Astilbic acid induced COLO 205 cell apoptosis by regulating Bcl-2 and Bax expression and activating caspase-3[1]

Xiao-liang ZHENG, Hong-xiang SUN[2], Xue-li LIU, Yun-xiang CHEN[3], Bo-chu QIAN[4]

Institute of Materia Medica, Zhejiang Academy of Medical Sciences; [2]College of Animal Sciences, Zhejiang University; [3]State Key Laboratory for New Drug Safety Evaluation (Zhejiang), Hangzhou 310013, China

KEY WORDS astilbic acid; COLO 205 cells; apoptosis; cell cycle; bcl-*2* genes; Bax protein; mitochondria; caspase 3

ABSTRACT

AIM: To investigate the effect of astilbic acid (3β, 6β-dihydroxyolean-12-en-27-oic acid, AA) on human colorectal carcinoma COLO 205 cell proliferation and apoptosis. **METHODS:** Proliferation of COLO 205 cells was measued by MTT assay. Content of DNA in COLO 205 cell was measued by modified diphenylamine assay. AA-induced morphological changes was observed with fluorescence microscope and transmission electron microscope . DNA fragmentation was visualized by agarose gel electrophoresis. Apoptosis rate and cell cycle distribution were determined by flow cytometric analysis. Expressions of Bcl-2 and Bax proteins were visioned by immunohistochemical analysis. The change of relative mitochondral transmembrane potential (MTP) in COLO 205 cell was analyzed with FCM after rhodamine 123 staining. **RESULTS:** The IC_{50} (96 h) of AA for inhibiting COLO 205 cell proliferation was 61.56±0.34 μmol/L. AA induced a marked concentration- and time-dependent inhibition of COLO 205 cell proliferation and reduced the DNA content in COLO 205 cell. Cells treated with AA 64 μmol/L showed typical morphological changes of apoptosis and DNA "ladder" pattern. The cell cycle was arrested in G_0/G_1 phase, and the apoptosis rate was 28.25 % for COLO 205 cells treated with AA 64 μmol/L for 48 h. Meanwhile the expression of Bcl-2 protein was decreased while that of Bax was increased and relative MTP was decreased as well. DEVD-CHO 1 μmol/L could increase the viability of COLO 205 cells treated with AA for 48 h. **CONCLUSION:** AA showed potent inhibitory activity on COLO 205 cells proliferation, and could induce COLO 205 cells apoptosis through disturbing DNA replication, down-regulating Bcl-2 expression, and up-regulating Bax expression, lowering relative MTP, and activating caspase-3 pathway.

INTRODUCTION

Astilbic acid (AA, Fig 1) is a natural product extracted from *Astilbe chinensis* (Maxim) Franch *et* Savat, which belongs to Saxifragaceae[1]. *Astilbe chinensis*, a traditional Chinese medical herb, has been widely used in treating cold, headache, fever, and cough, *etc*. Recently researchers have found that *Astilbe chinensis* possesses anticancer activity *in vivo*[2]. AA was found to be the active component from the rhizomes of *Astilbe chinensis* for inhibiting tumor cell proliferation for the first time. In this study, the effects of AA on COLO 205 cell proliferation and apoptosis were investigated, and its possible mechanism was explored.

[1] Project supported by the Natural Science Foundation of Zhejiang Province, No 300456.
[4] Correspondence to Prof Bo-chu QIAN.
Phn 86-571-8821-5627. Fax 86-571-8886-9045.
E-mail qianbochu@yahoo.com.cn
Received 2003-08-26 Accepted 2004-03-22

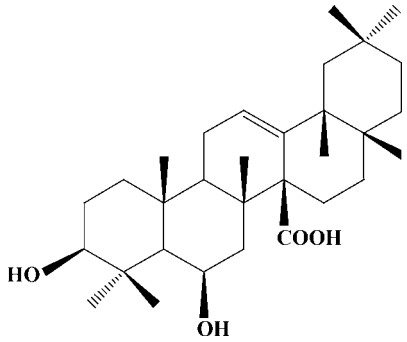

Fig 1. Chemical structure of Astilbic acid, $C_{30}H_{48}O_4$, M_r=472.

MATERIALS AND METHODS

Reagents Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from Gibco. New-born calf serum was purchased from Hangzhou Sijiqing Co. MTT, propidium iodide (PI), and rhodamine123 (Rh123) were obtained from Sigma. Acridine orange (AO) was the product of Edward Gurr. DNA Ladder Plus 100 bp, proteinase K, and Rnase A were obtained from MBI, MERCK, and AMRESCO, respectively. Bcl-2 and Bax IH kits were purchased from Wuhan Boster Biological Technology Ltd. Cell-permeable DEVD-CHO was the product of Biomol. All other reagents were of analytical reagent quality.

Drug AA was extracted by Prof Hong-xiang SUN[1]. AA was dissolved in Me_2SO and the final concentration of Me_2SO was less than 0.1 % in all experiments.

Cell culture Human colorectal carcinoma cell line COLO 205 was obtained from ATCC (American Type Culture Collection) and cultured in DMEM supplemented with 10 % heat-inactivated new-born calf serum at 37 ºC in a humidified 5 % CO_2 incubator. PC_3 (human androgen-independent prostate adenocarcinoma), Bcap37 (human breast cancer), and K562 (human chronic myelogenous leukemia) cell were cultured in the same condition.

MTT assay and cell growth curve After incubation with various concentrations of AA, the IC_{50} on COLO 205, PC_3, Bcap37, and K562 cell proliferation were measured with MTT assay and calculated by NDST software[3]. The viable COLO 205 cells were counted with hemocytometer every day in the first 4 d by trypan blue dye exclusion method for cell growth curve[4].

Cell DNA determination The content of DNA in COLO 205 cells treated with AA 16, 32, and 64 μmol/L was measured by modified diphenylamine assay[5]. COLO 205 cell DNA was extracted with trichloroacetid acid, then dissolved in $HClO_4$. After being combined with diphenylamine, DNA was measured at 595 nm with UV-754 ultraviolet spectrophotometer (Shanghai Third Analysis Instrument Factory).

Morphological studies of apoptosis

Fluorescence microscope observation After a 48-h exposure to AA 64 μmol/L, the cover slides with COLO 205 cells in each culture dish were taken out and COLO 205 cells were stained with 0.01 % AO, then observed under fluorescence microscope (XSJ-2, Chongqing Optical Instrument Factory)[6].

Transmission electron microscope observation After a 48-h exposure to AA 64 μmol/L, COLO 205 cells were fixed with glutaraldehyde and osmium tetroxide. After dehydration, the samples were embedded in Epo812, then ultramicrotomed[7]. The sections were routinely stained and examined by electron microscope (H-600A, HITACHI).

DNA agarose gel electrophoresis After incubation with AA 16, 32, and 64 μmol/L for 48 h, DNA fragmentation was analyzed by electrophoresis as previously described[8].

Flow cytometric analysis of cell cycle and apoptosis After a 48-h treatment with AA 16, 32, and 64 μmol/L, quantitative detection of apoptotic cells and analysis of cell cycle distribution in cultures were performed by FCM (FACSCalibur, Becton Dickson) using a FACScan and repeated for at least 4 times[7].

SABC immunohistochemical analysis After a 48-h exposure to AA 16, 32, and 64 μmol/L, the expressions of Bcl-2 and Bax proteins in COLO 205 cells were visualized by immunohistochemical assay kit, as recommended by the manufacturer[9]. Briefly, cultured cell sections were fixed with 4 % paraformaldehyde and endogenous peroxidase activity was blocked with H_2O_2 and normal goat serum. Then, the sections were incubated with the rabbit anti-human Bcl-2 or Bax polyclonal antibody respectively, biotinlyated goat anti-rabbit IgG, and avidin-biotin-peroxidase complex in turn. After stained by DAB, the sections were observed under light microscopy. Photos were taken and quantitative analysis was conducted with td2000 pathology cell image analysis system in 5 areas of each slide.

Measurement of relative mitochondrial transmembrane potential (MTP) After treatment with AA 16, 32, and 64 μmol/L for 48 h, the change of relative MTP in COLO 205 cell was analyzed[10]. Briefly, after

trypsinization, cells were washed twice with PBS then the concentration of cell suspension was adjusted to 1×10^9/L. Rh123 (20 mg/L) 100 μL was added to cell suspension 100 μL. After an incubation at 37 ºC for 30 min, the cells were washed with PBS again and suspended in PBS finally. The fluorescence of individual cell was measured with FCM and analyzed with Cellquest 3.1f Analysis Software.

Detection of viability of COLO 205 cell treated with AA or DEVD-CHO and AA After an incubation with DEVD-CHO 1 μmol/L or DMEM for 4 h, COLO 205 cells were administered with AA 55, 68, 85, or 106 μmol/L, respectively and the viability was measured by MTT assay at 48 h later[11].

Statistics Data were expressed as mean±SD, and analyzed with *t*-test. $P<0.05$ was considered to be significant.

RESULTS

Effects of AA on COLO 205 and other tumour cells proliferation According to MTT assay, the IC_{50} (96 h) of AA for inhibiting COLO 205, PC_3, Bcap37, and K562 cell proliferation was 61.56±0.34, 64.76±5.42, 61.20±1.61, and 65.80±1.95 μmol/L, respectively. AA induced a marked concentration- and time-dependent inhibition of COLO 205 cell proliferation (Fig 2).

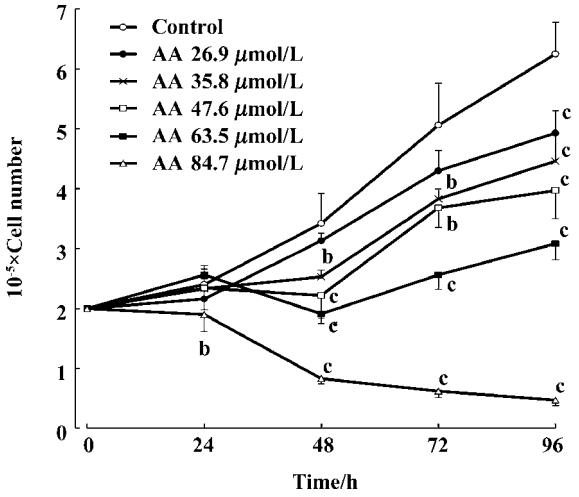

Fig 2. Effect of AA on COLO 205 cell growth. *n*=4. [b]*P*<0.05, [c]*P*<0.01 *vs* control.

Effects of AA on DNA content in COLO 205 cells After an exposure to AA for 48 h, the DNA content in COLO 205 cell was reduced significantly ($P<0.01$, Tab 1).

Tab 1. Effects of Astilbic acid (AA) on DNA content of COLO 205 cells. *n*=3. Mean±SD. [c]*P*<0.01 *vs* control.

AA Concentration/μmol·L^{-1}	DNA content/μg per 1×10^6 cells
0 (control)	63.55±5.15
16	41.15±3.13[c]
32	37.96±5.13[c]
64	30.82±1.43[c]

Effects of AA on morphology of COLO 205 cells After an exposure to AA, COLO 205 cells showed typical apoptosis characterized by volume reduction, chromatin condensation, nuclear fragmentation, and appearance of apoptotic bodies (Fig 3).

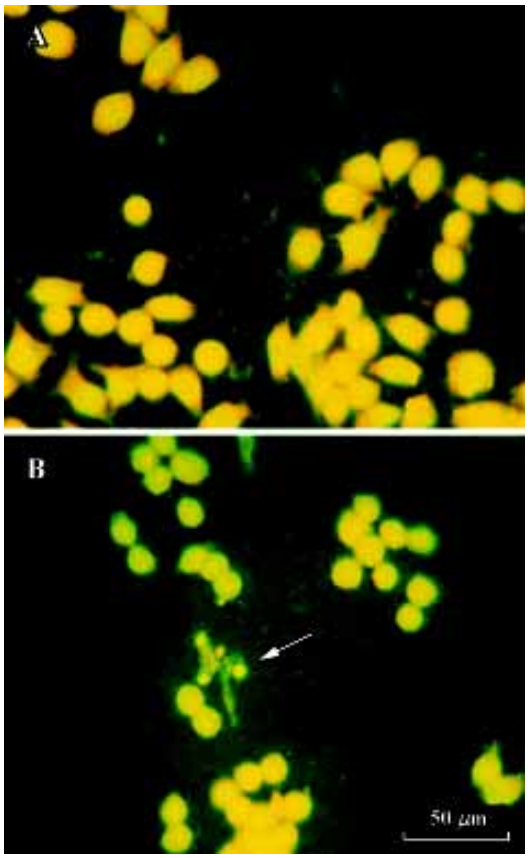

Fig 3. Morphological changes of COLO 205 cells stained with AO under fluorescence microscope. A) control; B) Cells treated with AA 64 μmol/L for 48 h. ×100.

Under electron microscope, the reduced cell volume, shranked cytoplasm could be observed. But plasma membrane remained well defined and condensed chromatin located along nuclear envelope or formed

irregularly shaped crescents at nuclear edges (Fig 4).

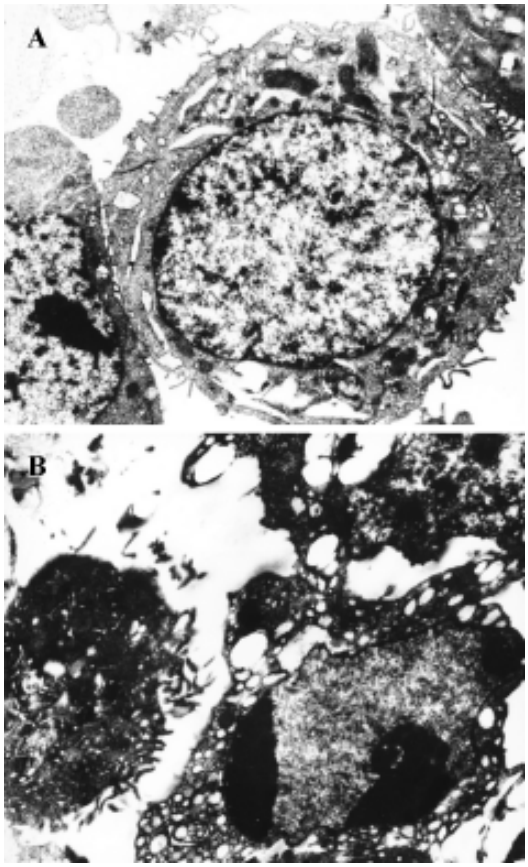

Fig 4. Characteristic morphology of AA-induced apoptosis of COLO 205 cells under electron microscope. A) control (×5000); B) Cells treated with AA 64 μmol/L for 48 h (×6000).

Effects of AA on COLO 205 cell DNA fragmentation DNA isolated from COLO 205 cells cultured with AA 64 μmol/L for 48 h showed the characteristic "ladder" pattern of apoptosis. A comparison with molecular weight markers indicated that the fragments were multiples of approximately 180 bp (Fig 5).

Apoptosis rate and cell cycle distribution After treatment of COLO 205 cells with AA 64 μmol/L for 48 h, a subdiploid peak (apoptotic peak) of DNA of apoptosis characteristics was observed, and the apoptosis rate was 28.25 %±2.89 % for AA 64 μmol/L. The obvious changes in cell cycle distribution of COLO 205 cells treated with AA were characterized by increase of G_0/G_1 phase and decrease of S and G_2/M phase cells, suggesting AA led to accumulation of COLO 205 cells in G_0/G_1 phase (Tab 2).

Expressions of Bcl-2 and Bax proteins Expression of Bcl-2 protein, was reduced in AA-treated COLO 205 cells, while that of Bax protein was increased, suggesting AA down-regulated Bcl-2 protein level and up-regulated Bax protein level (Tab 3).

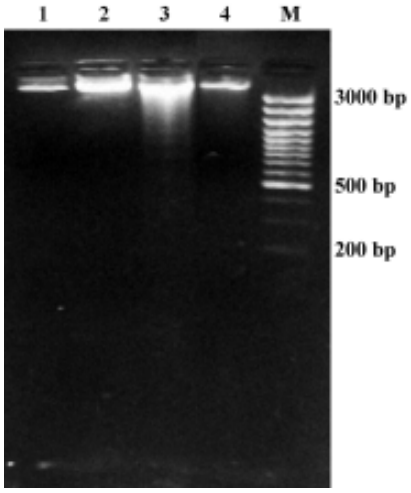

Fig 5. Agarose gel electrophoresis for detecting DNA fragmentation in COLO 205 cells treated with AA for 48 h (lane 1: AA 16 μmol/L; lane 2: AA 32 μmol/L; lane 3: AA 64 μmol/L; lane 4: control; lane M: DNA marker).

Tab 2. Effects of AA on cell cycle distribution and apoptotic index of COLO 205 cells after 48 h treatment. *n*=4-5. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control .

Concentration/ μmol·L^{-1}	Cell cycle/% G_0/G_1	S	G_2/M
0 (control)	76.65±1.12	18.15±3.12	5.20±2.38
16	82.51±1.98^c	13.33±1.94^b	4.15±0.22^a
32	87.02±1.11^c	9.74±0.57^c	3.24±0.88^a
64	90.79±1.32^c	8.60±1.80^c	0.62±0.75^c

Changes of relative MTP in COLO 205 cells The results of FCM measurement showed that relative

Tab 3. Effects of AA on the expression of Bcl-2 and Bax of COLO 205 cells after 48 h treatment. *n*=5. Mean±SD. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control .

Concentration/ μmol·L^{-1}	Bcl-2/%	Bax/%	Bcl-2/Bax value
0 (control)	37.93±2.19	19.74±2.91	1.92
16	33.90±2.94^b	23.49±4.26^a	1.44
32	25.17±5.32^c	32.78±3.28^c	0.77
64	19.07±1.97^c	34.60±3.90^c	0.55

MTP in COLO 205 cell treated with AA 64 μmol/L for 48 h was decreased significantly (Fig 6), suggesting structural and functional alterations in mitochondria.

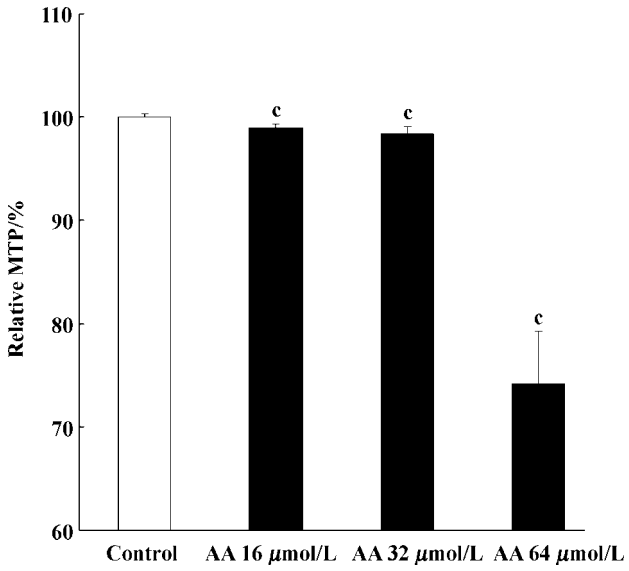

Fig 6. Change of relative MTP in COLO 205 cells treated with AA for 48 h. *n*=4. $^{c}P<0.01$ *vs* control.

Effects of selective caspase-3 inhibitor DEVD-CHO on the viability of COLO 205 cells DEVD-CHO 1 μmol/L could significantly increase the viability of COLO 205 cells treated with AA (≤85 μmol/L) for 48 h (Tab 4).

DISCUSSION

AA isolated from *Astilbe chinensis* rhizome is a pentacyclic triterpenoid. Researchers have demostrated some triterpenoids could inhibit tumor cell growth through induction of cell apoptosis[12,13]. However there was no report on antitumor effect of AA. Our results showed AA inhibited the proliferation of human colorectal carcinoma COLO 205 cells in concentration- and time-depended manner, reduced cell DNA content and induced apoptosis. It was indicated AA possessed antitumor activity through inducting cell apoptosis[14]. Furthermore, we explored the mechanism of apoptosis induced by AA. We examined the expression of Bcl-2 and Bax proteins in AA-treated COLO 205 cells. As we know that Bcl-2 and Bax are two kinds of apoptosis related protein[15,16], and caspase-3 was in the downstream of Bcl-2 and Bax. They all play important role in the control of apoptosis initiation and execution. According to the results, the expression of Bcl-2 was decreased, while that of Bax was increased, and the ratio of Bcl-2 and Bax was reduced significantly. Recent researches showed that Bax could form heterodimers with Bcl-2 to inhibit the anti-apoptotic function of Bcl-2[17]. Hence Bcl-2/Bax ratio is important to apoptosis induced by several agents and the decline of this ratio contributes to apoptosis induction. In addition, Bax dimers or oligomers directly form channel in mitochondrial outer membrane in apoptosis [16]. Mitochondria also plays a central role in the regulation of apoptosis [18]. Our experiment showed that the relative MTP in COLO 205 cell treated with AA was decreased significantly, and it was an evidence for the alterations of permeability of outer membrane of mitochondria. The lowered level of MTP is the upstream event of caspase activation, which will lead cell to apoptosis at last[18]. Furthermore we examined the effect of DEVD-CHO[19], a highly specific and potent inhibitor of caspase-3, on inhibition of AA-treated COLO 205 cell proliferation and found that DEVD-CHO blocked AA-induced inhibition. It suggested that the activation of caspase-3 was necessary in the execution of apoptosis induced by AA. Therefore AA induced COLO 205 cell apoptosis through down-regulating Bcl-2 and up-regulating Bax protein expression and activating caspase-3 pathway.

REFERENCES

1 Sun HX, Zhang JX, Ye YP, Pan YJ, Shen YA. Cytotoxic pentacyclic triterpenoids from the rhizome of *Astilbe chinensis*. Helv Chim Acta 2003; 86: 2404-13.

2 Chen PF, Lai PF, Zhang P, Chen LL, Li AP. Studies on anticancer activity of Astilbe chinensis. China J Chin Mater Med 1996; 21: 302-3.

Tab 4. Effects of DEVD-CHO on the viability of COLO 205 cells treated with AA for 48 h. *n*=3. Mean±SD. $^{c}P<0.01$ *vs* control.

DEVE-CHO/ μmol·L^{-1}	Cell viability/%			
	AA 55 μmol·L^{-1}	AA 68 μmol·L^{-1}	AA 85 μmol·L^{-1}	AA 106 μmol·L^{-1}
0 (control)	63.6±3.3	46.8±1.4	8.2±0.7	7.1±1.7
1	85.0±1.9^{c}	73.3±4.4^{c}	15.1±2.0^{c}	8.3±0.5

3 Liu XL, Chen K, Ye YP, Peng XY, Qian BC. Glutathione antagonized cyclophosphamide and acrolein induced cytotoxicity of PC_3 cells and immunosuppressive actions in mice. Acta Pharmacol Sin 1999; 20: 643-6.
4 Fu XL, Zheng XL, Liu XL, Qian BC. Inhibition in *in vitro* proliferation of human prostate cancer PC_3 cells induced by melatonin. Chin Pharmacol Bull 2003; 19: 883-6.
5 Wang JZ, Wu YJ, Rao CM, Gao MT, Li WG. Effect of recombinant human basic fibroblast growth factor on stomach ulcers in rats and mice. Acta Pharmacol Sin 1999; 20: 763-8.
6 Yang SF, Yang ZQ, Wu Q, Sun AS, Huang XN, Shi JS. Protective effect and mechanism of *Ginkgo biloba* leaf extracts for Parkinson disease induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine. Acta Pharmacol Sin 2001; 22: 1089-93.
7 Liu XL, Zhang L, Fu XL, Chen K, Qian BC. Effect of scopoletin on PC_3 cell proliferation and apoptosis. Acta Pharmacol Sin 2001; 22: 929-33.
8 Shi JS, Haglid GK. Effect of rhynchophylline on apoptosis induced by dopaminein NT2 cells. Acta Pharmacol Sin 2002; 23: 445-9.
9 Leng Y, Feng Y, Cao L, Gu ZP. Effects of droloxifene on apoptosis and Bax, Bcl-2 protein expression of luteal cells in pseudopregnant rats. Acta Pharmacol Sin 2001; 22: 155-62.
10 Chen XC, Zhu YG, Chen LM, Fang F, Zhou YC, Zhao CH. Nitric oxide induced PC12 cells apoptosis and the protective effect of ginsenoside Rg1. Chin Pharmacol Bull 2002; 18: 516-9.
11 Wright SC, Schellenberger U, Wang H, Kinder DH, Talhouk JW, Larrick JW. Activation of CPP32-like proteases is not sufficient to trigger apoptosis, inhibition of apoptosis by agents that suppress activation of AP24, but not CPP32-like activity. J Exp Med 1997; 186: 1107-17.
12 Pec MK, Aguirre A, Moser-Thier K, Fernandez JJ, Souto ML, Dorta J, *et al.* Induction of apoptosis in estrogen dependent and independent breast cancer cells by the marine terpenoid dehydrothyrsiferol. Biochem Pharmacol 2003; 65: 1451-61.
13 Pedersen IM, Kitada S, Schimmer A, Kim Y, Zapata JM, Charboneau L, *et al*. The triterpenoid CDDO induces apoptosis in refractory CLL B cells. Blood 2002; 100: 2965-72.
14 Evan G, Littlewood T. A matter of life and cell death. Science 1998; 281: 1317-22.
15 Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, *et al.* Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74: 597-608.
16 Yin C, Knudson CM, Korsmeyer SJ, Van Dyke T. Bax suppresses tumorigenesis and stimulates apoptosis *in vivo*. Nature 1997; 385: 637-40.
17 Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 hetero-dimerizes *in vivo* with a conserved homolog, bax, that accelerates programmed cell death. Cell 1993; 74: 609-19.
18 Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281: 1309-12.
19 Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, *et al.* Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995; 376: 37-43.