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Effects and mechanisms of emodin on cell death in human lung squamous cell carcinoma

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1 Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is an active component from the root and rhizome of *Rheum palmatum* that has been reported to exhibit antitumour effects, but the mechanism is not known. The study investigated the effects and mechanisms of emodin-induced cell death in human lung squamous carcinoma cell line CH27.

2 Emodin (50 μM)-induced CH27 cell apoptosis was confirmed by cell morphological change, sub-G_1 formation in flow cytometry analysis, viability assay and degradation of focal adhesion kinase in this study.

3 Emodin-induced apoptosis of CH27 cells does not involve modulation of endogenous Bcl-X_L protein expression, but appears to be associated with the increased expression of cellular Bak and Bax proteins. This study also demonstrated the translocation of Bak and Bax from cytosolic to particulate fractions.

4 This study has shown that emodin-treated CH27 cells revealed the increases in the relative abundance of cytochrome *c* for the indicated time intervals in cytosolic fraction.

5 This study demonstrates that the activation of caspase-3, caspase-9 and caspase-8 is an important determinant of apoptotic death induced by emodin.

6 These results suggested that emodin induces CH27 cell death by Bax death pathway and Fas pathway.

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Keywords: Emodin; human lung squamous carcinoma cell line CH27; apoptosis; Bcl-X_L; Bak; Bax; cytochrome *c*; caspase

Abbreviations: CH27 cell, human lung squamous carcinoma cell line; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethylsulphoxide, DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(*β*-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FAK, focal adhesion kinase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween; Tris, tris (hydroxymethyl) aminomethane

Introduction

Emodin, an active component contained in the root and rhizome of *Rheum palmatum l.* (Polygonaceae) (Tsai & Chen, 1992; Liang *et al*., 1993), was found to have antitumour, antibacterial, diuretic and vasorelaxant effects (Koyama *et al*., 1988; Zhou & Chen, 1988; Huang *et al*., 1991). Emodin has also been reported to sensitize *HER-2/neu*-overexpressing lung cancer cells to chemotherapeutic drugs (Zhang & Hung, 1996) and repress transformation and metastasis-associated properties of *HER-2/neu* overexpression breast cancer cells (Zhang *et al*., 1995; 1998). However, the reason why the molecular mechanisms of emodin produced its biological effects remained unknown.

Apoptosis is a major form of cell death characterized by a series of stereotypic molecular features, such as expression and translocation of Bcl-2 family proteins, release of cytochrome *c*, and activation of caspases. Cytochrome *c*, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following the induction of apoptosis by many different stimuli including Fas, tumour necrosis factor (TNF) and chemotherapeutic agents (Liu *et al*., 1996; Kluck *et al*., 1997; Reed, 1997). Caspases, a family of cysteine proteases, play a critical role during apoptosis. There are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspases (caspase-3, -6 and -7) may be initiated by the most apical caspase, one involving caspase-8 and the other involving caspase-9 (Srinivasula *et al*., 1996; Zou *et al*., 1997; Srinivasula *et al*., 1998). Therefore, two typical apoptosis pathways, receptor (Fas)-mediated (involving caspase-8) and chemical-induced (involving caspase-9) apoptosis, have been suggested (Deveraux *et al*., 1999; Sun *et al*., 1999). The Bcl-2 family proteins, such as Bcl-2, Bcl-X_L, Bax and Bak, are the best-characterized regulators of apoptosis (Adams & Cory, 1998; Evan & Littlewood, 1998; Nomura *et al*., 1999). Many reports have indicated that the release of mitochondrial cytochrome *c* and activation of caspase-3 is blocked by anti-apoptosis members of the Bcl-2 family, such as Bcl-2 and Bcl-X_L, and promoted by proapoptotic members, such as Bax and Bak (Kluck *et al*., 1997; Yang *et al*., 1997; Jurgensmeier *et al*., 1998).

The major purpose of this study was to investigate whether emodin induced cancer cell death. Since the anticancer mechanisms of emodin were unclear, this study also investigated the mechanisms of emodin-induced cell death

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in the human lung squamous carcinoma cell line CH27. The results of the present study suggest that emodin induces CH27 cell death by the Bax death pathway and the Fas pathway. In other words, the pathway of emodin-induced CH27 cell apoptosis involved receptor-mediated and chemical-induced apoptosis.

Methods

Materials

Antipain, aprotinin, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), dithiothreitol, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)-N,N, N′,N′-tetraacetic acid (EGTA), emodin (1,3,8-trihydroxy-6-methylanthraquinone), leupeptin, pepstatin, phenylmethylsulphonyl fluoride, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide, and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.); anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibody were purchased from Amersham (Buckinghamshire, U.K.). Antibodies to various proteins were obtained from the following sources: caspase-3 and focal adhesion kinase were obtained from Transduction Laboratory (Lexington, KY, U.S.A.); Bcl-2, Bcl-X_L and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); Bak, caspase-8 and cytochrome *c* were purchased from PharMingen (San Diego, CA, U.S.A.). Caspase-9 was purchased from Oncogene Research Products (Boston, MA, U.S.A.). Enhanced chemiluminescent (Renaissance) detection reagents were obtained from NEN Life Science Products (Boston, MA, U.S.A.).

Cell culture

The human lung squamous cell line CH27 was kindly provided by S.L. Hsu (Taichung Veterans General Hospital, Taiwan). The cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 5% foetal bovine serum, antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) and 2 mM glutamine at 37°C in a humidified atmosphere comprised of 95% air and 5% CO_2. When CH27 cells were treated with emodin, the culture medium containing 1% foetal bovine serum was used. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

Cell viability assay

Cells were seeded at a density of 1×10^5 cells per well onto 12-well plate 24 h before drug treatment. Drugs were added to medium, at various indicated times and concentrations. The control cultures were treated with 0.1% DMSO (dimethylsulphoxide). After incubation, cells were washed with PBS (phosphate-buffered saline). The number of viable cells was determined by staining cell population with Trypan blue. One part of 0.2% Trypan blue dissolved in PBS was added to one part of the cell suspension, and the number of unstained (viable) cells was counted.

Mitochondrial reductase activity

Cells were seeded at a density of 1×10^5 cells per well onto a 12-well plate 24 h before drug treatment. Drugs were added to medium, at various indicated times and concentrations. The control cultures were treated with 0.1% DMSO. After incubation, cells were washed with PBS. Cellular mitochondrial reductase activity of live CH27 cells was determined by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). At each end point, the treatment medium was replaced with fresh, serum-free medium containing 2.4×10^{-4} M MTT at pH 7.4. Cells were incubated with MTT medium for 1 h at 37°C. After solubilization in DMSO, absorbance was measured at 550 nm.

4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining

Cells were seeded at a density of 1×10^5 cells per well onto a 12-well plate 24 h before drug treatment. CH27 cells were cultured for 16 h in 1% serum medium with vehicle alone (0.1% DMSO) or 50 μM emodin. After treatment, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg ml^{-1} DAPI for 5 min at 37°C. The cells were then washed with PBS and examined by fluorescence microscopy (Olympus IX 70).

Flow cytometry analysis

The percentage of hypodiploid cells was determined as previously described (Hsu *et al*., 1999). Briefly, 2×10^6 cells were trypsinized, washed twice with PBS, and fixed in 80% ethanol. Fixed cells were washed with PBS, incubated with 100 μg ml^{-1} RNase for 30 min at 37°C, stained with propidium iodide (50 μg ml^{-1}), and analysed on a FACScan flow cytometer (Becton Dickinson Instruments). The percentage of cells that had undergone apoptosis was assessed to be the ratio of the fluorescent area smaller than the G_0-G_1 peak to the total area of fluorescence. The average of the results from at least three samples of cells for each experimental condition is presented.

Protein preparation

Preparation of total protein Adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were resuspended in modified RIPA buffer (mM) (Tris-HCl 50, pH 7.5, NaCl 150, EGTA 1, DTT 1, phenylmethylsulphonyl fluoride 1, sodium orthovanadate 1, sodium fluoride 1, aprotinin 5 μg ml^{-1}, leupeptin 5 μg ml^{-1}, antipain 5 μg ml^{-1}, Nonidet P-40 1%, sodium deoxycholate 0.25%) for 30 min at 4°C. Lysates were clarified by centrifugation at $100{,}000 \times g$ for 30 min at 4°C and the resulting supernatant was collected, aliquoted (50 μg per tube) and stored at −80°C until assay. The protein concentrations were estimated with the Bradford method (Bradford, 1976).

Preparation of subcellular fractions Cell fractionation was performed as previously described (Jun *et al*., 1999) with some modifications. Briefly, adherent and floating cells were

collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were frozen at −80°C, thawed at 4°C and resuspended in cytosol extraction buffer (mM) (Tris-HCl 50, pH 7.5, EDTA 5, EGTA 10, phenylmethylsulphonyl fluoride 0.2, sodium orthovanadate 1, sodium fluoride 1, aprotinin 1 μg ml^{-1}, leupeptin 5 μg ml^{-1}, antipain 5 μg ml^{-1}, β-mercaptoethanol 0.3%) for 20 min at 4°C until >95% of the cells were Trypan blue positive. Lysates were clarified by centrifugation at 100,000 × g for 30 min at 4°C and the resulting supernatant was collected as the 'cytosolic' fraction, aliquoted (10 μg per tube for cytochrome c; 50 μg per tube for Bak and Bax). The pellet was resuspended in modified RIPA buffer for 30 min at 4°C and centrifuged as before. The supernatant was collected as the 'particulate' fraction, aliquoted (50 μg per tube) and stored at −80°C until assay.

Western blot analysis

Samples were separated by various appropriate concentrations (10, 12 and 15%) of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE). The SDS-separated proteins were equilibrated in transfer buffer (Tris 50 mM, pH 9.0–9.4, glycine 40 mM, 0.375% SDS, 20% methanol) and electrotransferred to Immobilon-P Transfer Membranes. The blot was blocked with a solution containing 5% nonfat dry milk in Tris-buffered saline (Tris 10 mM, NaCl 150 mM) with 0.05% Tween 20 (TBST) for 1 h, washed, and incubated with antibodies to focal adhesion kinase (1 : 1000) Bcl-2 (1 : 500), Bcl-X_L (1 : 500), Bax (1 : 500), Bak (0.2 μg ml^{-1}), cytochrome c (1 : 500), caspase-3 (1 : 1000), caspase-8 (1 : 1000) and caspase-9 (0.1 μg ml^{-1}). Secondary antibody consisted of a 1 : 20,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (for Bcl-2, Bcl-X_L, Bak, Bax, caspase-8 and cytochrome c) or HRP-conjugated goat anti-mouse IgG (for caspase-3, caspase-9 and focal adhesion kinase). The enhanced chemiluminescent (Renaissance) detection system was used for immunoblot protein detection.

Results

Morphological alterations by emodin in CH27 cells

The phenotypic characteristics of emodin-treated CH27 cells were evaluated by microscopic inspection of overall morphology. Treatment with 50 μM emodin for 4 h resulted in formation of vesicles in cells (Figure 1B). When cells were treated with emodin for 16 h, cells displayed a rounded morphology and eventually detached from the substratum (Figure 1D). In contrast, cells incubated in control medium were well spread, with flattened morphology (Figure 1C). Following 24 h emodin exposure, cellular fragmentation was extensive and few adherent cells remained (Figure 1F). To demonstrate whether the floating cells were dead, the detached cells were collected and incubated with fresh medium in the absence of emodin for 24 h. None of the cells were observed in the bottom of the petri-dish (data not shown), suggesting that emodin would induce the death of CH27 cells.

Emodin induces CH27 cell death in a dose- and time-dependent manner

This study determined the effect of emodin on cell viability by Trypan blue dye exclusion and mitochondrial reductase activity assay. The number of viable cells was counted by Trypan blue dye exclusion. As shown in Figure 2A, 72 h of continuous exposure to various concentrations of emodin resulted in time- and dose-dependent decreases in cell number relative to control cultures. The similar results of the effect of various concentrations of emodin for various indicated times on cell viability were obtained by mitochondrial reductase activity assay (Figure 2B). Mitochondrial reductase activity was assayed by reduction of MTT at 2, 4, 8, 16, 24, 48 and 72 h after the addition of control medium or various concentrations of emodin. The emodin-induced CH27 cell death was significant at 50 μM emodin. Therefore, 50 μM emodin was chosen for further experiments.

Emodin induces apoptosis of CH27 cells

Based on the emodin-induced morphological changes and cell death, the effects of emodin in CH27 cells were a clear indication of apoptosis. To obtain further support for the induction of apoptosis by emodin in CH27 cells, *in situ* DAPI staining and flow cytometry analysis were performed. Treatment with 50 μM emodin for 16 h resulted in changes in nuclear morphology, evidenced by the DAPI staining, a DNA binding dye (Figure 3). There was a gradual increase in the amount of nuclear condensation after treatment with emodin (Figure 3). Apoptosis was also confirmed on the appearance of a sub-G_1 peak of DNA content by flow cytometry, suggesting the presence of cells with fragmented DNA. According to the DNA histogram shown in Figure 4, a sub-G_1 peak was detected following 24 h of exposure to 50 μM emodin. During the emodin-induced apoptosis, normally flat cells became round in shape and detached from the extracellular matrix. Therefore, this study also demonstrated the degradation of focal adhesion kinase (FAK) during the emodin-induced apoptosis. When CH27 cells were treated with emodin (50 μM) for various indicated times, there was a significant decrease in the amount of FAK (Figure 5). These above data suggest that emodin-induced CH27 cell death is apoptosis.

Emodin-induced CH27 cell death is irreversible after 8 h of emodin-treatment

In order to determine if the inducement of emodin on cell death is reversible or irreversible, this study treated CH27 cells with 50 μM emodin in the presence of 1% serum for indicated time intervals, and washed off the reagents. These cells were then incubated with fresh serum-containing medium for 72 h. The viable cells were then measured by Trypan blue dye exclusion and mitochondrial reductase activity assay and the fraction of viable cells was calculated by defining the cells without treatment with emodin as 100%. When emodin was washed out after 1, 2, 4 and 8 h treatment, the cells started to grow and the growth rate was similar to the untreated cells. However, the fraction of viable cells decreased to 8% compared with untreated cells after cells were treated with 50 μM emodin for 16 h (Figure 6A). Similar

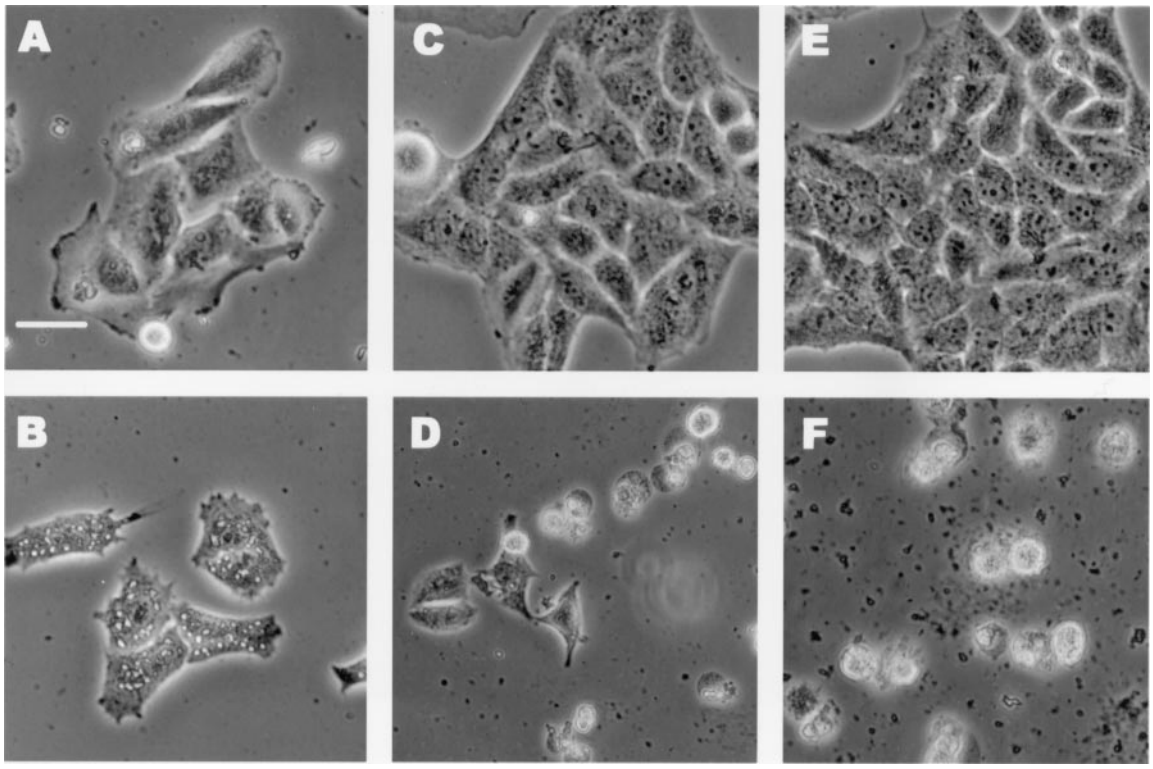

Figure 1 Changes in CH27 cell morphology during emodin-induced apoptotic cell death. Shown are phase-contrast views of CH27 cells cultured for 4 (A and B), 16 (C and D) and 24 h (E and F) in control (A, C and E) or 50 μM emodin treatment (B, D and F). Bar = 50 μm.

results of the effect of emodin for various indicated times on cell viability were obtained by MTT assay (Figure 6B). These results indicate that the emodin-induced CH27 cell death is irreversible after treatment with emodin for 8 h.

Effect of emodin on the expression of death-related proteins

The Bcl-2 family proteins are the best-characterized regulators of apoptosis. Some members of this family, such as Bcl-2 and Bcl-X_L, suppress apoptosis, whereas others, such as Bax and Bak, promote apoptosis. To elucidate whether the expressions of these cell death-related proteins are involved in emodin-induced apoptosis, this study examined the emodin regulation of Bcl-2, Bcl-X_L, Bax and Bak levels by Western blotting techniques during emodin-mediated apoptosis. Exposure of CH27 cells to 50 μM emodin resulted in increases in Bax and Bak levels after 8 h of treatment (Figure 7). Bcl-2 expression was up-regulated after 8 h treated with emodin and down-regulated after 16 h (Figure 7). Up to 24 h, there were no changes in Bcl-X_L protein levels (Figure 7). From these data, this study showed that the increase in Bax and Bak proteins expression was consistent with the onset of apoptosis in this cell line. This study also investigated the distribution of Bax and Bak in cytosolic and particulate fractions. Bax and Bak were clearly detected in cytosolic and particulate fractions. After the incubation of CH27 cells with 50 μM emodin for 2 h, cells exhibited extensive translocation of Bak (Figure 8) and Bax (Figure 9) from cytosolic fraction to particulate fraction. Densitometric analysis of the emodin-induced expression of Bak and Bax in cytosolic and particulate fractions was carried out on Table 1. From these data, this study showed that the expression of Bcl-2 family proteins was consistent with the onset of apoptosis in CH27.

Effect of emodin on the release of cytochrome c *in CH27 cells*

Since cytochrome *c* released from mitochondria is controlled by members of the Bcl-2 family of apoptosis regulators, this study characterized the effect of emodin on the release of cytochrome *c*. Western blotting analysis of the cytosolic fraction of emodin (50 μM)-treated CH27 cells revealed increases in the relative abundance of cytochrome *c* for the indicated time intervals (Figure 7). Furthermore, the amount of cytochrome *c* was significantly increased at 16 h treatment with emodin. This result suggested that the increase in cytochrome *c* released from mitochondria was associated with the emodin-induced apoptosis in CH27 cells.

Effect of emodin on the caspase-3, -8 and -9 in CH27 cells

To investigate the pathway of emodin-induced CH27 cell death, the activation of caspase-3, -8 and -9 was detected. Caspases are a family of cysteine proteases that are activated during apoptosis. Once activated, caspases cleave and activate downstream caspases. By Western blotting analysis, the proform of caspase-3 and caspase-8 were significantly decreased at 2 h of emodin treatment (Figure 10). However, emodin-treated made a significant change in proform and fragments of caspase-9 compared to control until 8 h (Figure 10). Untreated CH27 cells contained mainly the 46 kDa

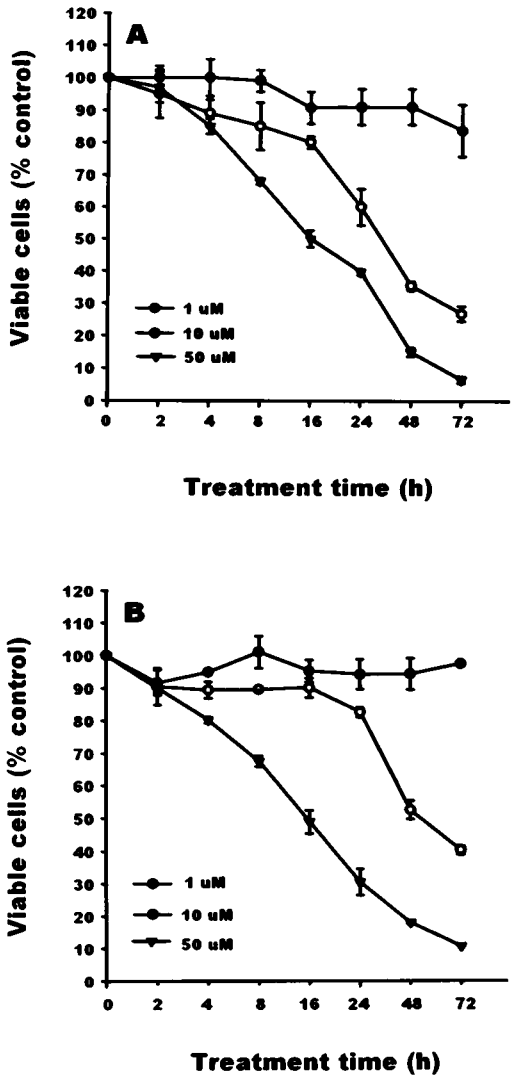

Figure 2 Effect of emodin on cell death in CH27 cells. (A) Cells were cultured 24 h before drug treatment in 12-well plates. Cells were treated without (control as 100%) or with emodin in the presence of 1% serum at 37°C for different times (2, 4, 8, 16, 24, 48 and 72 h), and cells were washed and counted by Trypan blue exclusion with a hemocytometer. All determinations are expressed as the mean ± s.d.mean of triplicate from three independent experiments. (B) Cells were cultured 24 h before drug treatment in 12-well plates. Cells were treated without (control as 100%) or with emodin in the presence of 1% serum at 37°C for different times (2, 4, 8, 16, 24, 48 and 72 h), the viable cells were measured by MTT assay and fraction of viable cells was calculated by defining the absorption of cells without treatment of emodin as 100%. All determinations are expressed as the mean per cent control ± s.d.mean of triplicate from three independent experiments.

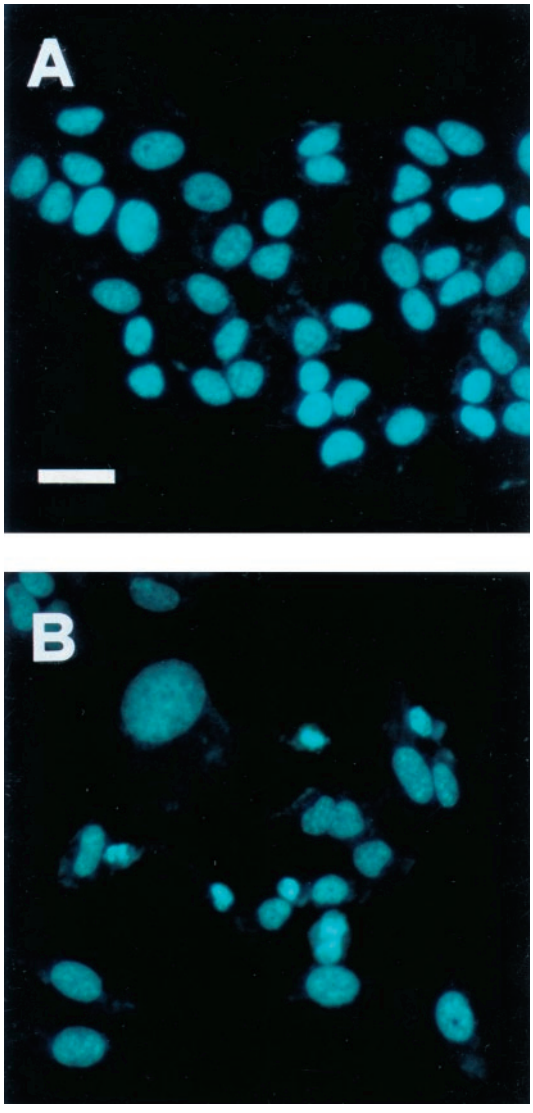

Figure 3 Emodin induced phenotypic changes in cell nucleus. CH27 cells were cultured for 16 h in 1% serum medium with vehicle alone (A) or 50 μM emodin (B). After treatment, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg ml^{-1} DAPI for 5 min at 37°C. The cells were then washed with PBS and examined by fluorescence microscopy. Results are representative of three independent experiments. Bar = 50 μm.

proform of caspase-9, which on stimulation of emodin was processed to yield a fragment of 32 kDa. After treatment with emodin for 8 h, the amount of the fragment of 32 kDa was more intense than that found in untreated cells. Caspase-3 was present in control CH27 cells primarily as 32 kDa protein. Treatment with 50 μM emodin resulted in a time-dependent processing of caspase-3 accompanied by the formation of two major products, 22 kDa and 17 kDa fragments. It is worthy of note that the quantity of these fragments of caspase-3 was significantly increased after treatment with emodin for 8 h. In control cells, a low level of processing of caspase-3 (22 and 17 kDa), caspase-8 (40 and 23 kDa) and caspase-9 (32 kDa) were observed; this may reflect basal caspase activity.

Discussion

The herb *Rheum palmatum* has been used as a Chinese medicine for a long time. Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is the major active constituent of *Rheum palmatum* (Tsai & Chen, 1992; Liang *et al*., 1993). Pharmacological studies have demonstrated that emodin possesses antitumour, antibacterial, diuretic and vasorelaxant effects (Koyama *et al*., 1988; Zhou & Chen, 1988; Huang *et*

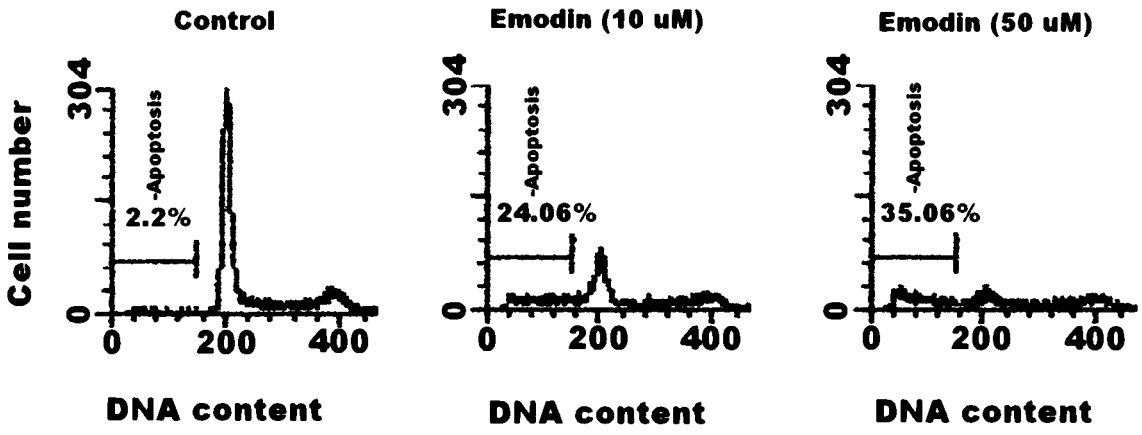

Figure 4 Emodin induced the appearance of a sub-G_1 peak in flow cytometry assay. Cells were treated with vehicle alone or 10 μM or 50 μM emodin in the presence of 1% serum for 24 h. After treatment, cells were harvested and subjected to cytometric analysis. Apoptosis was measured by cell cycle analysis with propidium iodide staining and the percentage of hypodiploid cells (apoptotic population of cells) was calculated. Results are representative of three independent experiments.

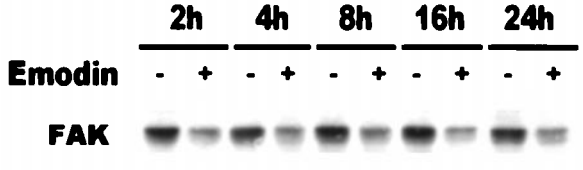

Figure 5 Emodin induced the degradation of focal adhesion kinase (FAK). Cells were treated with vehicle alone or 50 μM emodin in the presence of 1% serum for the indicated time periods (2, 4, 8, 16 and 24 h). Western blotting for FAK was performed on lysates prepared from cells incubated in control or emodin for 2, 4, 8, 16 and 24 h. Results are representative of three independent experiments.

al., 1991). Recently, some reports have also suggested that emodin can repress transformation and metastasis-associated properties of *HER-2/neu* overexpression breast cancer cells (Zhang *et al*., 1995; 1998). Since the mechanisms of emodin on anticancer were unclear, this study investigated the mechanisms of emodin-induced cell death in human lung squamous carcinoma cell CH27.

Apoptosis is a major form of cell death and essential for normal development and for the maintenance of homeostasis. In addition, current antineoplastic therapies, chemotherapy and radiation-therapy, are likely to be affected by the apoptotic tendencies of cells; thus this process has obvious therapeutic implications (Green *et al*., 1994). During apoptosis, certain characteristic morphologic events, such as nuclear condensation, nuclear fragmentation and cell shrinkage, and biochemical events, such as DNA fragmentation, occur (Hsu *et al*., 1999; Shinoura *et al*., 1999). In this study, the morphological change in emodin-induced CH27 cells, sub-G_1 peak formation in flow cytometry analysis and cell death (Trypan blue dye exclusion and MTT assay) were observed. Furthermore, Kook *et al*. (2000) have suggested that morphological changes, where normally flat cells became round in shape and detached from the extracellular matrix, appeared to be closely associated with degradation of focal adhesion proteins, including focal adhesion kinase (FAK) and paxillin, during apoptosis. In this study, degradation of FAK was significantly observed in emodin-induced cell death. Based on the above results, emodin-induced CH27 cell death was indicative of a typical apoptosis.

The Bcl-2 family proteins are the best-characterized regulators of apoptosis (Adams & Cory, 1998; Evan & Littlewood, 1998; Nomura *et al*., 1999). Some members of this family, such as Bcl-2 and Bcl-X_L, suppress apoptosis, whereas others, such as Bax and Bak, promote apoptosis (Hsu *et al*., 1999; Nomura *et al*., 1999). Emodin-induced apoptosis of CH27 cells does not involve modulation of endogenous Bcl-X_L protein expression, but appears to be associated with the increased expression of cellular Bax and Bak proteins. This result is consistent with previous observations in which Bax and Bak overexpression induced cell apoptosis due to a variety of stimuli, including chemotherapeutic agents such as etoposide and paclitaxel (Simonian *et al*., 1996; Strobel *et al*., 1996; Ibrado *et al*., 1997; Adams & Cory, 1998; Pastorino *et al*., 1998; Nomura *et al*., 1999). Furthermore, other investigators have also suggested that Bax and Bak exert their proapoptotic activity by translocation from the cytoplasm to the mitochondria, directly target the mitochondrial outer membrane channel, and allow cytochrome *c* to pass through this channel (Cosulich *et al*., 1997; Shimizu *et al*., 1999; Eilon *et al*., 2000). In this study, the translocation of Bax and Bak from cytosolic to particulate fractions was observed after treatment with 50 μM emodin. Emodin-induced apoptosis of CH27 cells appears to be associated with the increased expression of cellular Bax and Bak protein and translocated Bax and Bak from cytosolic to particulate fractions. This result is consistent with previous observations in which Bax and Bak overexpression or translocation were involved in cell apoptosis (Cosulich *et al*., 1997; Pastorino *et al*., 1998; Nomura *et al*., 1999; Shimizu *et al*., 1999; Eilon *et al*., 2000).

Cytochrome *c*, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following the induction of apoptosis by many different stimuli including Fas (CD95), tumour necrosis factor (TNF) and chemotherapeutic and DNA-damaging agents (Liu *et al*., 1996; Kluck *et al*., 1997; Reed, 1997). Since Bax and Bak directly target the mitochondrial outer membrane channel and allow cytochrome *c* to release from mitochondria (Eskes

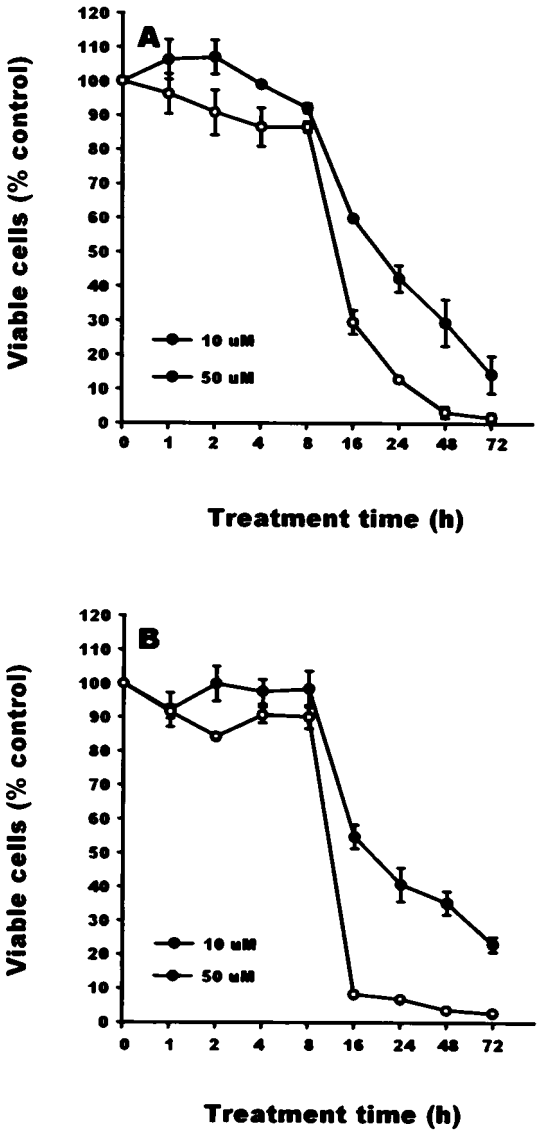

Figure 6 Inducing cell death by emodin is irreversible after 8 h treatment. CH27 cells were treated with or without 50 μM emodin in the presence of 1% serum for 1, 2, 4, 8, 16, 24, 48 and 72 h, and then washed off the reagents. After that, the cells were incubated with fresh medium without emodin for 3 days. The viable cells were measured by Trypan blue dye exclusion (A) and mitochondrial reductase activity assay (B) and the fraction of viable cells was calculated by defining the cells without treatment of emodin as 100%. The results are the mean $\pm$ s.d.mean of three independent experiments.

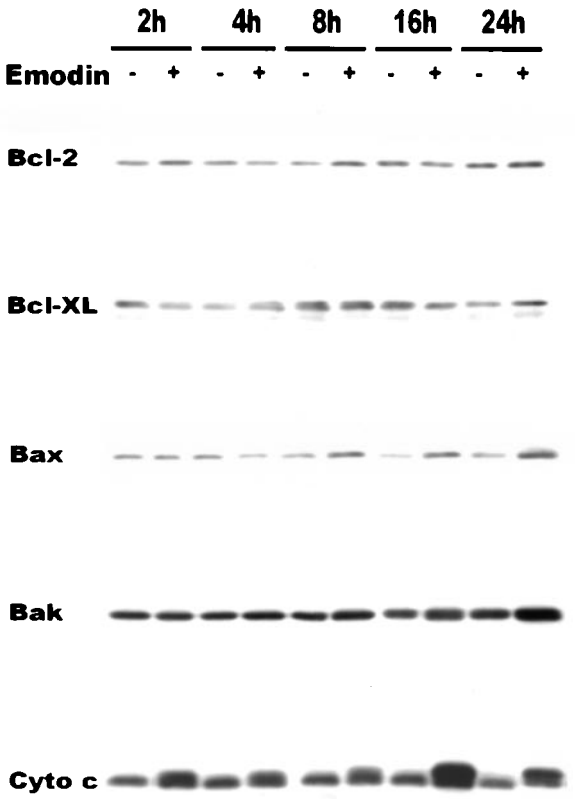

Figure 7 Effects of emodin on the expression of death-related proteins. The effect of emodin (50 μM) on death-related proteins was detected by Western blot analysis in CH27 cells. Cells were incubated with or without 50 μM emodin in the presence of 1% serum for 2, 4, 8, 16 and 24 h. Cell lysates were analysed by 12% (Bcl-2, Bcl-X_L, Bak and Bax) or cytosolic fractions were analysed by 15% (cytochrome *c*, Cyto c) SDS–PAGE and probed with primary antibody as described in Methods. Results are representative of three independent experiments.

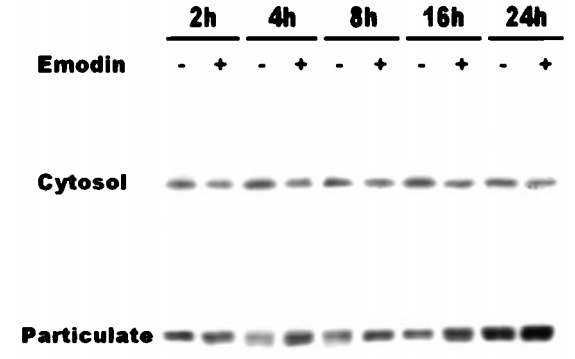

Figure 8 Western blot showing the effects of 50 μM emodin on Bak translocation in CH27 cells. Cells were incubated with or without 50 μM emodin in the presence of 1% serum for 2, 4, 8, 16 and 24 h. Cytosolic and particulate fractions were analysed by 12% SDS–PAGE and probed with primary antibody as described in Methods. Results are representative of three independent experiments.

et al., 1998; Green & Reed, 1998; Rosse *et al*., 1998; Susin *et al*., 1999), this study also characterized the effect of emodin on the release of cytochrome *c*. This study has shown that emodin (50 μM)-treated CH27 cells revealed increases in the relative abundance of cytochrome *c* in cytosolic fractions, particularly after treatment with emodin for 8 h. This indicated that the overexpression of Bax and Bak by emodin could be associated with the release of cytochrome *c* from mitochondria to cytosol. However, the increase in cytochrome *c* released from mitochondria during the 8 h treatment with emodin may be consistent with the onset of the translocation of Bak and Bax from cytosolic to particulate fractions in this cell line.

Caspases, a family of cysteine proteases, play a critical role in the apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis (Cohen, 1997; Cryns & Yuan, 1998). However,

other recent studies have shown that mitochondrial release of cytochrome *c* into the cytosol during apoptosis promotes the activation of caspase (Li *et al*., 1997; Reed, 1997; Zou *et al*., 1997). It has been proposed that 'initiator' caspases, such as caspase-8 and caspase-9, either directly or indirectly activate 'effector' caspases, such as caspase-3, -6 and -7 (Fraser & Evan, 1996; Srinivasula *et al*., 1996; Sun *et al*., 1999). Recently, many investigators have suggested that two prototypical pathways for induction of apoptosis in mammalian cells are induced by Bax (involving caspase-9) and Fas (involving caspase-8). Bax induced apoptosis by promotion of cytochrome *c* released from mitochondria. Once cytochrome *c* is released, together with cofactor nucleotide triphosphate (dATP or ATP), apoptosis promoting factor (Apaf-1) then binds and activates procaspase-9, which in turn cleaves and activates caspase-3 and other downstream caspases (Li *et al*., 1997; Reed, 1997; Zou *et al*., 1997). However, Fas is known to induce apoptosis through mitochondria-independent mechanisms. Fas (as termed CD95 and APO-1) is a member of the tumour necrosis factor (TNF) family of apoptosis-inducing receptors that induces apoptosis in sensitive cells upon oligomerization by the interaction with its ligand FasL, then activates procaspase-8 and possibly other initiator caspases (Boldin *et al*., 1996; Muzio *et al*., 1996; Wallach *et al*., 1997). Caspase-8 then cleaves and activates caspase-3 and other downstream caspases that function as the ultimate effectors of apoptosis. Therefore, caspase-8 and caspase-9 are the most apical caspases in receptor-mediated and chemical-indued apoptosis, respectively.

To investigate the pathway of emodin-induced CH27 cells death, the activation of caspase-3, -8 and -9 was detected. By Western blotting analysis, the proform of caspase-3 and caspase-8 were significantly decreased at emodin-treated 2 h. However, emodin-treated cells had a significant change in proform and fragments of caspase-9 compared to control

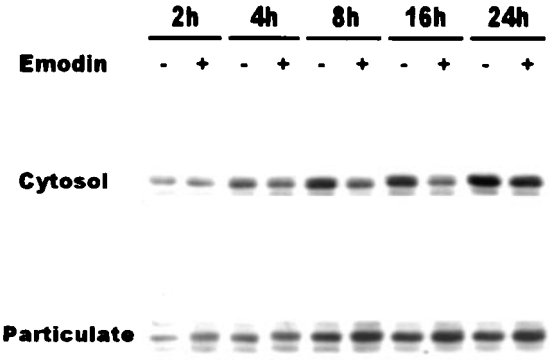

Figure 9 Western blot showing the effects of 50 μM emodin on Bax translocation in CH27 cells. Cells were incubated with or without 50 μM emodin in the presence of 1% serum for 2, 4, 8, 16 and 24 h. Cytosolic and particulate fractions were analysed by 12% SDS–PAGE and probed with primary antibody as described in Methods. Results are representative of three independent experiments.

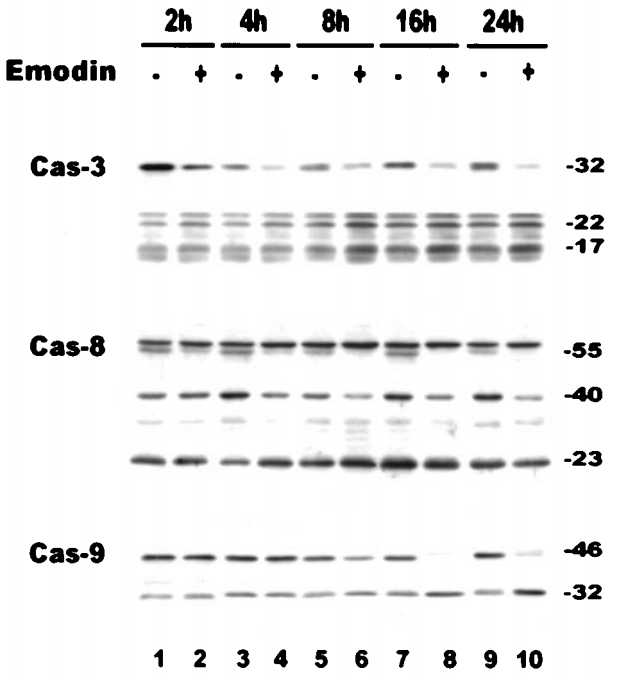

Figure 10 Effects of emodin on the expression of caspase-3 (Cas-3), -8 (Cas-8) and -9 (Cas-9). The effect of emodin (50 μM) on caspase-3, -8 and -9 was detected by Western blot analysis in CH27 cells. Cells were incubated with or without 50 μM emodin in the presence of 1% serum for 2, 4, 8, 16 and 24 h. Cell lysates were analysed by 10% (caspase-9) and 12% (caspase-3 and caspase-8) SDS–PAGE, and then probed with primary antibody as described in Methods. Results are representative of three independent experiments.

Table 1 Densitometric analysis of the emodin-induced expression of Bak and Bax in cytosolic and particulate fractions

		Densitometric intensity (% of control)			
		Bak		*Bax*	
Time	*Treatment*	*Cytosolic*	*Particulate*	*Cytosolic*	*Particulate*
2 h	Control	100	100	100	100
	Emodin	72.8±5.2	118.8±4.7	75.4±4.0	109.8±7.0
4 h	Control	100	100	100	100
	Emodin	62.0±3.5	151.0±7.2	74.9±3.6	132.7±5.6
8 h	Control	100	100	100	100
	Emodin	55.7±2.3	159.5±4.7	55.7±3.5	155.8±6.0
16 h	Control	100	100	100	100
	Emodin	48.7±3.0	167.5±5.6	49.5±4.0	181.6±5.3
24 h	Control	100	100	100	100
	Emodin	63.7±4.4	158.0±6.5	70.3±3.1	164.8±7.0

Densitometric analysis of the emodin-induced expression of Bak and Bax in cytosolic and particulate fractions in Figures 8 and 9 was carried out above. Data are plotted as the mean percentage of the relative control (absence of emodin)±s.d.mean ($n=3$).

after 8 h. Untreated CH27 cells contained mainly the 46 kDa proform of caspase-9, which on stimulation by emodin was processed to yield a fragment of 32 kDa. After treatment with emodin for 8 h, the amount of the fragment of 32 kDa was more intense than that found in untreated cells. Caspase-3 was present in control CH27 cells primarily as 32 kDa protein. Treatment with 50 μM emodin resulted in a time-dependent processing of caspase-3 accompanied by the formation of two major products, 22 kDa and 17 kDa fragments. It is worthy of note that the quantity of these fragments of caspase-3 was significantly increased after treatment with emodin for 8 h. This time point is consistent with the time of activation of caspase-9. It indicates that caspase-9 may be an upstream activator of caspase-3 of emodin-induced apoptosis in CH27 cells. The study also observed that the activation of caspase-8 was before the activation of caspase-9. Therefore, the activation of caspase-3 before the 8 h treatment with emodin may be induced by caspase-8. Based on the above results, the activation of caspase-3, caspase-8 and caspase-9 is an important determinant of apoptotic death induced by emodin. These results suggested that emodin induces CH27 cell death by the Bax death pathway and the Fas pathway.

In summary, the present study demonstrated that emodin induced apoptotic cell death in CH27 cells. Bax and Bak overexpression and translocation were involved in emodin-induced apoptosis of CH27 cells. The release of cytochrome *c* from mitochondria to cytosol by emodin was regulated in part by Bax and Bak regulation of mitochondrial function. Caspase activation in this model occurs, at least in part, *via* the mitochondrial death pathway, as evidenced by the translocation of cytochrome *c* from the mitochondria to the cytosol and the activation of both caspase-9 and caspase-3. This study has also demonstrated that the activation of caspase-8 is involved in emodin-induced CH27 cell death and is before the activation of caspase-9.

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