrome c release and caspase-3 activation in xenograft tumour cells, resulting in a significant decrease of tumour volume and tumour weight in the swainsonine-treated xenograft mice groups compared with that in the control group. Taken together, this study demonstrated that swainsonine inhibited Eca-109 cells growth through activation of mitochondria-mediated caspase-dependent pathway.

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1. Introduction

Human oesophageal carcinoma is one of the most aggressive malignancies and the sixth leading cause of death from cancer (Enzinger and Mayer 2003). In Asia and Europe, oesophageal squamous cell carcinoma is the dominant subtype of oesophageal carcinoma (Ekman *et al.* 2008). Despite significant advances in surgical resection, radiotherapy and chemotherapy since the 1980s, the overall 5-year survival rate remains at about 10% (Ekman *et al.* 2008). Therefore, research and exploration of new chemotherapeutic drugs against oesophageal carcinoma is necessary.

Swainsonine (1, 2, 8-trihyroxyindolizidine), an alkaloid isolated from locoweed, has shown potential antitumour properties. Swainsonine not only suppresses the growth of human melanoma, colon, gastric and lung cancer cells, as well as C6 glioma cells *in vitro* and *in vivo*, but also inhibits murine B16F10 melanoma and MDAY-D2 lymphoid tumour cells in syngeneic mice (Dennis *et al.* 1989a, b, 1990; Li *et al.* 2012; Sun *et al.* 2007, 2009). Clinical trials

Keywords. Apoptosis; caspase; Eca-109 cells; mitochondrial pathway; swainsonine

Abbreviations used: AIF, apoptosis inducing factor; Apaf-1, apoptotic protease activating factor 1; COX4, cytochrome c oxidase IV; $\Delta\psi_m$, mitochondrial membrane potential; MTT, 3-(4,5-Dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; Smac, second mitochondrial activator of caspases; PARP, poly(ADP-ribose) polymerase; PVDF, polyvinylidene difluouride

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with swainsonine, as an antitumour agent, have shown obviously curative effects with good tolerance in the patients with advanced malignancies (Goss *et al.* 1994, 1997). As for the mechanisms of swainsonine antitumour, some studies show that swainsonine inhibits the growth of human gastric carcinoma SGC-7901 cells and rat C6 glioma cells *in vitro* via induction of apoptosis involving in Ca^{2+} overloading and Bcl-2 decrease (Sun *et al.* 2007, 2009). Our previous study shows that swainsonine can induce apoptosis in several human lung cancer cell lines including A549, Calu3, SPC-A-1 and H1299, and that swainsonine induces apoptosis in A549 cells through activation of mitochondrial pathway both *in vitro* and *in vivo*. These data suggest that swainsonine might serve as an apoptotic inducer in some types of cancer cells. Till date, however, the effects of swainsonine on oesophageal squamous cell carcinoma cells have not yet been examined. In the present study, we measured the effects of swainsonine on the growth of human oesophageal squamous cell carcinoma Eca-109 cells *in vitro* and *in vivo*, and investigated possible mechanisms involved in this process. The results suggested that human oesophageal squamous cell carcinoma cells were less sensitive to swainsonine as compared to adenocarcinoma cells, and that swainsonine induced apoptosis in oesophageal carcinoma Eca-109 cells through activation of a signal pathway similar to that of human lung cancer cells. This find may be favourable for the further expansion of clinical application of swainsonine.

2. Materials and methods

2.1 *Reagents*

Swainsonine was isolated from *Oxytropis kansuensis* Bunge (a kind of locoweed widely distributed in the west of China) and identified by interpretation of spectral data (1H NMR, 13C NMR and EI-MS) as described previously (Tong *et al.* 2008). Its purity was evaluated to be above 99%. The compound was dissolved in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS), at 10 mg/mL as stock solution, sterilized by ultrafiltration, and stored at −20°C. Mouse monoclonal antibodies against caspase-9, cytochrome c, Bcl-2, Bax, AIF, Smac, Apaf-1, COX4, poly (ADP-ribose) polymerase (PARP) and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, US). Horseradish peroxidase-conjugated secondary antibody was purchased from Wuhan Boster Bio-Engineering Co., Ltd. (Wuhan, China). 3-(4,5-Dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), z-VAD-fmk, z-DEVD-fmk, z-LEHD-fmk and z-IETD-fmk were all purchased from Sigma-Aldrich (St. Louis, MO, US). All of other chemicals and reagents were the highest quality and obtained from standard commercial sources.

2.2 *Cells culture and treatment*

Human oesophageal squamous cell carcinoma cell lines Eca-109, TE-1 and TE-10 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% new born bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C in a 5% CO_2 atmosphere incubator. Cells were treated with swainsonine from a prepared stock solution in PBS, added to the culture medium to obtain final concentrations indicated as in each experiment. Equivalent volume of PBS was used as vehicle.

2.3 *Electron microscopy observation*

The ultrastructural morphology changes were observed under a transmission electron microscope. After swainsonine treatment, the cells were fixed with 4% glutaraldehyde, and post-fixed with 1% OsO4. Then samples were dehydrated in graded ethanol solutions, followed by embedment and section. Ultra-thin sections were stained with uranyl acetate and lead citrate, and then observed under a transmission electron microscope (JEM-1230, Tokyo, Japan) at 60 kV.

2.4 *Flow cytometry analysis*

Annexin V-FITC/PI apoptosis detection kit (Becton-Dickinson, US) was used for apoptosis detection as described previously (Huang *et al.* 2011a). Cells were washed twice with PBS and re-suspended in 100 μL 1× Annextin V binding buffer containing 5 μL Annexin V-FITC and 5 μL PI. After incubation in the dark for 10 min at room temperature, cells were diluted with 400 μL 1× Annextin V binding buffer and analysed by flow cytometry (Beckman Coulter, Inc. Fullerton, CA. US).

2.5 *Caspase activity assay*

Caspases activities were measured by colorimetric assay kits (BioVision, Inc., Mountain View, California, US) according to the manufacture's recommendations. Briefly, cells from each group were lysed and protein concentrations were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, US). 200 μg of protein samples were incubated with each caspase substrate, respectively, at 37°C in a microplate for 4 h. Samples were then read at 405 nm in microplate spectrophotometer (BioTek Instruments, Inc., Winooski, US).

2.6 *Mitochondrial transmembrane potential ($\Delta\psi m$) assessment*

The transmembrane potential $\Delta\psi_m$ was analysed using a JC-1 Mitochondrial Potential Detection Kit (Biotium Inc.,

Hayward, CA, US). Eca-109 cells treated with 0, 2.5, 5 and 10 μg/mL of swainsonine for 48 h were harvested and stained by JC-1 in PBS for 15 min at room temperature in the dark, followed by flow cytometric analysis.

2.7 *Immunoblot analysis*

After swainsonine treatment, the proteins were prepared and separated in 8% or 12% SDS-PAGE as described previously (Huang *et al.* 2010). Samples were then electrotransferred to polyvinylidene difluouride (PVDF) membrane (Millipore Corp, Atlanta, GA, US). After the blocking step, the blots were probed with specific antibodies and then visualized with appropriate HRP-conjugated secondary antibody and an enhanced cheniluminescence (ECL) detection system (Pierce, Rockford, IL, US).

2.8 *Real-time RT-PCR analysis*

Total RNA was isolated from swainsonine-treated cells and reverse-transcribed with MMLV reverse transcriptase and oligo $(dT)_{18}$ primers. Quantitation of genes coding for Bcl-2 and Bax were performed using SYBR Premix Ex TaqTM II kit (Takara, Dalian, China) by Bio-Rad iQ 5 Real Time PCR System. The β-actin gene was used as an endogenous control. The primers sequence was: TTGAGGAAGTGAACATTTCGGTG (forward), AGGTTCTGCGGACTTCG GTC (reverse) for Bcl-2; GCGAGTGTCTCAAGCGCATC (forward), CCAGTTGAAGTTGCCGTCAGAA (reverse) for Bax; AGTTGCGTTACACCCTTTCTTG (forward), TCACCTT- CACCGTTCCAGTTT (reverse) for β-actin. The gene expression fold changes were calculated using cycle time (Ct) values as described previously (Livak and Schmittgen 2001; Huang *et al.* 2011b).

2.9 *Antitumour activity in vivo*

Female congenital athymic BALB/c nude (nu/nu) mice were purchased from Shanghai National Center for Laboratory Animals (Shanghai, China) and maintained in pathogen-free conditions. Exponentially growing Eca-109 cells suspended in serum-free medium were injected subcutaneously into the back of the 4-week-old mice (1×10^7 cells in 100 μL). After tumour transplantation for 1 week, mice were divided randomly into three groups ($n=6$) and orally administrated PBS (control group), 1 mg/kg/day or 2.5 mg/kg/day doses of swainsonine in PBS in a 0.2 mL volume, respectively, for 5 weeks. These doses are chosen as safe and effective in mice model according to the previous report (Dennis *et al.* 1990). The length (a) and width (b) of tumour were measured regularly by a caliper and the tumour volumes were then calculated according to the formula $\left[\left(a\times b^2\right)/2\right]$. At the termination of the experiment, xenograft tumours were excised and weighed to record wet tumour weight. A portion of the tumours from control and treated animals were used for histologic study, and that the rest were used for preparation of tumour lysate used in further experiments. The animal experiments were performed in accordance with the 'Guidelines for Animal Experi-mentation' of the Forth Military Medicine University.

2.10 *TUNEL assay*

Xenograft tumours were fixed in 4% paraformal-dehyde, embedded in paraffin and cut into 6 μm sections. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was conducted to study DNA fragmentation using the in situ cell death detection kit (Beyotime Inst. Biotech, Beijing, China) according to the manufacturer's instructions. After mounting the TUNEL positive cells, the sections were observed at ×400 magnification under a Nikon microscope (Nikon Inc., Japan).

2.11 *Immunohistochemistry analysis the expression of Bax and Bcl-2*

Fresh tumour tissue sections were incubated with primary antibody against Bax (1:100) or Bcl-2 (1:100) monoclonal antibodies (Santa Cruz Biotechnology, US), and biotinylated secondary antibody. The immunoreactivity was visualized with a SABC kit according to its protocol (Maixin Biological Technology Company, Fuzhou, China). The tissue sections were viewed under a microscope at ×400 magnification.

2.12 *Statistical analysis*

Data are expressed as the mean±SD. For each assay, Student's *t*-test was used for statistical comparison. A probability of $p<0.05$ was considered significant.

3. Results

3.1 *Swainsonine treatment inhibits the growth of oesophageal squamous cell carcinoma cell lines*

To evaluate the effects of swainsonine on oesophageal squamous cell carcinoma, we measured the growth of Eca-109, TE-1 and TE-10 cell lines in culture with different concentration of swainsonine using MTT assay. The results showed that swainsonine treatment significantly inhibited the growth of these cells when these cells were cultured with a concentration over 5 μg/mL of swainsonine for 48 h (figure 1A). Further observation found that 5 μg/mL of swainsonine did

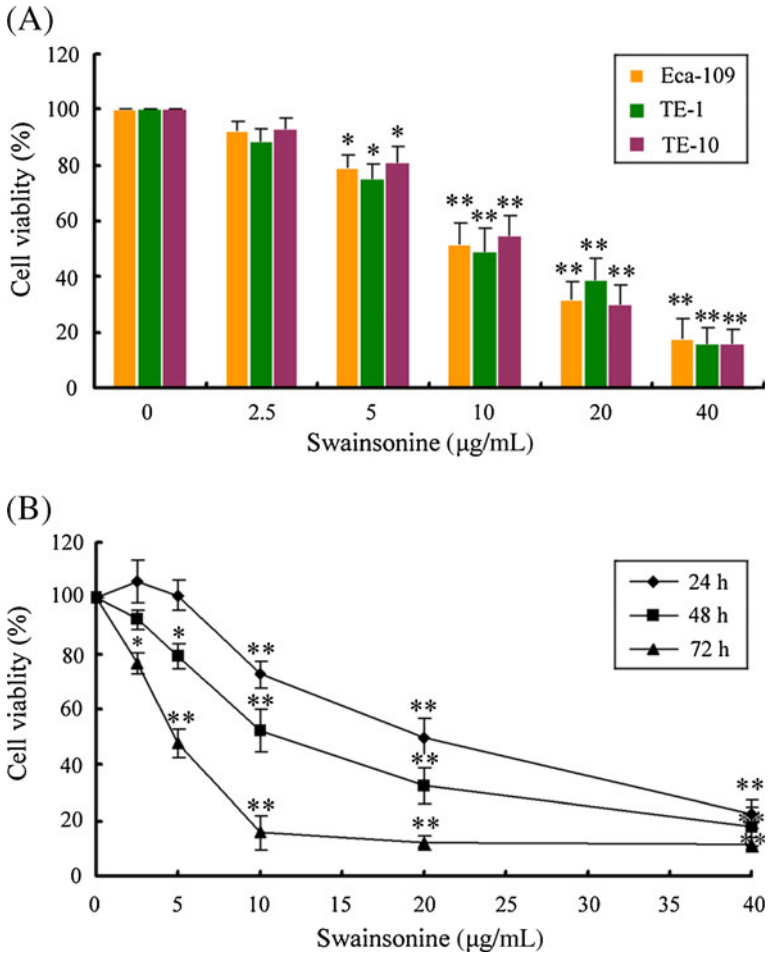

Figure 1. The effects of swainsonine treatment on different oesophageal squamous cell carcinoma cell lines. (**A**) Inhibitory effects of swainsonine on Eca-109, TE-1 and TE-10 cells. 1×10^4 cells per well were seeded in 96-well plates and treated with various concentrations of swainsonine (0–40 μg/mL) for 48 h. Then, cells were incubated with MTT (5 mg/mL) for 4 h at 37°C, and stopped by adding 100 μL DMSO. The absorbance was measured by microplate spectrophotometer (Bio-tek Instruments, Inc., US) at 570 nm. The cell viability values were calculated relative to the control group (0 μg/mL). Results were expressed as percentage of the controls, which were arbitrarily assigned 100% viability. (**B**) Concentration- and time-dependent inhibitory effects of swainsonine on Eca-109 cells. Data are mean±SD and representative of three independent experiments. *$p<0.05$, **$p<0.01$ versus the control group.

not significantly affect the growth Eca-109 cells at 24 h, but 10 μg/mL of swainsonine could inhibit the growth of Eca-109 cells at 24 h (figure 1B). These results suggested that swainsonine inhibited the growth of human oesophageal squamous cell carcinoma cells in a concentration- and time-dependent manner.

3.2 *Swainsonine treatment induces apoptosis in Eca-109 cells*

To determine whether apoptosis was involved in swainsonine-induced growth inhibition in Eca-109 cells, we first examined the cell morphological changes. Cells were treated with 0, 5, 10 and 20 μg/mL of swainsonine for 48 h, followed by DAPI and AO/EB staining. The control cells did not appear significant changes in cell nuclei and cell membrane integrity, but swainsonine-treated cells appeared different extent of chromatin condensation, nuclear fragmentation and destruction of cell membrane integrity after 48 h of incubation with different concentrations of swainsonine (figure 2A and B). Transmission electron microscope observation showed that Eca-109 cells displayed characteristically morphological changes after swainsonine treatment for 48 h, including cell shrinkage, volume reduction, chromatin condensation, cell blebbing and formation of membrane embedded apoptotic

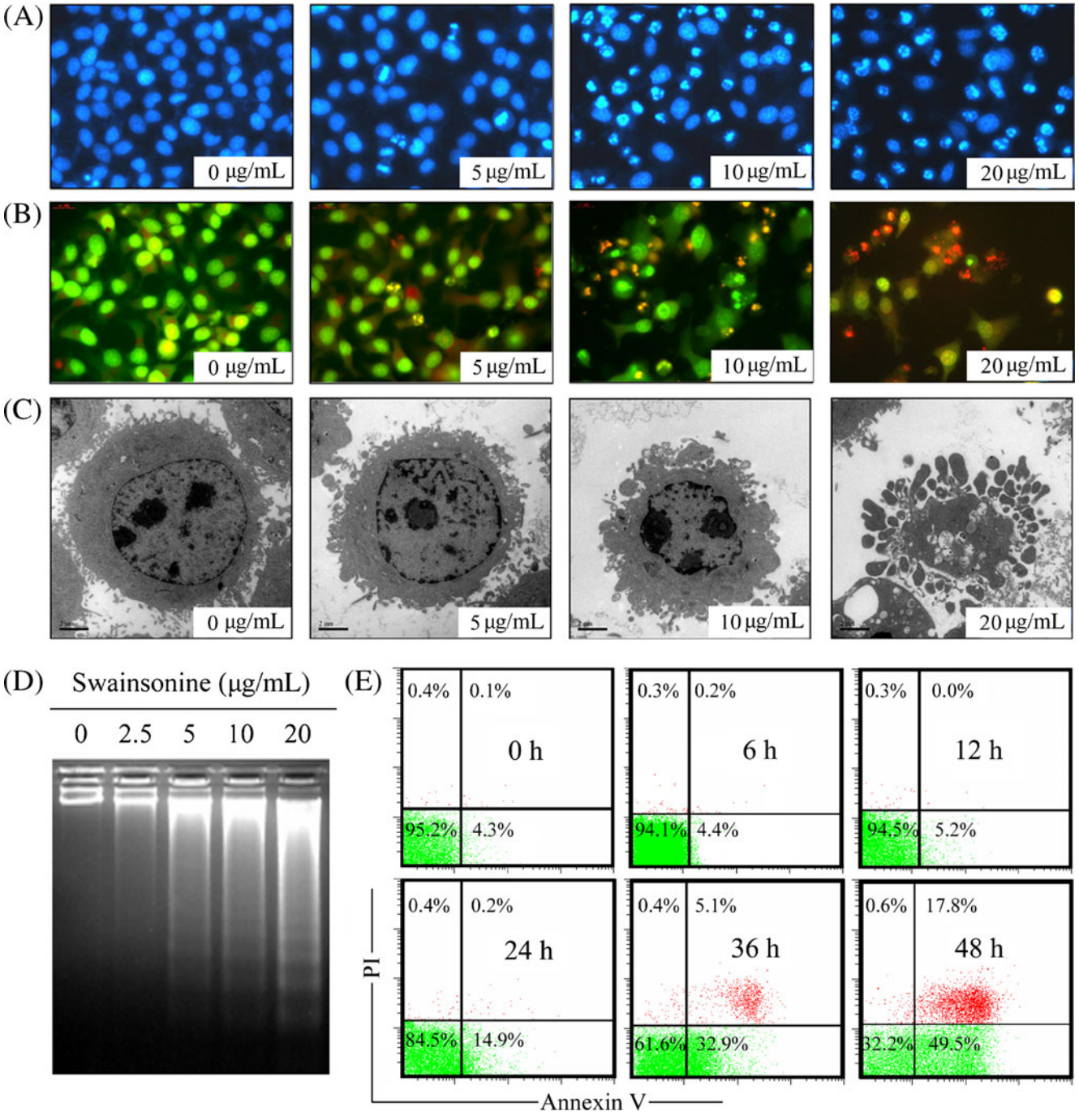

Figure 2. Swainsonine treatment induces apoptosis in Eca-109 cells. (**A**–**B**) Nuclear morphological changes in Eca-109 cells treated with swainsonine. Cells were treated with various concentrations of swainsonine for 48 h. Then cells were stained with DAPI (1 μg/mL) (**A**) or AO/EB dye mix (10 μg/mL acridine orange and 10 μg/mL ethidium bromide in PBS) (**B**). The nuclear morphology was observed under fluorescent microscope (400×). (**C**) Ultrastructural observation of swainsonine-treated cells. Eca-109 cells were treated with various concentrations of swainsonine for 48 h. After a series of washing, fixation, dehydration and stained, cells were visualized under transmission. (**D**) Induction of DNA fragmentation. DNA isolated from swainsonine-treated cells was subjected to 2% agarose gel electrophoresis, followed by visualization of bands and photography. (**E**) Apoptosis rate of Eca-109 cells. Cells were treated with 10 μg/mL of swainsonine for indicated times and stained with Annexin V-FITC and PI for 10 min at room temperature, followed by FCM analysis. All data shown are representative of three independent experiments.

bodies (figure 2C). Furthermore, chromosomal DNA fragmentation assay showed that DNA ladder started appearing and became more evident in cells when the concentration of swainsonine was over 5 μg/mL (figure 2D). Flow cytometry detection using Annexin V/PI dual staining showed that 10 μg/mL of swainsonine treatment significantly increased the percentage of apoptotic cells at 24 h and increased continuously with the time (figure 2E). These results suggested that swainsonine treatment inhibited Eca-109 cell growth via induction of cell apoptosis.

3.3 *Swainsonine treatment induces caspases activation in Eca-109 cells*

To gain insight into the mechanism underlying swainsonine-induced apoptosis, we addressed the contribution of caspases to the apoptosis induced by swainsonine in Eca-109 cells. Cells were cultured with 10 μg/mL of swainsonine for 48 h, and the enzymatic activities of initiator caspase (caspase-8 and caspase-9) and effector caspase (caspase-3) were evaluated by colorimetric assay. Addition of swainsonine into culture medium failed to affect caspase-8 activity, whereas significantly promoted caspase-9 and caspase-3 to activate by 24 h, and further increased their activities by 48 h (figure 3A).

To further confirm the role of caspases in the process of swainsonine-induced apoptosis, caspase-8, caspase-9, caspase-3 specific inhibitors and pan caspase inhibitor were used to block intracellular protease, and then the swainsonine-induced DNA fragmentation and apoptotic rate was analysed using agarose gel electrophoresis and flow cytometry assay. Consistent with the changes of caspases activities, incubation with z-VAD-fmk (pan caspase inhibitor), z-LEHD-fmk (caspase-9 specific inhibitor), or z-DEVD-fmk (caspase-3 specific inhibitor) significantly inhibited swainsonine-induced DNA fragmentation, whereas z-IETD-fmk (caspase-8 inhibitor) did not, when 10 μg/mL of swainsonine treated Eca-109 cells for 48 h (figure 3B). Furthermore, the apoptosis rate of swainsonine-treated cells was prevented in part by z-VAD-fmk, z-LEHD-fmk and z-DEVD-fmk, but not by z-IETD-fmk (figure 3C). Since caspase-8 is usually activated in the classical death receptor-mediated apoptotic pathway while caspase-9 is activated in the mitochondria-mediated apoptotic pathway, these results suggested that the swainsonine might be able to activate mitochondria-mediated apoptotic pathway to induce cell apoptosis.

3.4 *Swainsonine induces apoptosis through the activation of mitochondrial pathway*

Mitochondria are central to the apoptosis regulation in many physiological and pathological conditions. Under apoptotic stimuli, once mitochondrial integrity is damaged, mitochondria-mediated apoptotic pathway will be activated to release some pro-apoptotic proteins, followed by downstream signalling activation. Mitochondrial membrane potential ($\Delta\psi_m$) is determined by the balance of pro-apoptotic and anti-apoptotic Bcl-2 family members, such as Bax and Bcl-2. Bcl-2 protects cells from the induction of apoptosis by interacting with Bax to maintain $\Delta\psi_m$ and block the release of pro-apoptotic proteins. To determine whether swainsonine treatment activates mitochondria-mediated apoptotic pathway, we first used JC-1 as fluorescence probe to evaluate changes in $\Delta\psi_m$ by flow cytometry. JC-1 forms monomers and emits green fluorescence at low $\Delta\psi_m$ while it forms aggregates and emits red fluorescence at high $\Delta\psi_m$. Compared with control, $\Delta\psi_m$-depolarized cells increased about 30% and 39% in 5 μg/mL and 10 μg/mL of swainsonine-treated cells, respectively (figure 4A, lower right quadrants). Then, we investigated the effect of swainsonine treatment on the expression of Bcl-2 and Bax. After exposure to 10 μg/mL of swainsonine, the expression of Bax was up-regulated while Bcl-2 expression was down-regulated in both protein and mRNA levels with the treatment time (figure 4B and C), suggesting that swainsonine treatment might induce an increase of the Bax/Bcl-2 ratio, which was in favour of the collapse of $\Delta\psi_m$ and release of mitochondrial pro-apoptotic factors.

To further explore whether the loss of $\Delta\psi_m$ was associated with the translocation of Bax and release of mitochondrial pro-apoptotic molecules, the proteins extracts from both mitochondrial and cytosolic fractions of swainsonine-treated cells were subjected to Western blot analysis using primary antibodies against Bax, cytochrome c, Smac and AIF. COX4 and β-actin were introduced as internal controls for the mitochondrial and cytosolic fraction, respectively. The results showed that in swainsonine-treated cells, Bax levels increased while cytochrome c levels decreased in the mitochondrial fraction, concomitant with decreased Bax and increased cytochrome c levels in the cytosolic fraction (figure 4D). However, swainsonine treatment did not affect the expression levels of Smac and AIF in the mitochondrial fractions (figure 4D). Accordingly, no Smac or AIF was detected in the cytosolic fractions of swainsonine-treated cells (data not shown).

In cytoplasm, released cytochrome c usually combines with Apaf-1 and procaspase-9 to form the apoptosome in the presence of ATP, resulting in the activation of caspse-9 (Li *et al.* 1997). To detect whether swainsonine treatment promotes the formation of apoptosome, Eca-109 cells were cultured with 10 μg/mL of swainsonine for indicated times, and cell lysates were immunoprecipitated with an anti-Apaf-1 antibody and subsequently

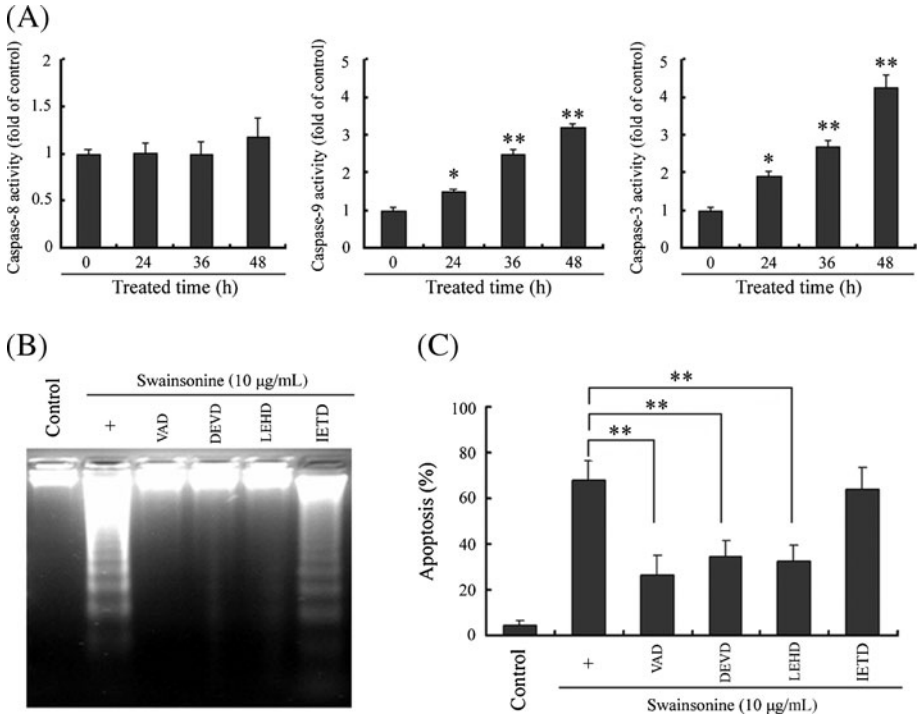

Figure 3. Swainsonine-induced apoptosis is mediated by activation of caspase-9 and caspase-3. (**A**) Caspase activities in cells treated with swainsonine. Cells were incubated with 10 μg/mL of swainsonine for indicated times and the enzymatic activities of caspases-8, -9, and -3 were measured using the colorimetric assay kits. The results are mean±SD and representative of three independent experiments. *$p<0.05$, **$p<0.01$ versus the control group (0 h). (**B**) Effects of caspase inhibitors on swainsonine-induced DNA fragmentation. Cells were incubated with 10 μg/mL of swainsonine for 12 h and then co-incubated with 20 μM of caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk or z-LEHD-fmk for another 36 h. The data shown are representative of three independent experiments. (**C**) Effects of caspase inhibitors on swainsonine-induced apoptosis. Eca-109 cells were treated as in (**B**). Rate of apoptosis was evaluated by FCM analysis. Data are mean±SD and representative of three independent experiments. **$p<0.01$ versus swainsonine alone without inhibitor.

subjected to Western blot with anti-Caspase-9 antibodies. The results showed that Apaf-1 began to be interacted with Caspase-9 in Eca-109 cells after 10 μg/mL of swainsonine treatment for 24 h (figure 4E, upper panel). In addition, PARP, an intracellular substrate of caspase-3, was shown to be cleaved from 116 to 85 kDa fragments in the swainsonine-treated cells (figure 4E, lower panel). These results suggested that swainsonine treatment-induced apoptosis was mainly through the activation of mitochondrial pathway.

3.5 *Swainsonine intake inhibits growth of Eca-109 xenografts in athymic nude mice*

Swainsonine has been shown to be effectively induced apoptosis in Eca-109 cells *in vitro*; thus, we further extended our study to determine whether these events occur *in vivo* using a xenograft mouse model. Athymic nude mice xenografted with Eca-109 cells were divided into control group and swainsonine-treated groups wherein swainsonine was administered at doses of 1 or 2.5 mg/kg/day for 35 days. In contrast to the control group, tumour volume was inhibited by 22.79% and 29.31% ($p<0.05$) and the wet weight of tumour was decreased by 19.96% and 31.67% ($p<0.05$) in 1 and 2.5 mg/kg/day swainsonine-treated group, respectively, at the termination of the experiment (figure 5A and B). Swainsonine administration did not seem to induce any adverse effects as judged by monitoring body weight and oesophagus, gastrointestinalinal tract, livers, lungs and kidneys (data not shown). Furthermore, we evaluated the effects of swainsonine intake on the induction of apoptosis and apoptosis-associated molecules in tumour xenografts. TUNEL assay showed evident *in situ* apoptosis in Eca-109 tumour sections at 1 and 2.5 mg/kg/day of swainsonine-treated groups, but not in the sections of control group (figure 5C, left panel). Further results showed that swainsonine

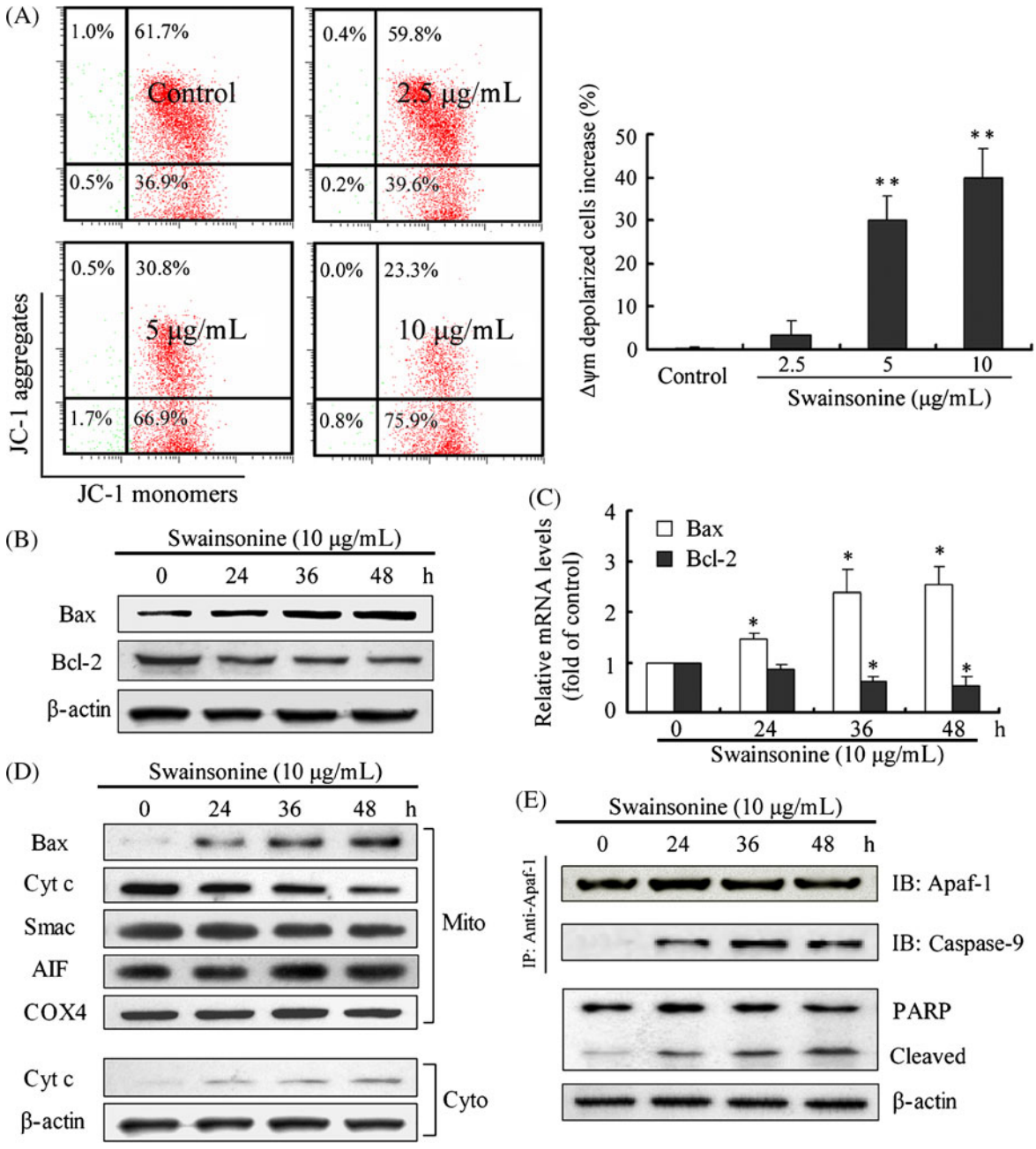

Figure 4. Sainsonine-induced Eca-109 cell apoptosis is mediated by the activation of mitochondrial pathway. (**A**) Swainsonine treatment induced collapse of $\Delta\psi_m$. Cells were treated with 0–10 μg/mL of swainsonine for 24 h, and stained with JC-1 for 15 min at room temperature, followed by FCM analysis. The results are mean±SD and representative of three independent experiments. $^{**}p<0.01$ versus the control group (0 μg/mL). (**B**–**C**) Swainsonine treatment up-regulated Bax while it down-regulated Bcl-2 both in protein and mRNA levels. Cells were incubated with 10 μg/mL of swainsonine for indicated time. The cell lysates were subjected to Western blot analysis, while total RNA was extracted and used for real-time RT-PCR assay. The results are mean±SD and representative of three independent experiments. $^{*}p<0.05$ versus the control group (0 h). (**D**) Swainsonine treatment promoted Bax translocation and cytochrome c release. Cells were treated as in (**B**). The cytosolic and mitochondrial fraction proteins were collected and then detected by western blot. COX4 and β-actin were used as internal controls for the mitochondrial fractions and the cytosolic fraction, respectively. All the data shown are representative of three independent experiments. (**E**) Swainsonine treatment induced apoptosome formation and PARP cleavage. Protein extractions from swainsonine-treated cells were collected and then used in immunoprecipitation assays against Apaf-1. Western blotting was then performed to detect the level of caspase-9 to indicate the formation of apoptosome complex. Cell lysates were subjected to western blot assay to detect the cleavage of PARP.

administration resulted in a decrease levels of Bcl-2, an increase Bax levels, with the translocation of Bax and cytochrome c release (figure 5C mid and right panels and D) in xenograft tumours, compared to that of control group. These results are consistent with our findings in cell culture. These data suggested that swainsonine administration inhibited Eca-109 xenograft tumours growth via induction of tumour cell apoptosis.

4. Discussion

Swainsonine is a natural alkaloid that is found in numerous poisonous plants, such as *Astragalus* and *Oxytropis* lentiginosus (known as locoweed) (Molyneux and James, 1982). Swainsonine shows chronic toxicity to domestic animals such as sheep, goat and bovine, and can cause specific poisoning symptoms. However, previous studies showed that rodents

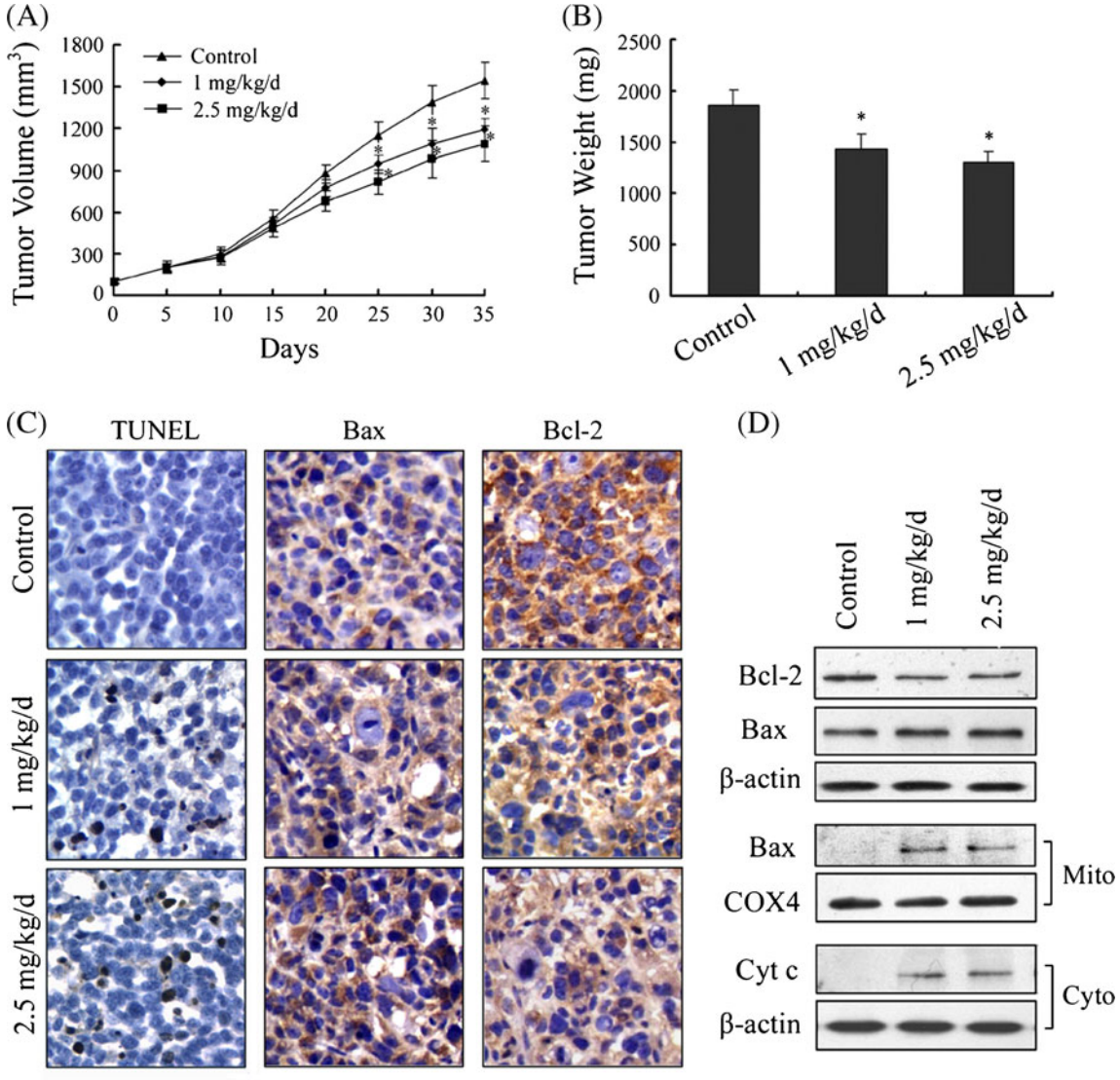

Figure 5. Swainsonine administration inhibits Eca-109 tumour growth in athymic nude mice via induction of apoptosis. Approximately 1×10^7 Eca-109 cells were injected into back of mouse, and swainsonine was provided to mice when the tumour reached 100 mm^3. Mice were divided into three groups (n=6): Group I received 0.2 mL PBS only as control; group II received 1 mg/kg swainsonine in 0.2 mL PBS; group III received 2.5 mg/kg swainsonine in 0.2 mL PBS daily for 35 days. (**A**) Tumour volumes (mm^3) were measured regularly in two dimensions throughout the study. *p<0.05 versus the control group. (**B**) Wet weight of tumours were measured in the end of this experiment, data are represented as the mean of 6 tumours from each group. *p<0.05 versus the control group. (**C**) TUNEL assay and immunohistochemistry staining with anti-Bax and Bcl-2 primary antibodies. The data are representative of three independent experiments. (**D**) Western blot analysis of Bax, Bcl-2, and cytochrome c, in tumour tissue lysates after intake of indicated doses of swainsonine. The data are representative of three independent experiments.

and human tolerate this alkaloid well (Novikoff *et al.* 1985; Goss *et al.* 1997). Swainsonine has been confirmed to have inhibitory and antimetastatic effects on several human and mouse cancer cell lines *in vitro* or *in vivo* studies (Dennis *et al.* 1990; Yagita and Saksela 1990; Goss *et al.* 1994; Sun *et al.* 2007, 2009). These studies provide rational for exploring swainsonine as an antitumour agent. In the present study, we evaluated the effects of swainsonine on human oesophageal squamous cell carcinoma cell lines Eca-109, TE-1, TE-10 *in vitro* and Eca-109 *in vivo*, and found swainsonine treatment inhibited these cells growth in a concentration-dependent manner. Interestingly, *in vitro* experiments showed that 10 μg/mL of swainsonine treatment for 24 h just has capability to induce early apoptosis in oesophageal squamous carcinoma cell Eca-109. However, our previous studies showed that 2 μg/mL (12 μM) of swainsonine treatment for 12 h could induce adenocarcinoma A549, Calu-3 and SPC-A-1 cells apoptosis (Li *et al.* 2012). Other report also showed that 1.5 μg/mL of swainsonine treatment for 24 h could induce adenocarcinoma SGC-7901 cells apoptosis (Sun *et al.* 2007). These results suggest the squamous carcinoma cells seem to be less sensitive to swainsonine as compared to adenocarcinoma cells. *In vivo* antitumour activity assay also showed swainsonine intake could effectively inhibit the growth of Eca-109 xenograft tumours through induction of apoptosis, but the effect of swainsonine on the squamous cell carcinoma still appeared a slower process compared with that in adenocarcinomas (Li *et al.*

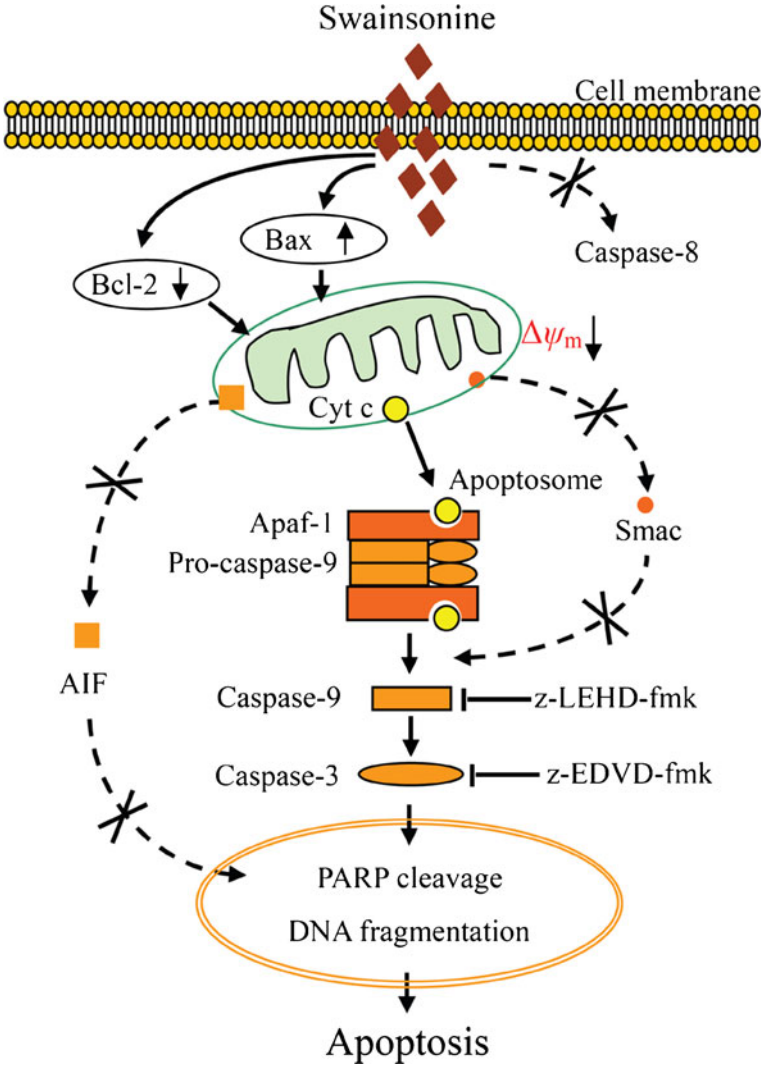

Figure 6. Proposed model of molecular mechanism of swainsonine-induced apoptosis in Eca-109 cells. Swainsonine treatment down-regulates Bcl-2, promotes Bax translocation into mitochondria, activates the mitochondria-dependent apoptotic pathway, resulting in cytochrome c release to cytosol, followed by activation of caspase-9 and caspase-3, and the cleavage of PARP.

2012; Sun *et al.* 2007). These studies suggest that carcinoma cells derived from different tissues exhibit different sensitivity to swainsonine treatment, and that the adenocarcinoma cells might be more sensitive to swainsonine than squamous carcinoma cells.

Many anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to prevent the promotion and progression of tumour (Surh 2003; Xu *et al.* 2009). Apoptosis is a tightly regulated process and its mechanisms, in most case, involve in the activation of caspases cascades. There are at least two major apoptotic pathways, extrinsic pathways (death receptors) and intrinsic pathways (mitochondria), which are initiated by caspase-8 and caspase-9, respectively (Ghobrial *et al.* 2005). The stimulation of the death receptor pathway, caspase-8 follows the recruitment of the procaspase to the death-inducing signalling complex. In contrast, the mitochondrial pathway requires the release of mitochondrial cytochrome c and the formation of a large multiprotein complex comprising cytochrome c, Apaf-1 and procaspase-9. Caspase-8 and caspase-9 will then proteolytically activate downstream caspases, in particular caspases-3 (Jänicke *et al.* 1998). The activation of caspase-3, a key and irreversible point in the development of apoptosis, is essential for DNA fragmentation and the morphological changes associated with apoptosis (Jänicke *et al.* 1998). In swainsonine-treated cells, caspase-9 and caspase-3, but not caspase-8, were activated. Co-incubation with the pan-caspase inhibitor z-VAD-fmk, caspase-3 specific inhibitor z-DEVD-fmk, and caspase-9 specific inhibitor z-LEHD-fmk effectively reduced swainsonine-induced DNA fragmentation and apoptosis, but the caspase-8 inhibitor z-IETD-fmk did not. These data suggest that apoptosis induced by swainsonine could occur through mitochondria-dependent caspase activation.

Mitochondria play an essential role in death signal transduction and amplification of the mitochondria-meidated apoptotic signalling. Several proteins with proapoptotic functions such as cytochrome c, Smac and AIF are localized in mitochondria (Liu *et al.* 1996; Susin *et al.* 1999; Verhagen *et al.* 2000). Under some apoptotic stimuli, the reduction of $\Delta\psi_m$ results in the opening of the permeability transition pore and redistribution of intermembranous pro-apoptotic proteins and triggers apoptosis through caspase-dependent or caspase-independent pathways. Swainsonine treatment increased the percentage of $\Delta\psi_m$-depolarized cells and subsequently promoted cytochrome c release but not Smac and AIF release, suggesting that mitochondria and cytochrome c release are involved in the process of swainsonine-induced apoptosis. It is known that mitochondrial integrity is controlled prominently by the members of Bcl-2 family, among which Bcl-2 and Bax have been identified as major regulators (Sharpe *et al.* 2004). In response to apoptotic stimuli, Bax translocates to the mitochondria and inserts into the outer mitochondrial membrane, resulting in the collapse of $\Delta\psi_m$ and release of mitochondrial pro-apoptotic proteins. In contrast, Bcl-2 blocks this process by binding to the outer mitochondrial membrane and forming a heterodimer with Bax resulting in neutralization of its proapoptotic effects (Huang *et al.* 2006). Here we showed that swainsonine treatment decreased the expression of Bcl-2 while increased that of Bax in both protein and mRNA levels, and promote the translocation of Bax from cytoplasm to mitochondria. The translocation of Bax may be associated to the collapse of $\Delta\psi_m$ and release of mitochondrial pro-apoptotic proteins.

In summary, swainsonine can significantly inhibit the growth of Eca-109 cells through induction of apoptosis *in vitro* and *in vivo*. Swainsonine treatment down-regulates Bcl-2, promotes Bax translocation into mitochondria, activates the mitochondria-dependent apoptotic pathway, resulting in cytochrome c release to cytosol, followed by activation of caspases-9 and -3, and the cleavage of PARP (figure. 6). This study provides us a new insight into swainsonine-induced apoptosis in carcinoma from different tissues. Further studies of the effects of swainsonine on some signalling upstream of mitochondria-dependent apoptotic pathway may be helpful for us to understand this issue.

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