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

Ming-Wei Lin a,b, An-Shen Lin d, Deng-Chyang Wu b,c, Sophie S.W. Wang b,c, Fang-Rong Chang b,d, Yang-Chang Wu d,e,f, Yaw-Bin Huang a,b,*

a Graduate Institute of Clinical Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC
b Cancer Center, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, ROC
c Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan, ROC
d Graduate Institute of Nature Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC
e Natural Medicinal Products Research Center and Center for Molecular Medicine, China Medical University Hospital, Taichung 402, Taiwan, ROC
f Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung 402, Taiwan, ROC

**A R T I C L E   I N F O**

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

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

**A B S T R A C T**

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 p27kip1 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.

© 2012 Published by Elsevier Ltd.

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 and Kelsen, 2010). Chemotherapy is the treatment of choice for gastric cancer, with metastatic and local cancer (Dangle et al., 2009). Extra-cellular signal-regulated kinase 1/2 (ERK1/2) belongs to one of the subgroups 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 inhibitroy 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...
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 (Russell 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; Russel 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/CHCl₃ (3:1, 2:1, and 1:1, at 800 ml each) and CHCl₃ (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/CH₂Cl₂ (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 keratinocyte-Serum-free medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum, N-acetyl-L-cysteine (360 μg/ml), and l-aspartic 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 × 10⁴ 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 detected 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 manufacturer’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 unbleached cells) were then analyzed by a FACSscan 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 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 FACSscan 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 FACSscan 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 PBS 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-p73 (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 immunoblotted proteins were detected using an enhanced chemiluminescence kit (GE Healthcare), followed by exposure to 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
The role of ERK1/2 in the euphol-induced apoptosis pathway.

ERK1/2 is a member of the mitogen-activated protein kinase (MAPK) family, which plays a crucial role in cell proliferation, differentiation, and apoptosis. In this study, the role of ERK1/2 in the euphol-induced apoptosis pathway was investigated in gastric cancer cells.

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 cells, 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 proteins (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 (Pulda 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 p27kip1 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 p27kip1 expression and inhibiting cyclin B1 expression. Furthermore, pretreatment with PD98059 markedly abolished the upregulation of p27kip1 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...
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 (Bal-lif 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 chemo- therapeutic 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 major 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 (Bionda 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

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

Euphol induced apoptosis in CS12 cells, as evidenced by annex-
in V-binding assays, flow cytometric detection, and Western blot-
ting. 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 re-
sponse to the euphol treatment, indicating that the ERK1/2 path-
way 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 apopto-
sis 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 prolif-
eration by euphol was found to be mediated by ERK1/2-dependent
p27kip1 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 prolifera-
tion by activating ERK1/2 signaling, inhibiting the survival AKT
pathway and inducing p27kip1 expression, leading to gastric cancer
cell cycle arrest (Park et al., 2011). P27kip1, 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
p27kip1 has been reported to promote apoptosis in gastric cancer
(Zheng et al., 2005), and low p27kip1 levels may promote carcino-
genesis 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. Cy-
clin 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 (And-
rico 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 p27kip1 in tumor cells in diffuse large B-cell lym-
phomas was reported to be strongly associated with increased
expression of cyclin B1 (Bai et al., 2001). Knockdown of the tumor
suppressor FH1L1 in lung cancer cells also suppressed p27kip1
expression and elevated the expression of cyclin B1 simultaneously
(Niu et al., 2011). In contrast, overexpression of cyclin B1 and
downregulation of p27kip1 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 p27kip1 protein levels.

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 induc-
Conflict of Interest

Funding

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

Acknowledgments

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 p27kip1 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.

References


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 induc-


