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Casticin induces caspase-mediated apoptosis via activation of mitochondrial pathway and upregulation of DR5 in human lung cancer cells

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ABSTRACT

Objective: To assess if casticin induces caspase-mediated apoptosis via activation of mitochondrial pathway and upregulation of DR5 in human lung cancer cells. **Methods:** Human non-small-cell lung carcinoma cell lines H460, A549 and H157 were cultured *in vitro*. The cytotoxic activities were determined using MTT assay. The apoptotic cells death was examined by flow cytometry using PI staining and DNA agarose gel electrophoresis. The activities of caspase-3, -8 and -9 were measured via ELISA. Cellular fractionation was determined by flow cytometry to assess release of cytochrome c and the mitochondrial transmembrane potential. Bcl-2/Bcl-XL/XIAP/Bid/ DR5 and DR4 proteins were analyzed using western blot. **Results:** The concentrations required for a 50% decrease in cell growth (IC_{50}) ranged from 1.8 to 3.2 μM. Casticin induced rapid apoptosis and triggered a series of effects associated with apoptosis by way of mitochondrial pathway, including the depolarization of the mitochondrial membrane, release of cytochrome c from mitochondria, activation of procaspase-9 and -3, and increase of DNA fragments. Moreover, the pan caspase inhibitor zVAD-FMK and the caspase-3 inhibitor zDEVD-FMK suppressed casticin-induced apoptosis. In addition, casticin induced XIAP and Bcl-XL down-regulation, Bax upregulation and Bid clearage. In H157 cell line, casticin increased expression of DR5 at protein levels but not affect the expression of DR4. The pretreatment with DR5/Fc chimera protein effectively attenuated casticin-induced apoptosis in H157 cells. No correlation was found between cell sensitivity to casticin and that to p53 status, suggesting that casticin induce a p53-independent apoptosis. **Conclusions:** Our results demonstrate that casticin induces caspase-mediated apoptosis via activation of mitochondrial pathway and upregulation of DR5 in human lung cancer cells.

1. Introduction

Lung cancer is the leading cause of cancer deaths in the

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world with over one million cases diagnosed every year. Multiple options for the treatment of lung cancer have been described, including surgery, chemotherapy, and radiation, however, therapeutic effect is typically transient and mostly absent with advanced disease[1,2]. Therefore, intense efforts are being mounted to find effective new agents and treatments against lung cancer.

Casticin is one of the main components from *Fructus viticis* (Manjingzi in Chinese name), a traditional Chinese medicine prepared from the fruit of *Vitex trifolia* L. (family Verbenaceae) that is also used as a folk medicine to be an anti-inflammatory agent and treat certain cancers in

China[3]. Its chemical structure is shown in Figure 1. Casticin has been showed to inhibit lymphocyte proliferation *in vitro*[4] and has an anti−inflammatory effect *in vivo*[5]. In recent years, many studies have demonstrated its anti−carcinogenic activity in various cancer cells, including lung cancer[6−8]. However, whether casticin induces apoptosis of carcinoma cells and its precise mechanisms are unclear.

In this report, we examined the effects of casticin on apoptosis of human non−small cell lung carcinoma (NSCLC) cell lines. We show for the first time that casticin is a potent inducer of apoptosis in NSCLC cells and this effect is associated with activation of caspase through mitochondrial signaling and DR5 upregulation.
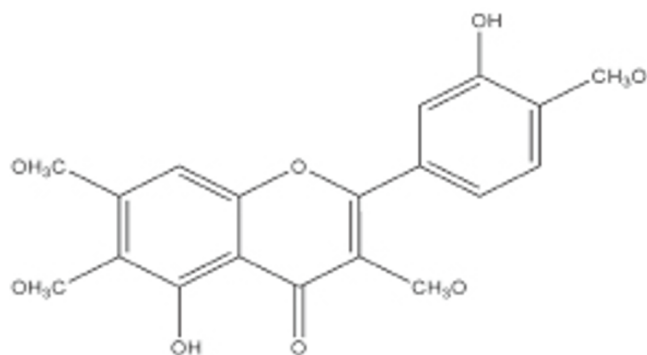

Figure 1. Chemcial constitution of casticin.

2. Materials and methods

2.1. Reagents

Casticin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Casticin has a molecular weight of 374.3 ku, appears as yellow crystals and has a purity of 98.0%. Casticin was prepared in dimethyl−sulfoxide at a concentration of 10 mmol/L, and aliquots were stored at −80 ℃. Stock solutions were diluted to the desired final concentrations with growth medium just before use. The following were purchased from Hunan Clonetimes Biotech Co., Ltd. (Changsha, China): RPMI−1640 medium (Invitrogen), fetal bovine serum (Invitrogen), Cell Apoptosis ELISA Detection Kit (Roche), Caspase 3 Activity Detection Kit (Millipore), Caspase 8 Colorimetric Activity Assay Kit 25 (Millipore), Caspase 9 Colorimetric Activity Assay Kit (Millipore), DR5/Fc chimera protein (R&D Systems), CBZ−Val−Ala−Asp−fluoromethyl ketone (zVAD−fmk: Livermore), CBZ−Asp−Glu−Val−Asp−fluoromethyl ketone (zDEVD−fmk: Livermore), Z−lle−Glu(Ome)−Thr−Asp(OMe)−fluoromethyl ketone (zIETD−fmk: Livermore), and Z−Leu−Glu(OMe)−His−Asp(OMe)−fluoromethyl ketone (zLEHD−fmk: Livermore), rhodamine 123 (Eugene), Mouse anti−human Bcl−2, Bax, XIAP, Bcl−XL, cytochrome c, DR5, DR4 and β

−actin antibodies (Santa Cruz Biotechnology), horseradish peroxidase−conjugated anti−mouse secondary antibody (Cell Signaling Technology).

2.2. Cell lines and cell culture

Human non−small−cell lung carcinoma cell lines H460 and A549 which possess wild−type p53, and H157 which express mutant p53[9,10], were obtained from the American Type Culture Collection (Manassas, VA). Human embryo lung WI−38 cells were purchased from cell bank, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI 1640 supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L L−glutamine, and 10% heat−inactivated fetal bovine serum at 37 ℃ in a humidified atmosphere consisting of 5% CO_2 and 95% air.

2.3. MTT assay

Cells were seeded in a 96−well plate at a density of 0.5×10^4 cells/well for 24 h, followed by treatment with various concentrations of casticin or cisplatin for the indicated time. MTT colorimetric analysis was performed as described previously[11]. The IC_{50} value, at which 50% of the cell growth inhibition compared with DMSO control, was calculated by nonlinear regression analysis using GraphPad Prism software (San Diego, CA).

2.4. Flow cytometry using PI staining

Cells were seeded at a density of 4×10^6 cells/well in 250 mL culture flasks for 24 h and then treated with the medium containing various concentrations of casticin and cisplatin for the indicated time. Propidium iodide staining for DNA content analysis was performed as described previously[10].

2.5. DNA fragmentation assay

Cells were seeded at a density of 4×10^6 cells/well in 250 mL culture flasks for 24 h and treated with medium containing various concentrations of casticin or cisplatin for 24 h. This assay was performed as described previously[12,13].

2.6. Histone/DNA ELISA for detecting apoptosis

Cells were seeded in a 96−well plate at a density of 1×10^4 cells/well for 24 h, added the tested agents and then cultured in RPMI−1640 medium containing 10% fetal bovine serum. After 24 h, the cytoplasm of the control and treatment group was transferred to the 96−well plate peridiumed by the streptavidin, incubated with the biotinylated histone antibody and peroxidase−tagged mouse anti−human DNA for 2 h at room temperature. The absorbance at 405

nm was measured with EXL–800 type Enzyme–Linked Immunosorbent apparatus.

2.7. Analysis of caspase–3, –8 and –9 activities

To evaluate caspase activity, cell lysates were prepared after their respective treatment with the tested agents. Assays were performed in 96–well plates by incubating 20 μg cell lysates in 100 μL reaction buffer (1% NP–40, 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 10% glycerol) containing a 5 μM caspase–3 substrate Ac–DEVD–*p*NA or caspase–8 substrate Ac–IETD– *p*NA or caspase–9 substrate Ac–LEHD– *p*NA. Lysates were incubated at 37 ℃ for 2 h. Thereafter, the absorbance at 405 nm was measured with an enzyme– labeling instrument (ELX–800 type). In the caspase inhibitors assay, cells were pretreated with a caspase inhibitor (20 μM, zVAD–fmk or zDEVD–fmk or zIETD–fmk or zLEHD–fmk) for 1 h prior to the addition of test agents.

2.8. Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential was determined by flow cytometry after cell loading with rhodamine 123, as described by Troyano *et al*[14].

2.9. Cellular fractionation

For assay of release of cytochrome c, cells were fractionated into cytosolic and mitochondrial fractions as described by Ling *et al*[15] In brief, cells were incubated in buffer containing 20 mM HEPES–KOH, pH 7.2, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin at 4 ℃ for 10 min, and then cells were homogenized with a Dounce homogenizer for 20 strokes. After addition of buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM EGTA, and 5 mM Tris–HCl, pH 7.5, the homogenates were centrifuged at 1 000 *g* for 10 min at 4 ℃. The supernatants were further centrifuged at 15 000 *g* for 30 min at 4 ℃, and collected as the cytosolic fraction. The pellet was further dissolved with lysis buffer containing with 1% SDS as the mitochondrial fraction.

2.10. Western blot analysis

Total cell extracts were obtained as previously described[12]. Cell lysate containing 50 μg of protein was separated on a 7.5%–12.5% SDS–polyacrylamide gel for electrophoresis and then blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Anti– Bcl–2, Bax, XIAP, Bcl–XL, cytochrome c, DR5, DR4 and β –actin (1:1 000 dilutions for each) were used as primary antibodies. Signals were detected using an ECL kit (Amersham Pharmacia Biotech, Piscataway,

NJ). Images were scanned followed by densitometry analysis with Alphazmager 2200 software (Silk Scientific). The ratios of the specific antibodies/β –actin were determined for the expression level of the proteins.

2.11. Statistical analysis

The database was set up with the SPSS 15.0 software package (SPSS Inc, Chicago, IL) for analysis. Data were represented as mean±SD. The means of multiple groups were compared with one–way ANOVA, after the equal check of variance, and the two–two comparisons among the means were performed using the LSD method. Statistical comparison was also performed with two–tailed *t*–test when appropriate. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effects of casticin on cytotoxicity of NSCLC cell lines

To characterize the effect of casticin on cytotoxicity, three kinds of human NSCLC cell lines, including H460, A549, H157 cells and human embryo lung WI–38 cells were treated with various concentrations of casticin and cisplatin for 24 h, and the cytotoxicity was assessed by MTT assay. Casticin and cisplatin were toxic to H460, A549, H157 and WI–38 cells with IC_{50} values as shown in Table 1. The potency of casticin to NSCLC cells was stronger than that of cisplatin. The selective index of cytotoxicity to NSCLC cells of casticin was 37 (86.7/2.3), 27(86.7/3.2), 48(86.7/2.3) which was higher than that of cisplatin with a SI of 7.8(38.5/9.3), 2.3(38.5/16.4), 1.7(38.5/22.6).

Table 1

IC_{50} values of casticin and cisplatin in H460, A549, H157 and WI–38 cells.

Cell lines	IC_{50} (μ mol/L)*	
	Casticin	Cisplatin
H 460	2.3±0.2	4.9±0.5
A549	3.2±0.4	16.4±1.4
H157	1.8±0.1	22.6±2.3
WI–38	86.7±5.6	38.5±3.3

*Means and standard deviations are calculated from triplicates of three independent experiments.

3.2. Induction of apoptosis by casticin in human NSCLC cells

To elucidate whether casticin inhibits cell growth through the induction of apoptosis in the NSCLC cells, we then examined the effects of casticin on apoptosis in three NSCLC cell lines (A549, H460, and H157) using different approaches.

Using flow cytometric analysis to detect increases in hypodiploid cell populations, casticin increased the percentage of the sub−G_1 population in H460, A549 and H157 cells (P < 0.05), respectively reaching 37.5%±5.7%, 26.8%± 4.8% and 42.2%±6.1% after 24 h of treatment with casticin at 4 μ mol/L (Figure 2A). The sub−G_1 population in H157 cells by casticin was increased at 12 h and peaked at 24 h (Figure 2B). Histone/DNA fragments in H460, A549 and H157 cells, as measured by the cell apoptosis ELISA detection kit, were increased in dose−dependent manner (P<0.05) after treatment with casticin (Figure 2C). Furthermore, DNA fragmentation analysis by agarose gel electrophoresis showed a typical ladder pattern of internucleosomal DNA fragments in A549, H460, and H157 cells treated with 4 μ mol/L casticin for 24 h (Figure 2D). These results suggest that casticin inhibited human NSCLC cells growth through a mechanism involving the induction of apoptosis.
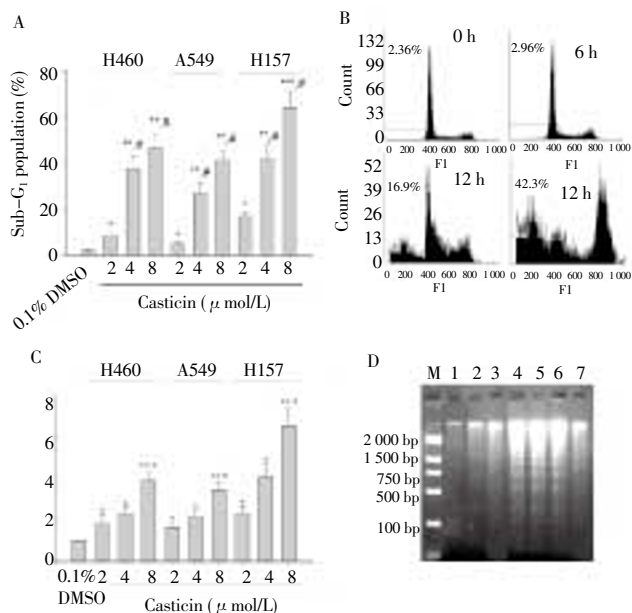
were actually activated during casticin induced cell death of human NSCLC cells. Figure 3A, B and C shows that treatment of human NSCLC cells with casticin for 24 h increased the levels of active caspase−3, −8. and −9 (P<0.05) in a concentration−dependent manner in H460, A549 and H157 cells respectively.

We further examined the role of caspases activated during apoptosis induced by casticin treatment using the pan−caspase inhibitor zVAD−fmk, the caspase−3 inhibitor zDEVD−fmk, the caspase−8 inhibitor zIETD−fmk and the caspase−9 inhibitor zLEHD−fmk. Figure 3D shows that zVAD−fmk and zDEVD−fmk completely abrogated apoptosis induced by casticin and zIETD−fmk and zLEHD−fmk attenuated casticin−induced apoptosis in H460, A549 and H157 cells respectively. These data indicate that casticin induced apoptosis was essentially dependent on activation of caspase−3, −8 and −9.

Figure 2. Apoptotic effects of casticin detected by flow cytometric analysis (A and B), cell apoptosis ELISA detection kit (C), and DNA agarose gel electrophoresis (D).
*P<0.05, ** P<0.01 vs. treatment with DMSO; #P<0.05 vs. treatment with 2.0 μ mol/L casticin. M, DNA marker (DL2000); 1, H460 cells were treated with 0.1% DMSO for 24 h; 2, A549 cells were treated with 0.1% DMSO for 24 h; 3, H157 cells were treated with 0.1% DMSO for 24 h; 4, H460 cells were treated with 4.0 μ mol/L casticin for 24 h; 5, A549 cells were treated with 4.0 μ mol/L casticin for 24 h; 6, H157 cells were treated with 4.0 μ mol/L casticin for 24 h; 7, H157 cells were treated with 20.0 μ mol/L zVAD−fmk for 1 h, followed by in combination with 4.0 μ mol/L casticin for 24 h.

3.3. Effects of casticin on caspases activities of human NSCLC cells

To determine the effectors active in casticin−induced apoptotic pathways, we next examined whether caspases
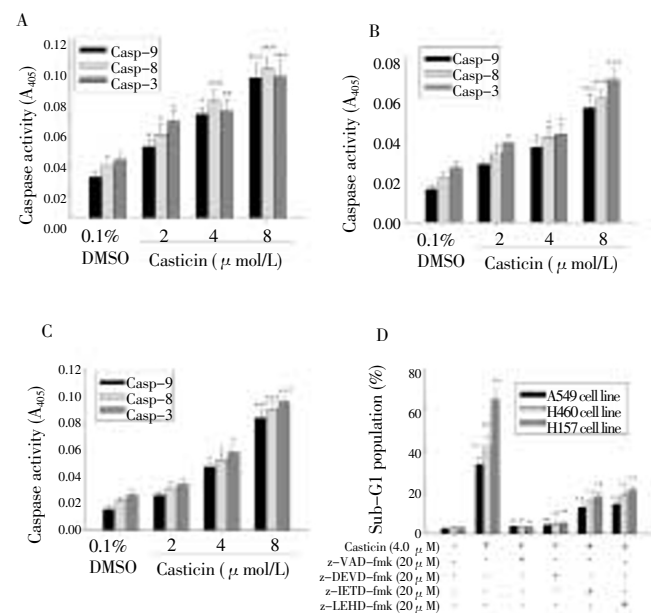
Figure 3. Effects of casticin on the caspase activities of lung cancer cells.
A: H460, B: A549, C: H157 cell lines. D, Cells were pretreated with specific caspase inhibitor (20.0 μ mol/L zVAD−fmk or zDEVD−fmk or zIETD−fmk or zLEHD−fmk) for 1 h, followed by co−treatment with 4.0 μ mol/L casticin for 24 h. *P<0.05, ** P<0.01 vs. treatment with DMSO; #P<0.05 vs. treatment with 2.0 μ mol/L casticin.

3.4. Effects of casticin on apoptosis induction for mitochondrial signaling of human NSCLC cells

Because casticin induced caspase−9 activation (Figure 3A), we wondered whether it affected casticin−induced apoptosis associated with mitochondrial signaling as well. Therefore, we next examined the effects of casticin on several important events for apoptosis such as mitochondrial membrane depolarization, cytochrome c release, and expression of some vital protein substrates in this pathway in H157 cells. The

results in Figure 4 indicate that: (i) treatment with casticin clearly elicited mitochondrial transmembrane potential ($\triangle\Psi$m) dissipation, as indicated by the decrease in rhodamine 123−derived fluorescence in flow cytometry assays (Figure 4A). (ii) Casticin triggered a rapid release of cytochrome c from mitochondria to cytoplasm, as determined by immunoblot using cytosolic extracts (Figure 4B). The release of cytochrome c occurred as early as 3 h after treatment, and, therefore, preceded the increase of sub−G_1 populations, which occurred after a 12−h treatment in NSCLC cells (Figure 2B). (iii) Treatment with castisin did not affect total Bcl−2 expression but caused a decrease in Bcl−XL and XIAP expression and a increase in Bax expression (Figure 4C). The treatment also caused a decrease in the amount of Bid proform (21 kDa), which represents an indirect evidence of protein truncation/activation (Figure 4D). These data demonstrate casticin induced apoptosis was involved in the mitochondrial executioner pathway in H157 cells.
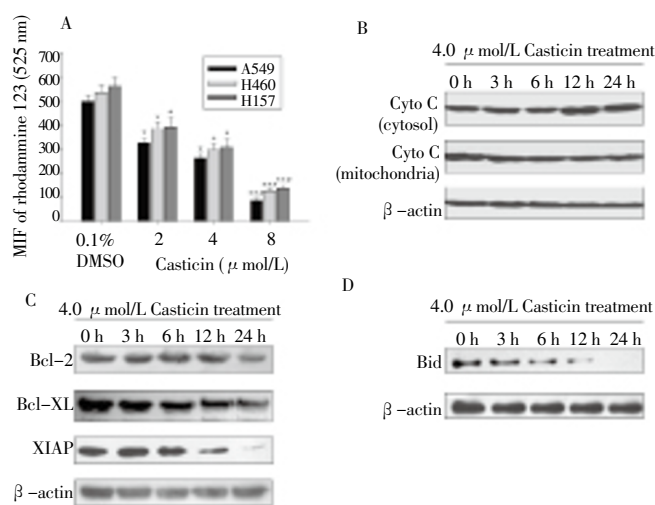

Figure 4. Effects of casticin on mitochondrial membrane potential in lung cancer cells (A), and cytochrome c release (B), Bcl−2/Bcl−XL/XIAP (C) and Bid protein expression in H157 cells.
*P<0.05, ** P<0.01 *vs.* treatment with DMSO; #P<0.05 *vs.* treatment with 2.0 μ mol/L casticin.

3.5. Effects of casticin on DR5 expression of human NSCLC cells

Because casticin induced caspase−8 activation (Figure 3A) and a decrease in the amount of Bid proform (Figure 4D), the observation led us to investigate the effect of casticin on expression of DR4 and DR5 proteins. In H157 cell line, casticin increased expression of DR5 at protein levels but did not affect the expression of DR4 (Figure 5A).

To further definite the role of DR5 upregulation in casticin−induced apoptotic cell death, we examined the effects of DR5/Fc chimera protein, a blocking antibody, on induction of apoptosis by casticin. Figure 5B shows that the pretreatment with DR5/Fc chimera protein effectively

attenuated casticin−induced apoptosis in H157 cells. These data suggest casticin induced apoptosis was associated with DR5 upregulation in human lung cancer H157 cell line.
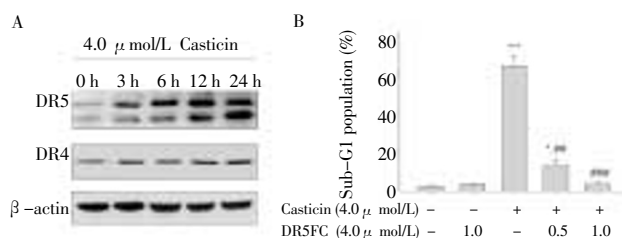

Figure 5. Casticin increases DR5 protein levels in lung cancer cells. A, H157 cells were treated with 4.0 μ mol/L casticin for the indicated time, and Western blotting of DR5, DR4 and β −actin as a loading control was done. B, Effect of DR5−specific blocking chimera antibody on casticin−induced apoptosis.
*P<0.05, ***P<0.01 *vs.* treatment with DMSO; ##P<0.01, ###P<0.05 *vs.* treatment with 4.0 μ mol/L casticin alone.

4. Discussion

Casticin, a polymethoxyflavone from *Fructus viticis*, widely used as an anti−inflammatory agent in Chinese traditional medicine[5], was reported to have anticancer activities[6,7]. In the present study, we investigated the effects of casticin on the cell growth and apoptosis using a panel of human NSCLC cell lines. We have demonstrated that casticin was a potent agent in decreasing the growth of human NSCLC cells. Therefore, casticin may be a promising candidate for use as a therapeutic agent for cancer treatment. Casticin inhibited the growth of human NSCLC cells through induction of apoptosis. Therefore, our results warrant additional studies on the therapeutic activity of casticin *in vivo*[6,7,16−20].

Caspases play important roles in apoptosis triggered by various proapoptotic signals[21,22]. In general, activation of the caspase cascade requires both initiator caspases such as caspase−8, and −9 and effector caspases such as caspase−3. The effector caspases cleave several vital substrates leading to apoptosis[21,22]. It has been well documented recently that the dissipation of $\triangle\Psi$m, cytochrome c release from mitochondria and its activation of caspase−9 through binding to the protein Apaf−1 is thought to mediate apoptosis triggered by signals such as chemotherapeutic agents[21−24].In this study, casticin triggered ($\triangle\Psi$m) dissipation, a rapid release of cytochrome c from mitochondria to cytosol, activated caspase−9 and caspase−3, followed by DNA fragment. Moreover, the pan−caspase inhibitor zVAD−FMK and the caspase−3 inhibitor zDEVD−FMK blocked and the caspase−9 inhibitor zLEHD−fmk suppressed casticin−induced apoptosis. Therefore, we conclude that casticin induces mitochondrial dysfunction and a cytochrome c−mediated, caspase−dependent apoptosis in human NSCLC cells.

Bcl–2, Bcl–XL, and Bax have been implicated as major players in the control of apoptosis[21–23]. Bcl–2 and Bcl–XL promote cell survival, whereas Bax promotes cell death[25,26]. More recently, these proteins have been found to regulate apoptosis by controlling cytochrome c release from mitochondria and activation of caspase–9[25,27]. Casticin did not alter the expression levels of Bcl–2, but it did down–regulate Bcl–XL and upregulate Bax. Whether the increase ratio of the Bax/Bcl–XL plays a role in casticin–induced apoptosis of human NSCLC cells remains to be elucidated. It has been reported that the release of cytochrome c from mitochondria to the cytosol, which is required for apoptosome assembly and subsequent activation of caspase–9/–3, and XIAP down–regulation, which relieves caspases from the inhibitory action exerted by that protein[28]. Moreover, IAPs are degraded by the ubiquitin–proteasome system[29], and earlier studies reported an increase in ubiquitin–conjugated proteins in flavonoid–treated cells[30]. Therefore, XIAP down–regulation probably reflects an increase in protein degradation. Our findings demonstrate that casticin–induced apoptosis of human NSCLC cells is involved in mitochondrial executioner pathway.

Of note, in addition to the mitochondrial pathway, casticin succeeded in activating the caspase–8/Bid pathway. The possibility that caspase–8 activation by casticin is a death receptor–mediated event, or alternatively represents a secondary event derived from mitochondrial activation[26,27]. Our findings demonstrated that casticin increased expression of DR5 at protein levels but did not affect the expression of DR4 in H157 cell line. Apoptosis by casticin is totally abrogated by the pan–caspase inhibitor z–VAD–fmk but only partially reduced by the caspase–8–specific inhibitor z–IETD–fmk and DR5/Fc chimera protein, a blocking antibody, suggesting that the caspase–8/Bid activation functions as an amplification loop for the final apoptotic result.

The cancer suppressor p53 is another important factor that affects the cell response to drug effects on growth inhibition and apoptosis induction[31,32]. The majority of evidence supports the notion that cells with wild–type p53 exhibit increased sensitivity to radiation or chemotherapeutic agents, whereas cells lacking wild–type p53 expression still undergo apoptosis but need a relatively high dose of radiation or chemotherapeutic drugs[31,32]. The investigation by Haĭdara et al[18] demonstrated that casticin induced the apoptotic cell death in p53 mutant or null breast cancer cell lines. In the present study, we found that casticin inhibited cell growth and induced apoptosis regardless of p53 status in human lung cancer cells. Therefore, we conclude that casticin–induced apoptosis in human lung cancer cells is p53–independent.

In conclusion, we have demonstrated that the polymethoxyflavone from *Fructus viticis*, casticin is a potent apoptosis–inducing agent in human NSCLC cells, which acts through both pathways involving mitochondria and death receptor, and subsequent activation of caspases. Moreover, casticin induces apoptosis independent of p53 status. Based on these findings, we suggest that casticin may be a good candidate for additional evaluation as a cancer therapeutic agent for human lung cancer as well as other types of cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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