

Active extracts of wild fruiting bodies of *Antrodia camphorata* (EEAC) induce leukemia HL 60 cells apoptosis partially through histone hypoacetylation and synergistically promote anticancer effect of trichostatin A

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Abstract The endemic species of *Antrodia camphorata* (AC) is a promising chemotherapeutic drug for cancer. We found that the ethanol extract from wild fruiting bodies of *Antrodia camphorata* (EEAC) could induce HL 60 cells apoptosis via histone hypoacetylation, up-regulation of histone deacetyltransferase 1 (HDAC 1), and down-regulation of histone acetyltransferase activities including GCN 5, CBP and PCAF in dose-dependent manner. In combination with histone deacetylase inhibitor, trichostatin A (TSA), did not block EEAC-induced apoptosis. Interestingly, combined

treatment (100 nM of TSA and 100 µg/ml EEAC) caused synergistic inhibition of cell growth and increase of apoptotic induction. EEAC could effectively increase the cytotoxic sensitivity of TSA through the up-regulation of DR5 and NFκB activation. In this present study, bioassay-guided fractionation of EEAC led to a major active compound, zhankuic acid A, as the bioactive marker. Moreover, our findings may represent an experimental basis for developing EEAC as a potential chemotherapeutic adjuvant.

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Abbreviations

DR	Death receptor
EEAC	Extracts of fruiting bodies of <i>Antrodia camphorata</i>
FLIP	FLICE inhibitory protein
HAT	Histone acetyltransferase
HDACIs	Histone deacetylase inhibitors
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor kappa B
PARP	Poly(ADP-ribose) polymerase
TRADD	Tumor necrosis factor (TNF)-receptor 1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TSA	Trichostatin A

Introduction

The endemic species of *Antrodia* are one of the difficult-to-classify and obscure groups of poroid Aphyllphorales

based on morphological appearance. However, it is becoming increasingly important to reliably identify the entire suite of *Antrodia camphorata* (AC) strains and *Antrodia* species due to the potential pharmaceutical value of their biologically active ingredients (Chiu 2007). The fruiting body of AC is a popular and an expensive medicinal health food. Grounded or sliced wild fruiting bodies are sold for about US \$8,000 per Kg in Taiwan due to host specificity, rarity in nature, and the failure of artificial cultivation. The high price of the original material makes the use of pure components of AC difficult. Now, many mycelia products are sold in the market and these may promote conventional modalities' anti-cancer effects as well as reduce treatment-related symptoms and other side effects. Mycelia products of AC have recently been reported to have antioxidant, antihypertensive, and immunostimulatory effects (Liu et al. 2007). On the markets, it has been claimed of these mycelia products that they contain active components similar to wild fruiting bodies with cytotoxic triterpenes, steroids, as well as immunostimulatory polysaccharides reported previously (Chen et al. 1995; Yang et al. 1996). We used NMR spectra to provide a visible method to clarify major compositions of the extracts from wild fruiting bodies to establish the truth of these claims. Using this simple experimental method, we attempt to understand the approximate differences in chemical composition of different extracts. Thus, a systemic extraction method in conjunction with NMR spectra as chemical profiles was proposed for a prototype design in quality control in the future.

Traditionally AC has been used as a health food to prevent inflammation, hypertension, itchy skin, and liver cancer (Tsai and Liaw 1982). Some reports have suggested that extracts of mycelia and fruiting bodies of AC could be a potential chemotherapeutic agent against hepatoma, as well as prostate, breast, bladder, and lung cancer cells (Chen et al. 2007; Hsu et al. 2007; Peng et al. 2007; Song et al. 2005; Wu et al. 2006). Although recent studies have greatly advanced our understanding of the regulatory mechanisms of histone acetylase inhibitors (HDACIs) in chromatin modification, little is known about the anticancer effects of EEAC on histone acetylation. However, detailed studies have not yet been performed to explore the extracts of fruiting bodies of AC on combined chemotherapy. Recent studies have reported on the chemical characterization of fruiting bodies; organic-solvent extracts from fruiting bodies of AC are rich in steroids and triterpenoids and these possess anticancer and anti-inflammatory activities (Chen et al. 1995; Hsu et al. 2005; Lin et al. 2003). With biological analysis, we investigated the effect of EEAC on histone acetylation in HL 60 cell and examined the efficiency of combined treatment of TSA and EEAC on anti-cancer activity. In the chemical study, we characterized the active extracts and identified the structure of the major active component.

Materials and methods

Bioassays materials

RPMI 1640 medium, fetal calf serum (FCS), trypan blue, penicillin G, and streptomycin were obtained from Gibco BRL (Gaithersburg, MD). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were from Sigma-Aldrich (St Louis, MO). Trichostatin A (TSA), GCN5, SRC-1, CBP, and PCAF were purchased from Upstate Biotechnology (Temecula, CA). Antibodies against HDAC1, histone H3, acetyl-H3, H3K9, and H3K18 were purchased from Cell Signaling Technologies (Beverly, MA, USA) and Bax and Bcl-2 from BD Pharmingen. Antibodies of DR5, p50, p65, PARP, and Actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-mouse and rabbit IgG peroxidase-conjugated secondary antibody were from Pierce, Rockford, IL. Hybond ECL transfer membrane and ECL Western blotting detection kits were obtained from Amersham Life Sciences (England).

Preparation of ethanol (EEAC) and water (WEAC) extracts from wild fruiting bodies

The species (Fig. 1) was identified by Dr. Hung-Liang Lay at National Pingtung University of Science and Technology. Fifty-two grams of dried fruiting bodies (Nan-Yan Farm, Jia-Yih, Taiwan) were ground into a fine powder with an electrical mill and then refluxed with ethanol at 75°C in a 1:10 (w/v) ratio for 2 h. The extracts were cooled and then allowed to precipitate overnight at 4°C. The supernatant of extracts was further filtered by a filter paper, centrifuged at 3,000 rpm for 30 min to remove the precipitate, and then the extracts were lyophilized and stored at -70°C before (EEAC). The residue was boiled and the reflux extracted with water for 6–8 h at a ratio of 1:10 (w/v). The superna-



Fig. 1 Morphology of wild fruiting bodies of *Antrodia camphorata* (AC)

tants were filtered further, centrifuged at 3,000 rpm for 30 min to remove the precipitate, and then lyophilized. The extracts were stored at -70°C before (WEAC).

Fractionation of EEAC

To identify the active fraction of EEAC, 1 g of dried fruiting bodies of AC was ground and extracted as described above. After MTT bioassay, the EEAC extract (IC_{50} : 104.82 $\mu\text{g}/\text{mL}$) was selected for further investigation. The extracted WEAC was inactive ($\text{IC}_{50} > 200 \mu\text{g}/\text{mL}$). Lyophilized parts of EEAC (203.1 mg) were sequentially extracted with solvents of increasing polarity [*n*-hexane, ethyl acetate (EA), and ethanol], yielding three different extracts, respectively. The yields in weight percentage of residues, referring to the weight of dry material extracted, were 12.8% (*n*-hexane, 26.0 mg), 61.6% (EA, 125.1 mg), 8.4% (ethanol, 17.0 mg), and 10.0% (the insoluble residue, 20.2 mg), respectively.

Isolation and purification of zhankuic acid A

EA fraction (95.4 mg) was chromatographed using Sephadex LH-20 with CHCl_3 -MeOH (1:3) to get three fractions. Fr. 2 (65.1 mg) was separated by prep. TLC with CHCl_3 -MeOH (25:1) to obtain eight fractions. Fr. 2-1 (10.3 mg) was purified using ODS HPLC column (250 \times 10 mm, Hypersil[®], MeOH- H_2O , 85:15) to give zhankuic acid A (4.3 mg, R_f 10 min, flow rate 2 ml/min).

General experimental procedures

^1H and ^{13}C NMR spectra were recorded on Varian Unity Plus 400, or Varian Gemini 200 NMR spectrometers. Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hertz. LRESIMS was measured on a VG Biotech Quattro 5022 mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) and Sephadex LH-20 were used for column chromatography, while TLC analysis was carried out on Silica gel GF₂₅₄ pre-coated plates with detection using 50% H_2SO_4 followed by heating on a hot plate. HPLC was performed with a Hitachi L-7100 pump and D-7000 interface equipped with a Bischoff RI detector using ODS (Hypersil[®], 250 \times 10 mm) columns.

Cell culture, cell viability and cytotoxicity

All components for cell culture were obtained from Invitrogen, Inc. (Carlsbad, CA, USA). Human leukemia HL 60 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100units/ml peni-

cillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 .

Cell viability was determined by the Trypan blue dye exclusion method and cytotoxicity was assessed by the MTT assay. Cells (1×10^5) were plated in 96-well plates and treated for 24 h with different concentrations (0–150 $\mu\text{g}/\text{ml}$) of EEAC dissolved in DMSO. Cells exposed to 0.2% Trypan blue were counted in a hemocytometer. MTT solution was added to each well (1.2 mg/mL) and incubated for 2 h. MTT was reduced by mitochondrial dehydrogenases of viable cells to a purple formazan product. The MTT-formazan product dissolved in DMSO was estimated by measuring absorbance at 570 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader.

Flow cytometric analysis

These cells (1×10^6) were seeded onto 10 cm dishes, treated with or without the indicated doses of EEAC for 24 h and then washed twice with ice-cold PBS and collected by centrifugation at $200 \times g$ for 5 min at 37°C . The cells were fixed in 70% (v/v) ethanol at 37°C for 30 min. After fixation, cells were resuspended in 1 ml propidium iodide staining buffer (0.1% TritonX-100, 100 $\mu\text{g}/\text{ml}$ RNase A, 500 $\mu\text{g}/\text{ml}$ propidium iodide in PBS) at 37°C for 30 min. Cells were detected using a cytofluorometer, and analyzed by FACScan and Cell Quest program (Becton Dickinson).

Assessment of DNA fragmentation

Apoptosis was also evaluated by examining the characteristic pattern of DNA laddering generated in the apoptotic cells using gel electrophoresis. Briefly, HL-60 cells were seeded at a density of 1×10^6 cells onto 10-cm dishes, 24 h before drug treatment followed by treatment with EEAC (0, 50, 100, 150 and 200 $\mu\text{g}/\text{ml}$) for 24 h, and control cultures were treated with DMSO. After the treatment, cells were washed twice with PBS and harvested with incubation buffer (10 μM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% (v/v) sodium dodecyl sulfate and 1 $\mu\text{g}/\text{ml}$ proteinase K). The cells were incubated at 56°C for 3 h, and then RNase (final concentration of 50 $\mu\text{g}/\text{ml}$) was added to the incubation buffer for further 1 h incubation. The DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and then precipitated with ethanol. The extracted DNA was separated and stained by electrophoresis in 2% agarose gel with ethidium bromide.

Histone fraction from acid extraction

Histone isolation was performed as described previously (Taplick et al. 1998). The cells were harvested by centrifugation at $700 \times g$ and washed once with ice-cold PBS. The

pellet was resuspended in 1 ml lysis buffer (10 mM Tris–HCl pH 6.5, 50 mM sodium disulfite, 10 mM $MgCl_2$, 10 mM sodium butyrate, 8.6% sucrose, 1% Triton X-100) and centrifuged at $1,000\times g$. After three washes in lysis buffer, the pellet was resuspended in 10 mM Tris–HCl pH 7.4, 13 mM EDTA. The pellet was then resuspended in cold distilled water and H_2SO_4 was added to 0.4 N of concentration. The sample was centrifuged at $10,000\times g$ for 5 min after incubation on ice for 1 h. From the supernatant, total histones were precipitated with $10\times$ volumes of acetone at $-20^\circ C$ overnight. The precipitated histones were collected by centrifugation, dried and resuspended in distilled water. The protein content was measured by the Bio-Rad protein assay kit (BioRad laboratories GmbH, Munchen).

Nuclear and cytoplasmic protein extraction

Proteins from nuclear and cytoplasmic fractions were collected as described previously (Vandergeeten et al. 2007). Cells were washed with cold phosphate-buffered saline and resuspended in lysis buffer containing 10 mM HEPES–KOH pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 0.5% IGEPAL, 1 mM PMSF, 1 mM DTT and protease inhibitors (Complete, Roche). After incubation on ice for 10 min and centrifugation at $10,000\times g$ for 30 s, the supernatant containing cytoplasmic proteins was harvested and stored at $-80^\circ C$. The pellet was resuspended in saline buffer (50 mM HEPES–KOH, pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, 400 mM NaCl, glycerol 10% (v/v), 1 mM PMSF, 1 mM DTT and protease inhibitors) and incubated for 30 min on ice. After centrifugation at $10,000\times g$ for 15 min, the supernatant containing nuclear proteins was harvested and stored at $-80^\circ C$. The protein content was measured by the Bio-Rad protein assay kit (BioRad laboratories GmbH, Munchen).

Western blot analysis

Cell lysates were prepared by treating the cells for 30 min in RIPA lysis buffer (1 \times PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 100 $\mu g/ml$ phenylmethylsulfonyl fluoride and 30 $\mu g/ml$ aprotinin) (all chemicals were from Sigma) as described previously (Lu et al. 2006). The lysates were centrifuged at $20,000\times g$ for 30 min and the protein concentration in the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated, respectively, by 7.5, 10 or 12% of SDS-polyacrylamide gel electrophoresis and then these were electrotransferred to a PVDF membrane. The membrane was blocked with a solution containing 5% nonfat dried milk TBST buffer (20 mM Tris–HCl,

pH 7.4, 150 mM NaCl and 0.1% Tween 20) for 1 h and washed with TBST buffer. The protein expressions were monitored by immunoblotting using specific antibodies. These proteins were detected by an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

NF κ B transcription factor assay

To determine the effect of treatment on NF κ B activation, we prepared nuclear extracts and performed the TransAM[®] transcription factor assay (Active motif, Tokyo, Japan) following the manufacture's protocol as described previously (Schwab et al. 2007). In brief, 7 μg of nuclear extract was added to each well of a 96-well plate which had been pre-coated with NF κ B consensus binding oligonucleotide (5'-GGGACTTCC-3'). After 1 h of incubation with smooth agitation, wells were washed three times with washes buffer and then incubated with p50 or p65 antibody (dilution 1:1,000) for 1 h at room temperature. After three successive washings, the wells were incubated for 1 h with diluted horseradish peroxidase-conjugated antibody (dilution 1:1,000) followed by the addition of 100 μl of developing solution. After 5 min of incubation, the reaction was blocked by adding 100 μl of stop solution reagent. The optical density was determined by a spectrophotometer at 405 nm by keeping the reference wavelength at 655 nm.

Statistics

All data are expressed as mean \pm SD. The difference between the treated and the samples was analyzed by *t*-test. A probability of $P < 0.05$ was considered significant.

Results

EEAC induced apoptosis, hypoacetylation and down-regulation of histone

Acetyltransferase activity in HL 60 cells

AC is a highly valued polypore mushroom native only to Taiwan, and this orange to brown-red colored mushroom is very bitter in taste with a camphor aroma (Fig. 1). It has been reported that EEAC inhibited cell growth and induced apoptosis in cancer cells (Hsu et al. 2005; Peng et al. 2007). Genomic DNA was run on 2% agarose gel and then stained with ethidium bromide. The DNA ladder in Fig. 2a suggested that the DNA fragmentation of HL 60 cells occurs upon treatment with EEAC. Acetylation and deacetylation of histones are known to modulate chromatin organization and thus they play a key role in the regulation of gene expression. The several specific HDAC inhibitors found to

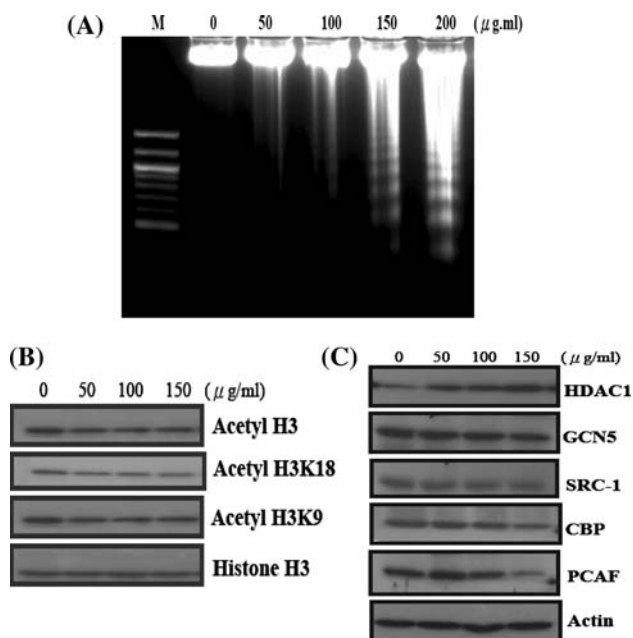


Fig. 2 Effects of EEAC on DNA fragmentation and histone acetylation in HL 60 cells. HL 60 cells were treated with EEAC at indicated doses for 24 h. **a** EEAC resulted in DNA fragmentation of HL 60 cells with electrophoresis analysis. **b** Histone acetylation and **c** histone acetyltransferases and HDAC1 were determined by western blot analysis

date represent not only valuable research tools, but also prospective drugs with a potential clinical use, especially in cancer therapy. The anticancer effect of EEAC on histone acetylation is still not completely understood. Therefore, we first tested the effect of EEAC on histone acetylation in HL 60 cells. The result of western blot showed that EEAC treatment leads HL60 cells to hypoacetylation of histone H3, H3K18 and H3K9 in a dose-dependent manner (Fig. 2b). To further assess whether the histone acetylation-associated enzymatic activities of EEAC treatment are functionally linked, we determined the histone acetyltransferases and HDAC 1 by western blot assay. These results showed a role for EEAC treatment in regulating deacetylation of H3 by histone acetyltransferases, and deacetylases, including the down-regulation of GCN5, CBP, SRC-1, PCAF and up-regulation of HDAC 1 in a dose-dependent manner (Fig. 2c).

EEAC participated in TSA-induced apoptosis through up-regulation of DR5 and NF κ B activation

To study histone hypoacetylation of EEAC, specific inhibitors of histone deacetylase have been used. TSA, a microbial metabolite, is a potent reversible inhibitor of HDAC. It was reported that the histone H3 acetylation can be enhanced by TSA. Moreover, TSA was found to strongly suppress the growth with accumulation of histone acetylation (hyperacetylation) in leukemia cells (Kang et al. 2004).

Therefore, in this study, we further tested whether TSA might block the effect of EEAC on cell growth and apoptotic induction in HL 60 cells by flow cytometric analysis. We found that EEAC and TSA synergistically induced apoptosis in HL 60 cells (Fig. 3a). One of the most important mechanisms by which EEAC inhibits tumor proliferation is the hypoacetylation of histone. Importantly, we show that the combination of 100 nM of TSA and 100 μ g/ml of EEAC causes a marked decrease in histone acetylation when compared with single treatment of TSA (Fig. 3b). EEAC treatment significantly diminished the acetylation of histone 3 induced by TSA. We presume that the cytotoxic effect of EEAC is partially mediated by histone hypoacetylation.

Research into the ability of TSA combined with chemotherapeutic drugs has become urgent. We found that EEAC enhanced the response of TSA effects in proliferation and apoptosis of HL 60. To understand the combined mechanism of TSA and EEAC which induced HL 60 cell apoptosis, we examined combination-induced alternations in the expression of several proteins. Among these, the expression of p21, PARP, Bax and Bcl-2 were determined, since these proteins were involved in the growth inhibition and apoptotic induction (Ruefli et al. 2001). These results indicated that combined treatment could increase the expression of p21 and decrease the expression of Bcl-2 as well as cleave PARP. A recent study has shown that AC is a potent FAS (TRAIL)-sensitizing agent in a variety of different TRAIL-resistant human cancer cells (Hsu et al. 2005). DR5 and TRADD are novel proteins that interact specifically with death domain of DR4. DR4 activation promotes the binding of TRADD and the subsequent recruitment of DR5 to promote apoptosis (Swarup et al. 2007). To determine whether DR5 and TRADD are involved in apoptotic induction, we explored the effects of combined treatment on the expressions of these proteins. The western blot analysis for TRADD and DR5 revealed two and fourfold increases over the corresponding control and TSA treatment alone. Combination-induced upregulation of DR5 is mediated by EEAC, but upregulation of TRADD is mediated synergistically by TSA and EEAC (Fig. 3c). Our results showed that combined treatment with TSA and EEAC cooperatively targeted the TRAIL pathway.

Certain cancer therapeutic agents induce the expression of DR5 in cancer cells and are thereby able to augment TRAIL-induced apoptosis or initiate apoptosis. Therefore, TRAIL is one of the promising new candidates for cancer therapeutics and is currently undergoing clinical trials. In addition, NF κ B activation can be triggered by direct stimulation of CD95 and the TRAIL death receptors as well as by overexpression of FLIP protein (Sheikh and Huang 2004). Additional findings suggest that the NF κ B transcription factor differentially regulates the DR5 expression involving

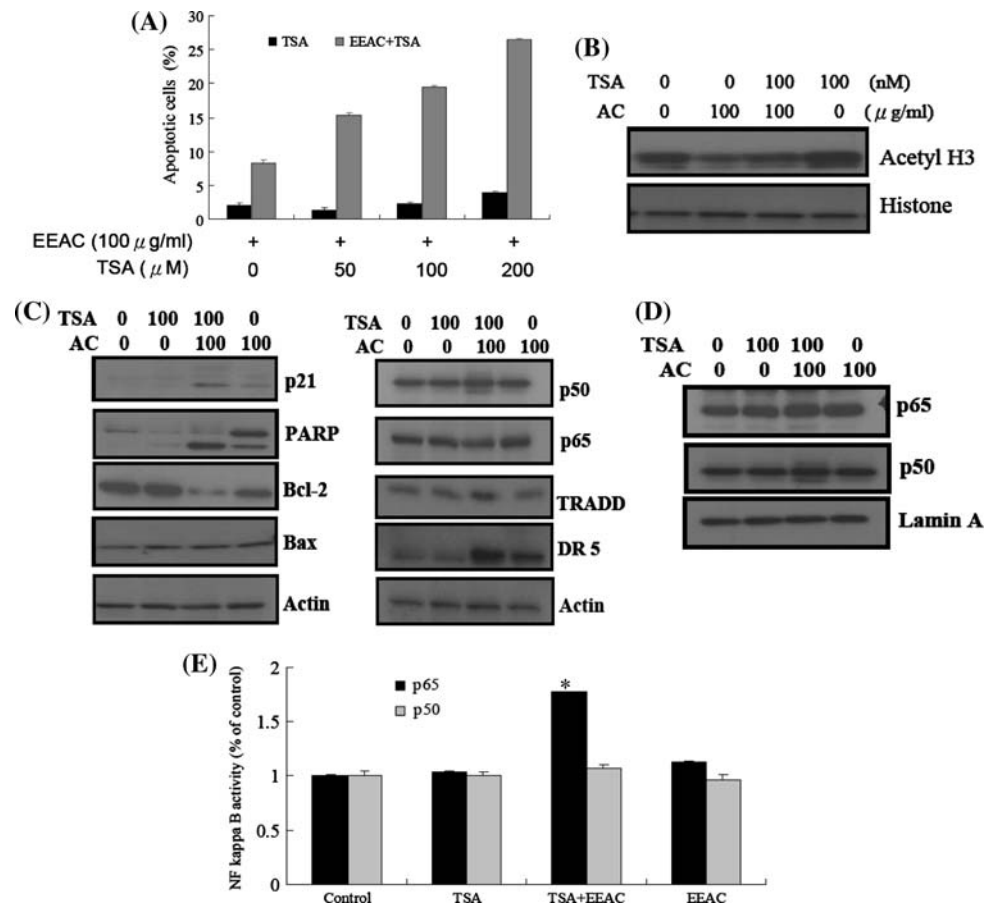


Fig. 3 Combination effects of TSA (100 nM) and EEAC (100 μg/ml) on cell viability and NF-κB activity in HL 60 cells. **a** EEAC promoted the apoptosis of HL 60 cells treated by TSA. All values are means of independent triple experiments. **b** Effect of TSA, EEAC or combined treatments on histone acetylation of acid extracts. Protein expressions of acid extracts were analyzed by western blot. **c** Effect of TSA, EEAC or combined treatment on the expression of total protein. Protein expressions of total extracts were analyzed by western blot. **d** Effect of

TSA, EEAC or combined treatment on NF-κB p50 and p65 expression of nuclear protein. Protein expressions of nuclear extracts were analyzed by western blot. **e** Effects of TSA, EEAC or combined treatment on NF-κB p65 activation. All values are means of independent triple experiments. The *asterisk* indicates significant difference in NF-κB activation of combined treatment when compared to the control ($P < 0.05$). NF-κB transcription factor binding DNA activities were determined with ELISA assay, described in “Materials and methods”

histone deacetylase 1 (Shetty et al. 2005). To examine the effect of NFκB activation on cellular apoptosis in response to combined treatment, western blot analysis was performed with the total and nuclear extracts of treated cells. It was found that combined treatment significantly increased the translocation of NFκB p65, but did not affect the expression with total extracts of cells. Obviously, combination-induced p65 translocation may be mediated by EEAC treatment alone (Fig. 3c, d).

To investigate the role of NFκB transcription factor activity in the promotion of apoptosis caused by combined treatment, this activity was examined in HL 60 cells. Treatment of HL 60 cells with TSA and EEAC resulted in an increase of basal NFκB p65 DNA binding activity in the nucleus (+78% vs. control, $P < 0.05$). In contrast, basal p50 NFκB DNA binding activity was not affected in response to different treatments (Fig. 3e), further supporting the sugges-

tion that nuclear translocation of p65 and activation are responsible for the combined treatment-induced NFκB p65 activation.

Zhankuic acid A is the major active component

We obtained the ethanol crude extract of fruiting bodies (EEAC), which was successively extracted by three different solvents of increasing polarity. The yields in weight percentage of residues, referring to the weight of dry material extracted, were 61.6% (EA fraction, FA), 8.4% (ethanol fraction, FB), and 12.8% (*n*-hexane fraction, FC), respectively. Three fractions labeled FA, FB and FC were collected and the effects of equal doses on cellular growth as well as apoptotic induction in HL 60 cells were compared with the effects of EEAC. The IC_{50} value for the antiproliferative effect of FA in HL 60 cells is about 80.53 μg/ml,

and the activity is better than EEAC (104.82 $\mu\text{g/ml}$). FB and FC are less cytotoxic, and the values of IC_{50} are more than 200 $\mu\text{g/ml}$. In addition, 100 $\mu\text{g/ml}$ of FA and EEAC could induce HL 60 cells apoptosis of about 26 and 10% (Fig. 4a, b). FA is an active fraction in EEAC.

Based on the above data, the use of standard active extracts should be developed. In this study, fractionation and biological testing of EEAC showed that EA fraction (FA) accounted for a significant fraction of cytotoxic activity of EEAC in causing antiproliferation, death, and apoptotic induction in HL 60 cells. Our results clearly demonstrated high activity of FA relative to EEAC. The NMR profiles of different extracts (3.0 mg/mL in CD_3OD) are

shown in supporting information. According to the general rules of chemical shifts in $^1\text{H-NMR}$, methyls of steroids or triterpenes will appear at δ 0.5–2 of NMR spectra. On the basis of NMR elucidation of these three extracts, FA mainly contains steroids or triterpenoids, FC mainly contains fatty acids and tri-glycerides, and FB mainly contains sugars or polysaccharides. As predicted, FA showed significant cytotoxicity and was the active fraction of EEAC. It is clear that steroids or triterpenoids mainly contributed to cytotoxic effects of EEAC, and they can be monitored by NMR together with MTT reduction and apoptotic assays (Fig. 4a, b). Moreover, the active fraction (FA) was chromatographed to yield zhankuic acid A, which was isolated

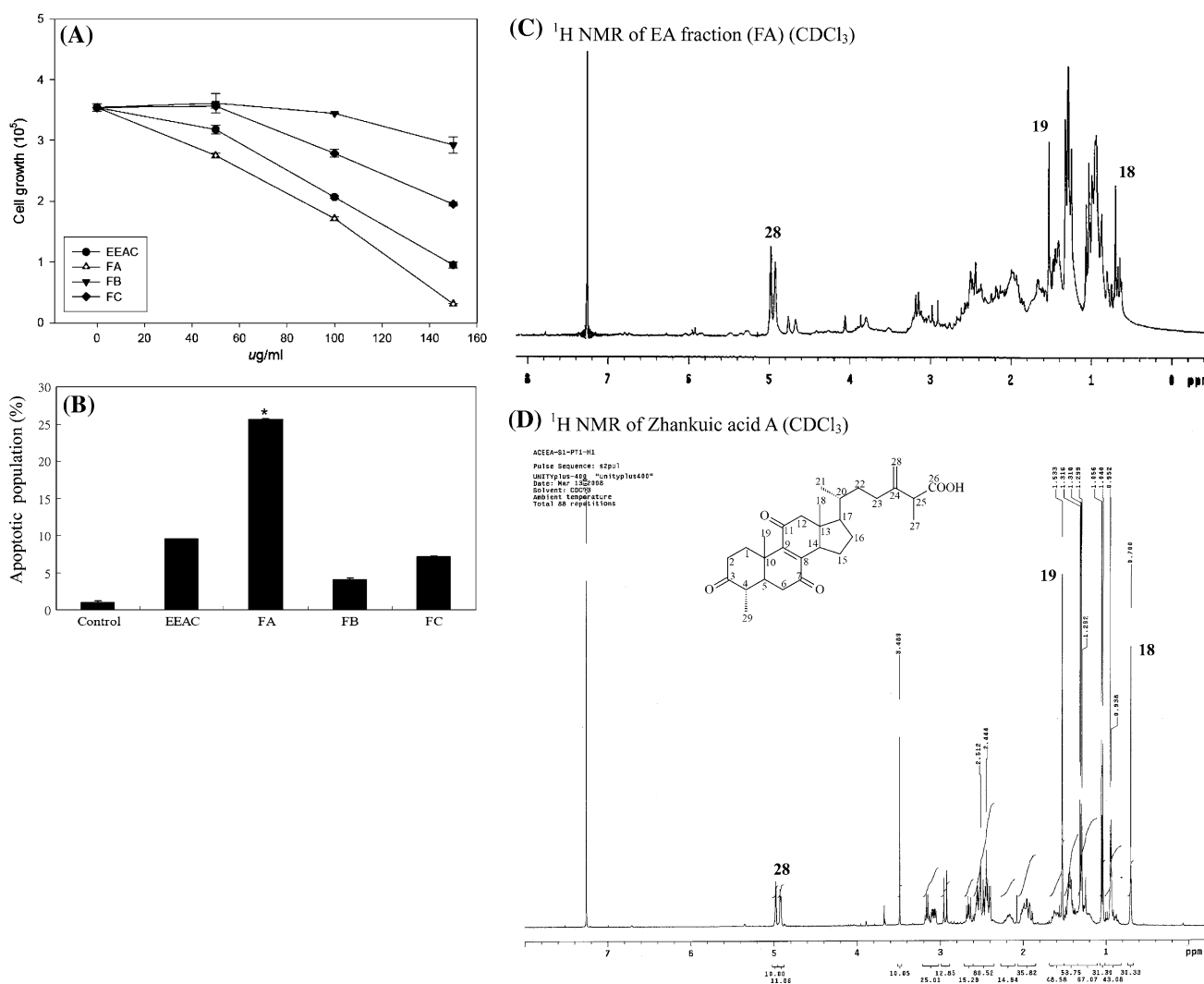


Fig. 4 Effects of three subfractions from EEAC on cell growth and apoptotic induction in HL 60 cells as well as on the NMR profiles of FA and zhankuic acid A. **a** Different extracts inhibited growth of HL 60 cells with MTT analysis. **b** Apoptotic cell population was determined with flow cytometric analysis. The effects of FA, FB and FC (100 $\mu\text{g/ml}$) on apoptosis in HL 60 cells were compared with the

effects of EEAC. Error bars represent standard error of the mean from three separate experiments. The asterisk indicates significant difference in apoptotic population compared to EEAC treatment ($P < 0.05$). **c, d** The $^1\text{H NMR}$ profiles of FA and zhankuic acid A (3.0 mg/mL in CD_3OD)

in a major quantity. Zhankuic acid A was obtained as pale-yellow amorphous solid. Its molecular formula was established as $C_{29}H_{40}O_5$ by ESIMS (m/z 469 $[M + H]^+$) and NMR spectra. The most significant signals of its 1H NMR spectrum were those corresponding to five methyls (three secondary at $\delta = 0.95$, 1.05, and 1.30, two tertiary at $\delta = 0.70$ and 1.53) and two terminal olefinic methylene protons at $\delta = 4.92$ and 4.98. The ^{13}C -NMR spectrum displayed three carbonyl groups (δ_C 200.8, 202.6, and 210.8), four olefinic carbons (δ_C 111.4, 145.5, 148.0, and 151.9), and one carboxylic acid (δ_C 178.8) (Chen et al. 1995). Comparing the characteristic 1H NMR signals of Zhankuic acid A with those of FA, our results suggested that zhankuic acid A was the major component of the active fraction, FA (Fig. 4c, d). Zhankuic acid A exhibited significant cytotoxicity to HL 60 cells (IC_{50} :5.45 $\mu g/ml$), and it could serve as a bioactive marker.

Discussion

Given the important role of acetylation as a regulatory mechanism, it is natural that inhibitors of both HDACs and HATs have been the focus of several studies. The inhibition of histone deacetylase leads to a growth inhibition of cancer cells by initiating differentiation or apoptosis. The manipulation of histone deacetylation inhibitor has therefore become an emerging therapeutic target for the treatment of cancer. Recently, many publications have been devoted to HAT inhibitor, including plitidepsin isolated from a sea tunicate, garcinol isolated from *Garcinia indica*, and curcumin derived from *Curcuma langa* (Balasubramanyam et al. 2003; Bravo et al. 2005; Kang et al. 2005). This compound-induced histone hypoacetylation mediated the suppression of histone acetyltransferases. These include GCN5 and PCAF, p300 and CBP, nuclear hormone co-activators SRC-1 and ACTR and TATA box binding protein-associated factor TAFII250 (Berger 2002). Acetylation of these factors alters their DNA/nucleosome binding and/or protein–protein interactions and consequently influences their effect in regulating gene transcription. Our results showed that EEAC could induce apoptosis mediated histone hypoacetylation and suppression of acetyltransferase activity in HL 60 cells (Fig. 2). To gain mechanical insights of combination-induced apoptosis, the PARP, p21 and apoptosis-related proteins Bax/Bcl-2 were investigated. We herein demonstrated that cleavage of PARP as well as up-regulation of ratio (Bax/Bcl-2) and p21 treated with combination is synergistically mediated by the EEAC effect (Fig. 3c).

Chemotherapeutic agents may exert their effects either by blocking or by metabolizing, or by inhibiting tumor cell growth. Another important advantage of chemopreventive

agents is their non-toxic nature. Therefore, recently chemopreventive agents have been used in cancer treatment with clinical chemotherapy. Chemotherapeutic drugs, including TSA, etoposide and doxorubicin in combination with TRAIL, provide a synergistic apoptotic response in cancer cells. The synergistic effect is partially due to the ability of chemotherapeutic drugs and TRAIL to induce DR4 and DR5 expression. TRAIL cross-links with two death receptors, DR4 and DR5. DR5 up-regulation results in the synergistic sensitization of soluble recombinant human TRAIL-induced apoptosis. Certain cancer therapeutic agents induce the expression of DR5 in cancer cells and are thereby able to augment TRAIL-induced apoptosis or initiate apoptosis (Sheikh and Huang 2004). Our results with western blot and ELISA analysis reveal that combination-induced apoptosis is through the up-regulation of DR5 and TRADD as well as through NF κ B activation mediated by the EEAC effect.

The fruiting bodies of *Antrodia camphorata* are well-known in Taiwan as a folk medicine and they have been used for treating food and drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer. Moreover, this report provides a concise view of major chemical compositions of natural and commercial AC products by NMR spectra. On the basis of the literature reports and the present finding, we conclude that more detailed mechanisms of proliferation inhibition and apoptotic induction of EEAC are partially involved in histone hypoacetylation. Furthermore, we further demonstrate that the association of HDAC inhibitor TSA with EEAC determines a significantly higher cytotoxicity when compared to single TSA, or EEAC treatment. This conclusion was confirmed by the finding that EEAC treatment not only diminished histone acetylation induced by TSA, but by the finding that the combined treatment also increased NF κ B activation and DR5 expression, showing that EEAC might synergistically enhance the anticancer effect of TSA (Fig. 3). Overall, we could confirm that zhankuic acid A is the main active component in EEAC, and there is a potential to develop *Antrodia camphorata* as a chemotherapeutic adjuvant. The ability of EEAC to potentiate the anticancer effects achieved by HDAC inhibitor TSA may have an important implication for the clinical treatment of cancer. Further experiments were aimed at identifying the key events of cellular survival pathways to further elucidate the mechanism of combined treatment. Therefore, our findings might provide an experimental basis for the clinical application of cancer chemoprevention.

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