Food and Chemical Toxicology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Euphol from *Euphorbia tirucalli* selectively inhibits human gastric cancer cell growth through the induction of ERK1/2-mediated apoptosis

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ARTICLE INFO

Article history:
Received 15 December 2011
Accepted 16 May 2012
Available online xxxx

Keywords:
Euphol
Human gastric cancer
ERK1/2
Anti-proliferation
Apoptosis

ABSTRACT

Gastric cancer is one of the most common malignancies worldwide, and the main cause of cancer-related death in Asia. The present study assessed the anticancer effects of euphol, a triterpene alcohol with anti-inflammatory and antiviral activities on human gastric cancer cells. Euphol showed higher cytotoxicity activity against human gastric CS12 cancer cells than against noncancer CSN cells. In addition, it up-regulated the pro-apoptotic protein BAX and down-regulated the prosurvival protein Bcl-2, causing mitochondrial dysfunction, possibly by caspase-3 activation. The anti-proliferative effects of euphol were associated with the increased $p27^{kip1}$ levels and decreased cyclin B1 levels. Inhibition of ERK1/2 activation by PD98059 reversed euphol-induced pro-apoptotic protein expression and cell death. Taken together, these findings suggest that euphol selectively induced gastric cancer cells apoptosis by modulation of ERK signaling, and could thus be of value for cancer therapy.

1. Introduction

Gastric cancer is one of the most common malignancies worldwide, accounting for nearly half of cancer-related mortality (Shah and Kelsen, 2010). Chemotherapy is the treatment of choice for gastric cancer, but the currently available therapeutic drugs for the treatment of gastric cancer have limited efficacy (Zhang et al., 2006). Combination chemotherapy is often associated with toxic side effects. Therefore, new agents that selectively target gastric cancer cells are urgently needed.

Recent studies have shown that the mitogen-activated protein kinase (MAPK) pathway may modulate cancer cell apoptosis and proliferation (Kim and Choi, 2010). The MAPK pathway is well-studied molecular targets for chemotherapeutic drug development, and several related clinical trials have been completed in patients with metastatic and local cancer (Dangle et al., 2009). Extra-cellular signal-regulated kinase 1/2 (ERK1/2) belongs to one of the sub-groups of MAPKs and important in a variety of signaling pathways that regulate multiple cellular processes. ERK1/2 mediates gene and protein expression changes in response to extracellular stimuli (Tibbles and Woodgett, 1999). The involvement of ERK1/2 in the regulation of cell proliferation has been extensively described (Ballif and Blenis, 2001). However, in some cell models, activation of ERK1/2 is associated with the induction of apoptosis (Lu et al., 2009; Wang et al., 2000).

Apoptosis is a form of cell death that can be triggered by several external or internal signals. The loss of mitochondrial membrane potential is the hallmark of the intrinsic apoptosis pathway. Mitochondria modulate the caspase–apoptosis cascade by regulating the translocation of cytochrome *c* from the mitochondrial inner-membrane space to the cytosol. Pro-apoptotic proteins, such as Bcl-2-associated X protein (BAX), can directly interact with the mitochondrial permeability transition pore complex. BAX displaces this complex from its inhibitory interaction with the pro-survival protein, B-cell lymphoma 2 (Bcl-2), disrupting the mitochondrial membrane potential and leading to the permeabilization of the mitochondrial membrane and the activation of the cytochrome

Abbreviations: BAX, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; ERK1/2, extra-cellular signal-regulated kinase 1/2; IC_{50}, 50% inhibitory concentration; FITC, fluorescein isothiocyanate; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; p-ERK1/2, phosphorylated extra-cellular signal-regulated kinase 1/2.

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0278-6915/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.fct.2012.05.029

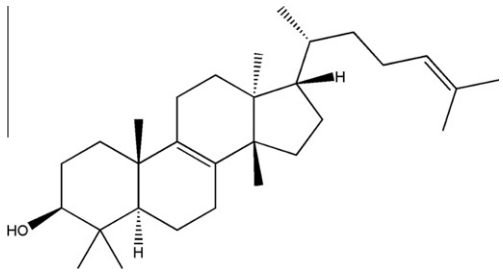

Fig. 1. Chemical structure of euphol.

c-caspase-dependent apoptosis pathway (Fulda et al., 2010; Tait and Green, 2010).

The latex of *Euphorbia tirucalli* (Euphorbiaceae), which is native to Madagascar, was used in indigenous medicine as a purgative and a remedy for rheumatism, neuralgia, and toothache in Africa and Asia (Rasool et al., 1989). In South Taiwan, its branches are boiled in water and used as one of ingredients of anticancer herbal drinks. However, the milky latex of this plant is considered to be poisonous (Lin et al., 2001) and possesses highly vesicant and irritant properties toward the skin and mucous membranes (Furstenberger and Hecker, 1977b). Studies have shown that the highly unsaturated irritant phorbol esters were the main constituents responsible for the toxicity of the latex (Furstenberger and Hecker, 1977a,b; Khan et al., 1988; Lin et al., 2001; Yoshida et al., 1991).

Euphol is a euphane-type triterpene alcohol (Fig. 1). It is isolated from the dichloromethane extract of *E. tirucalli*, and exhibits a variety of biological activities, such as anti-viral (Akihisa et al., 2002) and anti-inflammatory activities (Akihisa et al., 1997). In a recent study, a topical application of euphol was shown to markedly suppress the tumor-promoting effect in 2-stage carcinogenesis in mouse skin (Yasukawa et al., 2000). However, the mechanisms underlying this effect and the potential antitumor properties of euphol remain to be evaluated.

The results of the present study indicate that euphol has antiproliferative effects and selectively induces gastric cancer cell death in an ERK1/2-dependent manner. Moreover, euphol modulates the expression of cell cycle regulator proteins and promotes apoptosis by means of the mitochondrial apoptotic pathway.

2. Materials and methods

2.1. Isolation of euphol

The fresh aerial parts of *E. tirucalli* (Gildenhuys, 2006; Rasool et al., 1989) were collected in the Tainan County, Taiwan, in August 2002 and identified by botanist Dr. Ming-Hong Yen, Kaohsiung Medical University, Kaohsiung, Taiwan. The latex of the fresh plant was collected drop by drop, and the remaining aerial parts of the plant (15.0 kg) were extracted with MeOH. The evaporated latex MeOH extract (5.9 g) was separated by column chromatography on a silica gel (300 g) with a gradient system of *n*-hexane/$CHCl_3$ (3:1, 2:1, and 1:1, at 800 mL each) and $CHCl_3$ (1000 mL), yielding 20 fractions. Fractions of 7–9 (4.6 g) were combined and further purified by a silica gel column (200 g) with *n*-hexane/$CHCl_3$ (3:1, 1500 mL), yielding euphol (4.2 g) as the major constituent and triterpene.

2.2. Cell culture

The novel human gastric cancer cell line, KMU-CS12 (CS12) and human gastric cell line, KMU-CSN (CSN) were established in our previous studies (Yang et al., 2007, 2009). CSN and CS12 cells were cultured in kerotinocyte-Serum-free medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum, *N*-acetyl-L-cysteine (360 μg/mL), and L-ascorbic acid 2-phosphate (51.2 μg/mL). Human gastric adenocarcinoma AGS cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and MKN45 (a poorly differentiated human gastric adenocarcinoma) cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). The AGS and MKN45 cells were grown in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum.

2.3. WST-1 cell cytotoxicity assay

The cytotoxicity of euphol was assessed using a WST-1 cell proliferation kit (Roche. Applied Science, Basel, Switzerland). The cells were seeded for 72 h at a concentration of 5×10^4 cells/well in culture medium containing various amounts of euphol (2, 5, 10, 20, 40, and 60 μg/mL) in 96-well microplates. The reduction of the tetrazolium salt of the reagent to a formazan product by cellular dehydrogenases was detect by the generation of a yellow-color, which was measured at 440 nm with a microplate ELISA reader.

2.4. Detection of Annexin V-positive apoptotic cells

Apoptotic cells were detected by Annexin V staining (BioVision, Mountain View, CA, USA) according to the manufacture's instructions. Briefly, the cells were washed with phosphate buffered saline (PBS) and resuspended with Annexin binding buffer (Invitrogen). After treatment with annexin V-FITC (1:500) and propidium iodide (PI), the cells were incubated for 15 min in the dark. Annexin V-positive apoptotic cells (compared to unlabeled cells) were then analyzed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

2.5. Detection of mitochondrial transmembrane potential

The CS12 cells were pretreated with PD98059 (13.4 μg/mL) or vehicle (DMSO) for 30 min and incubated with euphol (20 μg/mL) for 72 h. The cells were washed with warm PBS and incubated with MitoTraker (Invitrogen) for 30 min at 37 °C in the dark. The cells were washed with warm PBS again, and the fluorescence intensity was determined by means of a FACScan flow cytometer (Becton Dickinson).

2.6. Caspase-3 activation assay

FITC-DEVD-FMK is cell permeable, nontoxic, and irreversibly binds to activated caspase-3 in apoptotic cells. Therefore, an anti-fluorescein isothiocyanate (FITC)-DEVD-FMK antibody was used to further confirm the role of the ERK1/2 MAPK pathway in the euphol-induced caspase-3 activation by flow cytometry. The CS12 cells were pretreated with PD98059 (13.4 μg/mL) or vehicle (DMSO) for 30 min and then incubated with euphol (20 μg/mL) for 72 h. The cells were washed with PBS and incubated with FITC-DEVD-FMK (BioVision) for 30 min at 37 °C in the dark. The cells were washed with warm PBS, and the fluorescence intensity was determined by means of a FACScan flow cytometer (Becton Dickinson) as described before (Carvalho et al., 2008).

2.7. Western blotting

For ERK1/2 phosphorylation assays, CSN, CS12, AGS and MKN45 cells were treated with euphol (20 μg/mL) for 4, 24, 48, and 72 h. For apoptotic protein expression level assays, the CS12 cells were pretreated with PD98059 (13.4 μg/mL) or vehicle (DMSO) for 30 min and then incubated with euphol (10 or 20 μg/mL) for 72 h. The cells were lysed using a commercially available lysis buffer, M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). Equal protein amounts were loaded onto 10% SDS–PAGE gels, and the separated proteins were transferred to PVDF membranes, blocked with 5% nonfat dried milk in PBST buffer, and incubated with anti-phospho-ERK1/2 (Cell Signaling, Beverly, MA, USA), anti-ERK1/2 (Cell Signaling), anti-BAX (StressGen, Victoria, BC, Canada), anti-Bcl-2 (Stressgen), anti-β-actin (Sigma–Aldrich, St. Louis, MO, USA), anti-p27 (Cell Signaling), or anti-cyclin B1 (Enzo Life Sciences, Farmingdale, NY, USA) primary antibody overnight. After probing with a horseradish peroxidase-conjugated secondary-antibody (GE Healthcare, Piscataway, NJ, USA) and thoroughly washing the membranes, the immunolabeled proteins were detected using an enhanced chemiluminescence kit (GE Healthcare), followed by exposure to an X-ray film.

2.8. Statistical analyses

The results were expressed as means ± SD. Statistical comparisons were performed with the Student *t*-test. The statistical significance was set at $P < 0.05$.

3. Results

3.1. Inhibition of gastric cancer CS12 cell proliferation by euphol

The antiproliferative effects of various concentrations of euphol (2, 5, 10, 20, 40 and 60 μg/mL) on CSN, CS12, AGS and MKN45 cells are shown in Fig. 2. The results of the WST-1 assay demonstrated that euphol inhibited the growth of CS12 cells and that of the commercially available AGS and MKN45 cell lines in a dose-dependent manner. To examine whether the growth inhibitory effect of euphol was mediated by apoptosis induction, the gastric cancer and

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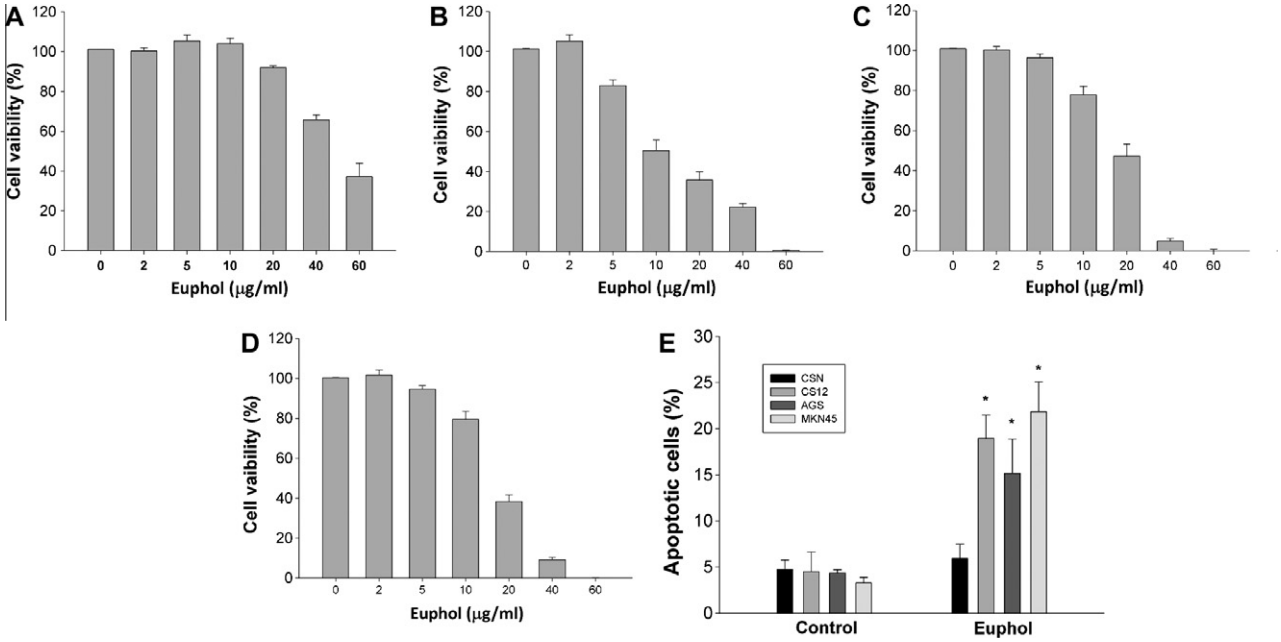

Fig. 2. Inhibitory effect of euphol on gastric cancer cell growth. The results of the WST-1 assays showing that euphol inhibited (A) CSN, (B) CS12, (C) AGS, and (D) MKN45 proliferation in a dose-dependent manner after 72 h of exposure to the drug. (E) The CSN, CS12, AGS, and MKN45 cells were treated with euphol (20 μg/mL) for 48 h. Euphol selectively induced apoptosis in 3 gastric cancer cells (CS12, AGS, and MKN45). The bars represent the mean ± SD of the 3 independent experiments (*$P < 0.01$, compared with the CSN cells).

normal cells were treated with euphol (20 μg/mL) and exposed to annexin V/PI staining.. As shown in Fig. 2E, the rate of apoptosis was greater in the euphol-treated gastric cancer cells than in the normal cells ($P < 0.01$). The euphol treatment significantly induced cell death in the gastric cancer CS12, AGS, and MKN45 cell lines but not in the CSN cells. The IC_{50} values for euphol in CSN, CS12, AGS and MKN45 cells were 49.6, 12.8, 14.7 and 14.4 (μg/mL), respectively.

3.2. Euphol induction of ERK1/2 phosphorylation in CS12 cells

The ERK1/2 MAPK pathway regulates many cellular activities, especially cell proliferation and apoptosis (Ballif and Blenis, 2001; Wang et al., 2000). To examine the role of ERK1/2 MAPK signaling in the apoptosis of gastric cancer cells induced by euphol, the CS12, AGS, MKN45 and CSN cells were treated with 20 μg/mL of euphol at various time points. As shown in Fig. 3B, the euphol treatment induced ERK1/2 activation in a time-dependent manner in the CS12 cells. Similar results were obtained in the AGS and MKN45 gastric cancer cell lines (Fig. 3C and D). In addition, the accumulation of phosphorylated ERK1/2 was significantly increased after 72 h in the euphol-treated gastric cancer cell lines, whereas no significant activation of ERK1/2 was observed in the CSN cells (Fig. 3A) under the same treatment conditions. To confirm the involvement of ERK1/2 in the euphol-induced growth inhibition, the CS12 cells were treated with the ERK1/2 inhibitor PD98059. As shown in Fig. 3E and F, PD98059 had a mild inhibitory effect on the euphol-induced apoptosis in this cell line, suggesting that the ERK1/2 MAPK pathway may participate play a role in euphol-induced CS12 apoptotic cell death.

3.3. Role of ERK1/2 in the euphol-induced mitochondrial-dependent apoptosis pathway

The role of ERK1/2 in the euphol-induced apoptosis pathway and the expression profiles of pro-apoptotic and prosurvival proteins in the euphol-treated CS12 cells were examined by Western blotting to measure the BAX and Bcl-2 protein expression levels. The treatment of the cells with euphol for 72 h markedly upregulated the BAX expression and downregulated Bcl-2 protein expression in a dose-dependent manner, and PD98059 reversed the effects of euphol on the expression of the apoptosis-related protein (Fig. 4A and B). The translocation of the pro-apoptotic protein BAX to mitochondria may result in the loss of mitochondrial membrane potential, and the induction of the caspase-mediated apoptosis pathway (Fulda et al., 2010). As shown in Fig. 4C, a shift in the euphol-treated cells toward the left compared with the vehicle-treated controls indicated that euphol (20 μg/mL) disrupted the mitochondrial membrane potential, as assessed by flow cytometry. In contrast, the pretreatment with PD98059 (13.4 μg/mL) resulted in a right shift of the MitoTracker fluorescent curves for the euphol-treated CS12 cells, indicating that the euphol-induced mitochondrial dysfunction was ERK1/2-dependent. Fig. 4D shows a shift of the euphol-induced FITC fluorescence to the right, which was inhibited by PD98059, suggesting that euphol-induced apoptosis in gastric cancer CS12 cells may be mediated by ERK1/2 regulation of the mitochondrial apoptotic pathway.

3.4. ERK1/2 contributes to the antiproliferative effect of euphol

To examine the mechanisms underlying the antiproliferative effects of euphol in gastric cancer CS12 cells, the expressions of $p27^{kip1}$ and cyclin B1 were assessed by Western blotting. As shown in Fig. 5, euphol altered the expression of these cell cycle regulatory proteins by inducing $p27^{kip1}$ expression and inhibiting cyclin B1 expression. Furthermore, pretreatment with PD98059 markedly abolished the upregulation of $p27^{kip1}$ and downregulation of cyclin B1 in response to the euphol treatment.

4. Discussion

The present results demonstrate that euphol has antiproliferative activity against CS12 gastric cancer cells and its mechanism of action involves the alteration of the expression of cell cycle

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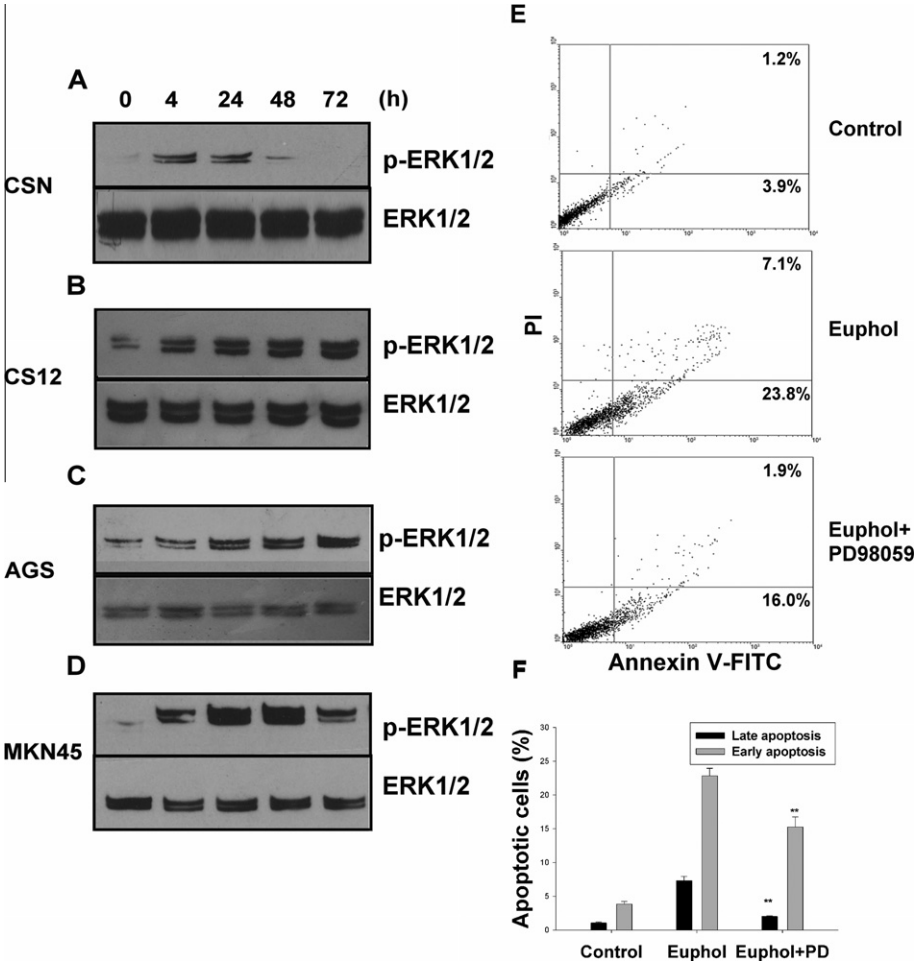

Fig. 3. Euphol-induced sustained ERK1/2 phosphorylation in gastric cancer cells. (A–D) The Western blot analyses of ERK1/2 phosphorylation. ERK1/2 level was used as internal control for phospho-ERK1/2. The euphol-induced ERK1/2 phosphorylation in the CS12, AGS, and MKN45 gastric cancer cells in a time-dependent manner. The sustained ERK1/2 activation was observed in the CS12, AGS, and MKN45 cells but not in the CNS cells. (E) The CS12 cells were pretreated with PD98059 (13.4 μg/mL) or vehicle (DMSO) for 30 min and then incubated with euphol (20 μg/mL) for 72 h. The percentage of apoptotic cells was quantified by flow cytometry. (F) Pretreatment with PD98059 reduced euphol-induced apoptosis. The bars represent the mean ± SD of the 3 independent experiments (**$P < 0.01$, compared with the euphol-treated cells).

regulatory proteins and the induction of apoptosis. The pretreatment with the ERK1/2 inhibitor PD98059 suppressed euphol-induced apoptosis, suggesting that the effect of euphol is participating mediated by an ERK1/2-associated pathway. Although ERK1/2 activation is generally related to cell proliferation and survival (Ballif and Blenis, 2001), increasing evidence indicates that ERK1/2 also transmits death signals. Its role in the promotion of apoptosis induced by anticancer drugs has been reported. The sustained activation of ERK1/2 for a period of 1–72 h has been reported to promote cell death in different cell types (Cagnol and Chambard, 2010). Long-term activation of the ERK1/2 pathway has been detected in association with cisplatin-, apiginin-, gemcitabine-, and adriamycin-induced apoptosis in HeLa, prostate, and pancreatic cancer cells (Wang et al., 2000; Zhao et al., 2006). Prolonged ERK1/2 activation has been associated with cell growth arrest and cell death (Martin et al., 2006; Martin and Pognonec, 2010; Tong et al., 2011). Previous studies have shown that the activities of platinum-based chemotherapeutic drugs are ERK1/2 dependent (Sheridan et al., 2010; Wang et al., 2000). However, the sustained ERK1/2 activation resulting in cell death remains poorly understood. Lu et al. (2009) demonstrated that ERK1/2 mediated the ubiquitination of the proto-oncogene MDM2, induced by the medical plant hispolon, indicating that it could be useful for the treatment of tumors with constitutive ERK1/2 activation. In the present study, enhanced ERK1/2 activation was observed in gastric CS12, AGS, and MKN45 cancer cells, but not in gastric CSN nontumorigenic cells, 72 h after the addition of 20-μg/mL euphol. The sustained activation of the ERK1/2 pathway in gastric cancer cells may play a significant role in the induction of apoptosis and growth arrest by euphol. ERK1/2 activation is tightly regulated in normal cells by ERK-specific phosphatases that ensure cellular homeostasis (Murphy and Blenis, 2006). However, the sustained activation of ERK1/2 triggers the production of ROS, which further inhibit ERK-specific phosphatases (Levinthal and Defranco, 2005). The dysregulation of ERK1/2 activation thus induces the progressive accumulation of death-promoting factors and cell death by apoptosis or necrosis.

Euphol is a cholesterol-like compound and therefore may possess toxic properties through its interaction with the plasma membrane and replacement of cholesterol. These effects should be investigated in future studies. Cholesterol is a key molecule in the cell membrane and is the main component of specialized lipid microdomains called lipid rafts, which are involved in the regulation of phosphorylation cascades (George and Wu, 2012). Depletion of cholesterol from the cell membrane alters signal transduction cascades and induces cancer cell death (Biondo et al., 2008). Cholesterol was reported to accumulate in a variety of tumor types (Freeman and Solomon, 2004), and high cholesterol levels in the cell membrane induced tumor cell proliferation through the lipid raft-AKT pathway (Zhuang et al., 2005). In addition, elevated levels of membrane cholesterol in cancer cells were

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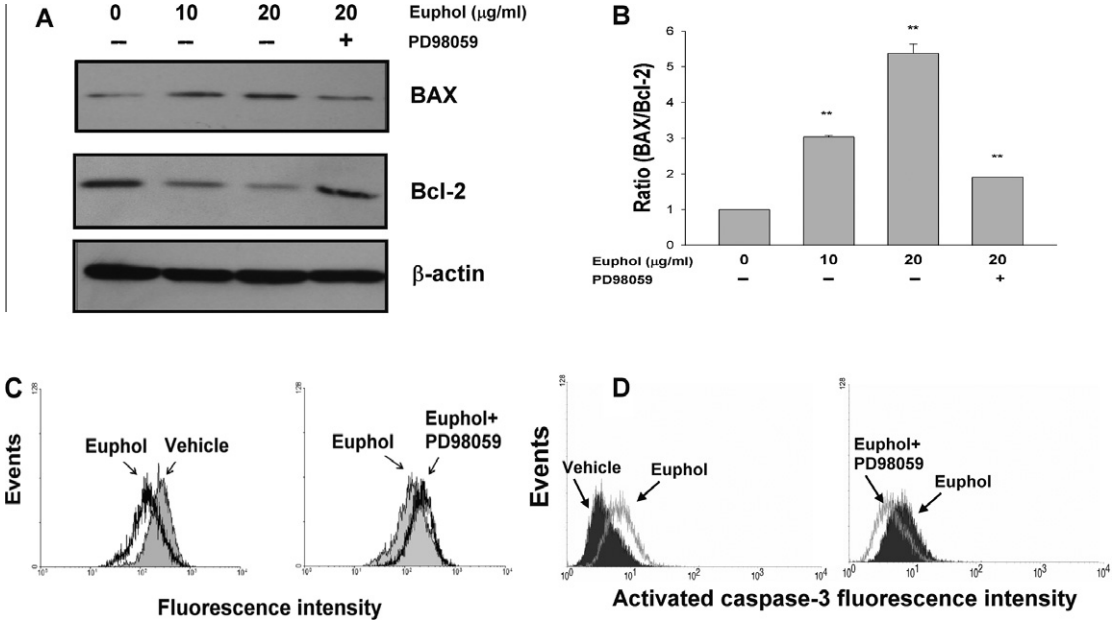

Fig. 4. Role of ERK1/2 in the euphol-induced mitochondrial membrane potential loss and apoptotic-related protein expression. (A) The Western blot analyses of the expressions of BAX and Bcl-2 in the euphol-treated CS12 cells. Beta-actin was used as an internal control. (B) Quantification of the Bcl-2 and BAX protein expressions from the Western blot analyses. Euphol increased the BAX/Bcl-2 ratio in a dose-dependent manner; however, PD98059 decreased the ratio in the euphol-treated cells. (C) The mitochondria membrane potential was analyzed by flow cytometry. PD98059 reversed the euphol-induced mitochondrial membrane potential loss in the CS12 cells. (D) Caspase-3 activation was determined by flow cytometry, using an anti-FITC-DEVD-FMK antibody. Each bar is the mean ± SD of the 3 independent experiments (**$P < 0.01$, compared with the control group).

correlated with apoptosis sensitivity induced by methyl-β-cyclodextrin, a cholesterol-depleting agent (Li et al., 2006). These findings suggest that differences in the potency of euphol between cancer and noncancer cells may be related to the membrane cholesterol content, lipid raft-related signal transduction and phosphatase regulation.

Euphol induced apoptosis in CS12 cells, as evidenced by annexin V-binding assays, flow cytometric detection, and Western blotting. Because gastric cancer cells show higher phosphatidylserine levels in the outer leaflet of the plasma membrane (Woehlecke et al., 2003), inhibition of ERK1/2 only slightly reduced annexing V binding in our results. However, the pretreatment with PD98059 markedly inhibited the downregulation of Bcl-2 in response to the euphol treatment, indicating that the ERK1/2 pathway may be involved in the antiproliferative effect of euphol. Moreover, euphol-induced apoptosis was associated with the upregulation of BAX, loss of mitochondrial membrane potential, and increased caspase-3 activity. BAX plays a critical role in the breakdown of the mitochondrial potential by translocating to the mitochondria in response to death stimuli (Tait and Green, 2010). The loss of mitochondrial membrane potential is associated with mitochondrial dysfunction, which is linked to apoptosis (Green and Reed, 1998). Therefore, euphol may play a critical role in the induction of apoptosis by altering the BAX/Bcl-2 ratio and activating caspase signaling, resulting in apoptotic cell death. Tong et al. (2011) demonstrated that the sustained activity of the ERK1/2 pathway modulates apoptosis by regulating the BAX/Bcl-2 ratio and caspase activation. Euphol-induced gastric cancer cell apoptosis may be mediated by a similar pathway leading to the activation of the caspase cascade.

In the present study, the inhibition of gastric cancer cell proliferation by euphol was found to be mediated by ERK1/2-dependent $p27^{kip1}$ upregulation and cyclin B1 inhibition. These results were in agreement with those of previous studies on gastric, breast, and colon cancers (Guo et al., 2011; Lin et al., 2010; Ollinger et al., 2007; Park et al., 2011). Icaritin, a prenyl-flavonoid derivative from the genus *Epimedium*, induced sustained ERK1/2 phosphorylation and the subsequent downregulation of Bcl-2 and cyclin B1 protein expressions in MDA-MB-453 and MCF7 breast cancer cells. It is interesting that an inhibitor of ERK1/2 activity abrogated icaritin-induced G2/M cell cycle arrest and cell apoptosis (Guo et al., 2011). Cannabinoids were reported to reduce cancer cell proliferation by activating ERK1/2 signaling, inhibiting the survival AKT pathway and inducing $p27^{kip1}$ expression, leading to gastric cancer cell cycle arrest (Park et al., 2011). $P27^{kip1}$, an important cell cycle regulatory protein and tumor suppressor, has been implicated in a variety of cellular processes, including the induction of cell cycle arrest and apoptosis (Said et al., 2001). Most important is that $p27^{kip1}$ has been reported to promote apoptosis in gastric cancer (Zheng et al., 2005), and low $p27^{kip1}$ levels may promote carcinogenesis associated with the *Helicobacter pylori* infection (Eguchi et al., 2004).

The cyclin B1 protein level has been shown to be a critical factor affecting survival, and cyclin B1 overexpression is correlated with the aggressiveness and metastatic potential of gastric cancer. Cyclin B1 overexpression was found in approximately 49% of gastric carcinomas (Begnami et al., 2010). Knockdown of cyclin B1 was shown to inhibit cancer cell proliferation *in vitro* and *in vivo* (Androic et al., 2008). A recent study provided evidence that the growth inhibitory and apoptosis induction effects of betulinic acid are mediated by targeting cyclin B1 protein downregulation in human gastric AGS cancer cells (Yang et al., 2010). Furthermore, null or low expression of $p27^{kip1}$ in tumor cells in diffuse large B-cell lymphomas was reported to be strongly associated with increased expression of cyclin B1 (Bai et al., 2001). Knockdown of the tumor suppressor FHL1 in lung cancer cells also suppressed $p27^{kip1}$ expression and elevated the expression of cyclin B1 simultaneously (Niu et al., 2011). In contrast, overexpression of cyclin B1 and downregulation of $p27^{kip1}$ protein were suggested to result in tumor progression and development (Begnami et al., 2010; Kim, 2007).

Our results suggest that euphol may inhibit cancer cell growth and tumor development by inhibition of cyclin B1 expression and elevation of $p27^{kip1}$ protein levels.

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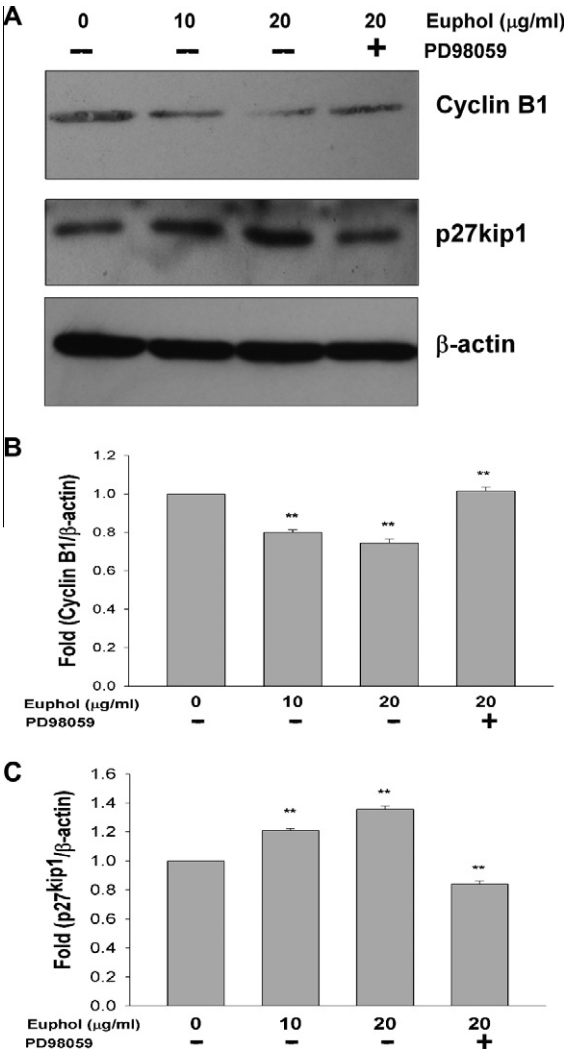

Fig. 5. Role of ERK1/2 in the expressions of the cell cycle regulatory proteins in the euphol-treated cells. (A) PD98058 suppressed euphol-induced $p27^{kip1}$ protein expression but elevated cyclin B1 expression. Beta-actin was used as an internal control. (B) Quantification of the cyclin B1 protein expression from the Western blot analyses. Euphol inhibited cyclin B1 expression in a dose-dependent manner, and PD98059 reversed this effect in the euphol-treated cells. (C) Quantification of $p27^{kip1}$ protein expression from the Western blot analyses. Euphol increased the $p27^{kip1}$ expression in a dose-dependent manner, and PD98059 reversed this effect in the euphol-treated cells. Each bar is the mean ± SD of the 3 independent experiments ($^{**}P < 0.01$, compared with the control group).

5. Conclusions

The present study demonstrated that euphol has antiproliferative effects and selectively promotes apoptosis in human gastric cancer cells. The mechanism underlying the effect of euphol involves mitochondrial-dependent caspase-3 activation and growth arrest through induction of $p27^{kip1}$ and inhibition of cyclin B1 in human gastric CS12 cancer cells. ERK1/2 participated in the euphol-induced apoptosis and growth inhibition. This study provides a mechanistic insight and supports the premise that euphol is a potentially promising agent for development as chemotherapy against gastric cancer in humans. The specificity of euphol in targeting cancer cells may lead to the reduction of toxic side effects in cancer patients.

Conflict of Interest

Authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by Grant from the Department of Health, Executive Yuan, ROC (Taiwan) (DOH100-TD-C-111-002).

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Please cite this article in press as: Lin, M.-W., et al. Euphol from *Euphorbia tirucalli* selectively inhibits human gastric cancer cell growth through the induction of ERK1/2-mediated apoptosis. Food Chem. Toxicol. (2012), http://dx.doi.org/10.1016/j.fct.2012.05.029

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