Induction of apoptosis by sanguinarine in C6 rat glioblastoma cells is associated with the modulation of the Bcl-2 family and activation of caspases through downregulation of extracellular signal-regulated kinase and Akt

Min Ho Han[a,d], Sung Ok Kim[a], Gi Young Kim[e], Taeg Kyu Kwon[f], Byung Tae Choi[b], Won Ho Lee[d] and Yung Hyun Choi[a,c]

Sanguinarine is a benzophenanthridine alkaloid that is derived from the root of *Sanguinaria canadensis* and other poppy fumaria species, and is known to have antimicrobial, antiinflammatory and antioxidant properties. This study investigated the possible mechanisms through which sanguinarine exerts its antiproliferative action in cultured C6 rat glioblastoma cells. The exposure of C6 cells to sanguinarine resulted in growth inhibition and the induction of apoptosis in a dose-dependent manner, as measured by the MTT assay, fluorescence microscopy, agarose gel electrophoresis and annexin-V-based assay. The sanguinarine treatment induced the proteolytic activation of caspases and ICAD/DFF45, which was associated with the modulation of the Bcl-2 family, concomitant degradation of poly(ADP ribose) polymerase and phospholipase C-γ1 protein, and DNA fragmentation. z-DEVD-fmk, a caspase-3-specific inhibitor, blocked poly(ADP ribose) polymerase degradation, DNA fragmentation and increased the survival rate of sanguinarine-treated C6 cells. Moreover, the activity of extracellular signal-regulated kinase and Akt was downregulated in sanguinarine-treated cells, and PD98059, a specific extracellular signal-regulated kinase inhibitor, and phosphatidylinositol 3′-kinase/Akt inhibitors, LY294002 and wortmanin, sensitized the cells to sanguinarine-induced apoptosis, indicating that the downregulation of the extracellular signal-regulated kinase and Akt signaling pathway may play a key role in sanguinarine-induced apoptosis in C6 cells. *Anti-Cancer Drugs* 18:913–921 © 2007 Lippincott Williams & Wilkins.

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[a]Department of Biomaterial Control (BK21 program), Dongeui University Graduate School, Departments of [b]Anatomy and [c]Biochemistry, Dongeui University College of Oriental Medicine, [d]Department of Biology, Pusan National University, Busan, [e]Faculty of Applied Marine Science, Cheju National University, Jeju and [f]Department of Immunology, School of Medicine, Keimyung University, Taegu, South Korea

Correspondence to Dr Yung Hyun Choi, PhD, Department of Biochemistry, Dongeui University College of Oriental Medicine, Busan 614-052, South Korea
Tel: +82 51 850 7413; fax: +82 51 853 4036; e-mail: choiyh@deu.ac.kr

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Introduction

Apoptosis, or programmed cell death, plays an important role in regulating the number of cells during development, in homeostatic cell turnover in adults and in many other settings. Apoptosis is characterized by a series of distinct morphological and biochemical alterations to cells such as DNA fragmentation, chromatin condensation, cell shrinkage and plasma membrane blebbing [1,2]. In recent studies, apoptosis has also been considered an ideal way of eliminating precancerous and/or cancer cells. Most cancer cells, however, can block apoptosis, which allows them to survive despite the genetic and morphologic transformations. The induction of apoptotic cell death is, therefore, an important mechanism in the anticancer properties of many anticancer drugs [3,4].

Sanguinarine (13-methyl (1,3) benzodioxolo [5,6c]-1,3-dioxolo[4,5–i]phenanthridinium) is a benzophenanthridine alkaloid that is derived from the root of *Sanguinaria canadensis* and from other poppy-*fumaria* species, and is widely used in toothpastes and mouth washes [5,6]. This compound is a structural homologue of chelerythrine, and has antimicrobial, antioxidant and antiinflammatory properties [7–9]. Many studies have indicated that sanguinarine, at the micromolar concentration, inhibits the growth of various cancer cell lines that are associated with cell-cycle arrest and the stimulation of apoptotic cell death [10–14]. The underlying mechanisms of its action, however, are not completely understood.

This study examined the antiproliferative activity of sanguinarine and its effect on the apoptosis of C6 rat glioblastoma cells. Furthermore, the levels of several important genes that are strongly associated with the signal-transduction pathway of apoptosis were measured to establish the anticancer mechanism of sanguinarine.

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Materials and methods

Cell culture and chemicals

C6 cells, a rat glioblastoma cell line, were obtained from American Type Culture Collection (Rockville, Maryland, USA). The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Gibco/BRL, Gaithersburg, Maryland, USA) and 1% penicillin–streptomycin. The cells were maintained under standard cell-culture conditions at 37°C in a humid environment containing 5% CO_2. Sanguinarine (Sigma, St Louis, Missouri, USA) was dissolved in methanol as a stock solution at a 10 mmol/l concentration and was stored in aliquots at −20°C.

Cell-viability assay

The C6 cells were plated at 1.5×10^5 cells/well in 2 ml of Dulbecco's modified Eagle's medium containing 0.5, 1.0, 1.5 and 2.0 μmol/l of sanguinarine in a six-well microtiter plate. After incubation for 24 h, the cell viability was determined using a MTT assay, which is based on the conversion of MTT into MTT-formazan by the mitochondria. Briefly, 200 μl of the MTT reagent (5 mg/ml in PBS) was added to each well and incubated for 2 h. The plate was then centrifuged at 2000 r.p.m. for 5 min at 4°C and the MTT solution was removed from the wells by aspiration. The formazan crystals were dissolved in 2 ml dimethyl sulfoxide and moved to a 96-well plate. The absorbance was recorded on a microplate reader at a wavelength of 540 nm. The effect of sanguinarine on the inhibition of cell growth was assessed as the percentage cell viability, where the vehicle-treated cells were considered to be 100% viable. For the morphological study, the C6 cells were treated with sanguinarine for 24 h and photographed directly using an inverted microscope (Carl Zeiss, Germany).

Nuclear staining with 4,6-diamidino-2-phenylindole

After treating the cells with sanguinarine for 24 h, the cells were harvested, washed in ice-cold PBS and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with a 4,6-diamidino-2-phenylindole (DAPI; Sigma) solution for 10 min at room temperature. The nuclear morphology of the cells was examined by fluorescence microscopy (Carl Zeiss).

DNA fragmentation assay

The C6 cells were treated with different sanguinarine concentrations for 24 h and lysed on ice in a buffer containing 10 mmol/l Tris HCl (pH 7.4), 150 mmol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100 for 30 min. The lysates were vortexed and cleared by centrifugation at 10 000 *g* for 20 min. The fragmented DNA in the supernatant was extracted using an equal volume of neutral phenol: chloroform:isoamylalcohol (25:24:1, v/v) and analyzed electrophoretically on 1% agarose gel containing ethidium bromide (Sigma).

Measurement of apoptosis by annexin-V–fluorescein isothiocyanate and propidium iodide double staining

The magnitude of the apoptosis elicited by sanguinarine was determined using an annexin-V fluoroscein isothiocyanate (FITC) apoptosis detection kit (Becton Dickinson, San Jose, California, USA). The cells were washed with PBS and resuspended in annexin-V binding buffer containing 10 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid (HEPES)/NaOH, pH 7.4, 140 mmol/l NaCl and 2.5 mmol/l $CaCl_2$ according to the manufacturer's protocol. Aliquots of the cells were incubated with annexin-V–FITC, mixed and incubated for 15 min at room temperature in the dark. Propidium iodide (PI) at a concentration of 5 μg/ml was added to identify the necrotic cells. The apoptotic cells (V^+/PI^-) were measured by fluorescence-activated cell-sorter analysis in a FACS analyzer (Becton Dickinson).

Protein extraction and Western blotting

The cells were harvested and lysed. The protein concentrations were measured using a BioRad protein assay (BioRad Laboratories, Hercules, California, USA) according to the manufacturer's instructions. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on sodium dodecyl sulfate–polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane (Schleicher & Schuell, Keene, New Hampshire, USA). The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody and visualized by enhanced chemiluminescence according to the recommended procedure (Amersham, Arlington Heights, Illinois, USA). The primary antibodies were purchased from Santa Cruz Biotechnology [Santa Cruz, California, USA: actin, SC-1615; Bcl-2, SC-509; Bad, SC-8044; inhibitor of apoptosis protein (IAP)-1, SC-7943; cIAP-2, SC-7944; XIAP, SC-11426; Fas, SC-715; FasL, SC-957; DR4, SC-7863; TRAIL, SC-7877; caspase-3, SC-7272; caspase-8, SC-7890; caspase-9, SC-7885; poly(ADP ribose) polymerase (PARP), SC-7150; phospholipase C (PLC)-γ1, SC-7290; extracellular signal-regulated kinase (ERK), SC-154; p38, SC-728], Cell Signaling Technology, [Boston, Massachusetts, USA: Akt, 9272S; pAkt, 9271S; pERK, 9106S; Jun N-terminal kinase (JNK), 9252S; pJNK, 9251S; pp38, 9211S] and Calbiochem (San Diego, California, USA: DR5, PC392; CAD/DFF40, PC380; ICAD/DFF45, PC366). The peroxidase-labeled donkey antirabbit immunoglobulin and peroxidase-labeled sheep antimouse immunoglobulin were purchased from Amersham.

In-vitro caspase activity assay

The caspase activity was determined by a colorimetric assay using a caspase-3, caspase-8 and caspase-9 activation kit according to the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota, USA). Briefly, the cells were lysed in a lysis buffer for 30 min on an ice bath. The

supernatants were collected and incubated at 37°C with the reaction buffer supplied, which contained dithiothreitol and substrates, Asp-Glu-Val-Asp (DEVD)-*p*-nitroaniline (pNA) for caspase-3, Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8 and Leu-Glu-His-Asp (LEHD)-pNA for caspase-9. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

Statistical analysis

The data are expressed as mean ± SD. A statistical comparison was performed using one-way analysis of variance followed by a Fisher test. The significant differences between the groups were determined using an unpaired Student's *t*-test. A *P*-value less than 0.05 was considered significant.

Results

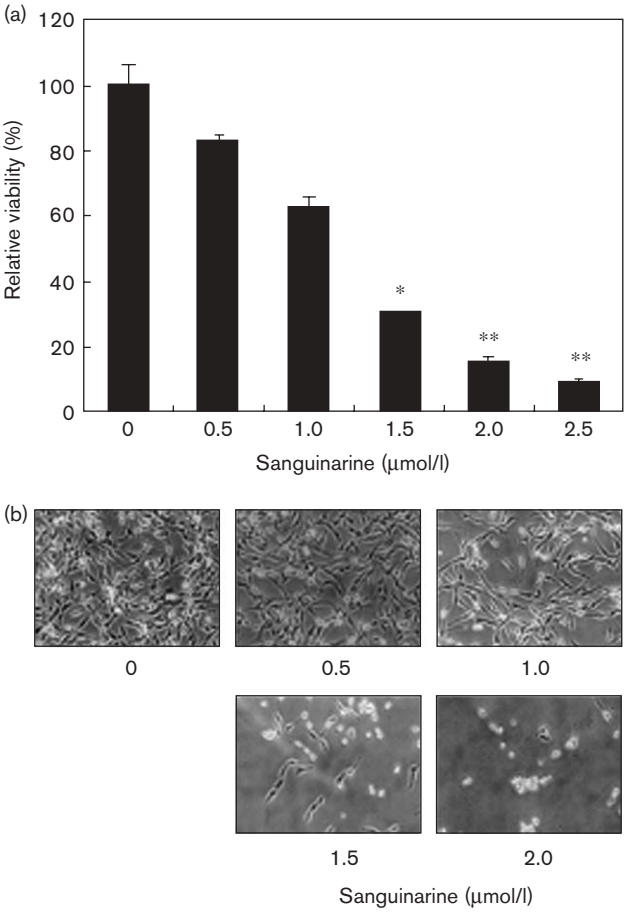
Inhibition of cell viability and induction of apoptosis by sanguinarin

To determine if sanguinarine decreases the cell viability, the C6 cells were stimulated with various concentrations of sanguinarine for 24 h and the cell viability was measured by an MTT assay. As shown in Fig 1a, the sanguinarine treatment significantly inhibited the cell viability in a concentration-dependent manner. After a 24-h treatment, at 1.0 and 2.0 µmol/l, sanguinarine inhibited the cell viability by approximately 35 and 82%, respectively, compared with the controls. Direct observations by inverted microscopy demonstrated that the C6 cells treated with sanguinarine showed many morphological changes compared with the control cells (Fig. 1b). In particular, cell shrinkage, cytoplasm condensation and formation of cytoplasmic filaments appeared after 1.5 µmol/l sanguinarine treatment for 24 h. Further experiments using fluorescence microscopy and flow-cytometry analyses were carried out to determine if the inhibitory effect of sanguinarine on the cell viability was the result of apoptotic cell death. Morphological analysis with DAPI staining showed nuclei with chromatin condensation and the formation of apoptotic bodies in the cells cultured with sanguinarine in a concentration-dependent manner. On the other hand, very few were observed in the control culture (Fig. 2a). Flow-cytometry analysis with annexin-V and PI staining was, therefore, used to determine the magnitude of apoptosis elicited by sanguinarine. As shown in Fig. 2b, the annexin V-positive cells increased in a concentration-dependent manner in the sanguinarine-treated C6 cells compared with the untreated control cells.

Modulation of the levels of the Bcl-2 and inhibitor of apoptosis protein family and activation of caspases by sanguinarin

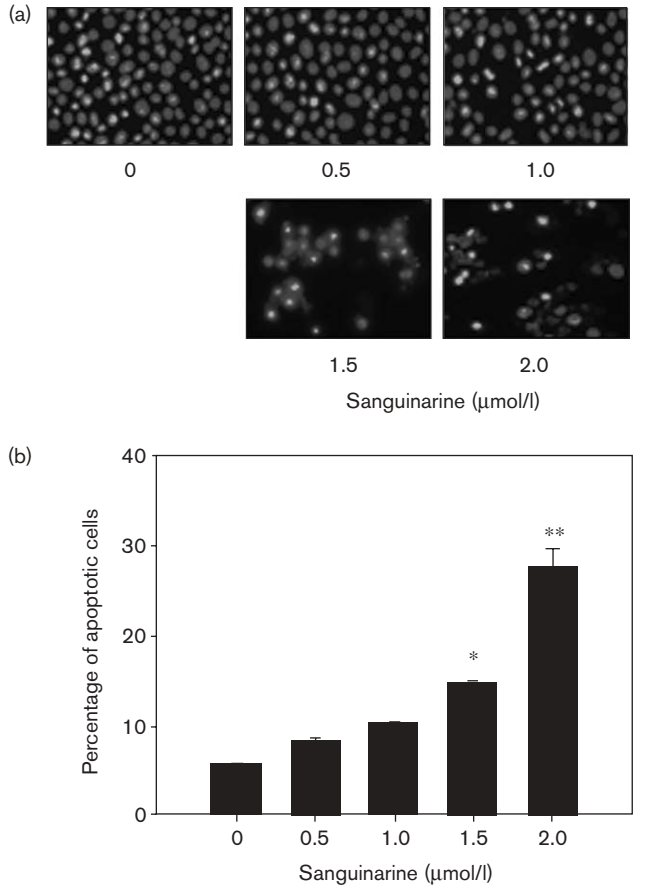
First, the apoptotic cascades in C6 cells caused by sanguinarine were examined by exposing the cells to sanguinarine and comparing the levels of Bcl-2 family members. Western immunoblotting showed that the levels of proapoptotic Bax and Bad expression were upregulated in the sanguinarine-treated cells, whereas the level of antiapoptotic Bcl-2 expression was markedly downregulated in response to the sanguinarine treatment (Fig. 3). The expression levels in the sanguinarine-treated cells were also examined to determine if sanguinarine induces C6 cell death through a change in the expression of the IAP family members, which binds the caspases and leads to caspase inactivation for an antiapoptotic effect. As shown in Fig. 3, sanguinarine induced a concentration-dependent decrease in the expression levels of the XIAP and cIAP-1 proteins, but had no effect on cIAP-2 expression. The levels of Fas, FasL, TRAIL, DR4 and DR5 protein expression, however,

Fig. 1



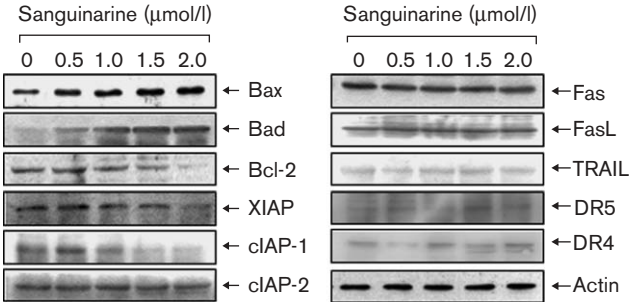
Growth inhibition and morphological changes of C6 cells by sanguinarine. The cells were seeded at an initial density of 2.5×10^5 cells per 60-mm plate, incubated for 24 h and treated with various concentrations of sanguinarine for 24 h. (a) The level of growth inhibition was measured using a MTT assay. Each point represents the mean ± SD of three independent experiments. The significance was determined by a Student's *t*-test (*$P<0.05$ vs. untreated control). (b) After 24-h incubation with sanguinarine, the cells were sampled and examined by inverted microscopy. Magnification, × 200.

Fig. 2



Induction of apoptosis by sanguinarine treatment in C6 cells. The cells were treated with various concentrations of sanguinarine for 24 h. (a) The cells were sampled, fixed, and stained with 4,6-diamidino-2-phenylindole. The stained nuclei were then observed under a fluorescent microscope using a blue filter. Magnification, $\times 400$. (b) After the sanguinarine treatment for 24 h, the cells were collected and stained with fluorescein isothiocyanate-conjugated annexin-V and propidium iodide (PI) for flow-cytometry analysis. The number of apoptotic cells are determined by counting the % of annexin V^+, PI^- cells and the % of annexin V^+, PI^+ cells. The results are expressed as the mean ± SD of three independent experiments. The significance was determined by a Student's *t*-test (*$P<0.05$ vs. untreated control).

Fig. 3



Effects of the sanguinarine treatment on the levels of Bcl-2 and inhibitor of apoptosis protein (IAP) family, and death receptor-related proteins in C6 cells. The cells were treated with the indicated concentrations of sanguinarine for 24 h. Equal amounts of the cell lysates (30 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose and probed with the indicated antibodies. Actin was used as the internal control.

which play important roles in the death receptor pathway, were similar in the sanguinarine-treated and sanguinarin-untreated control C6 cells (Fig. 3). To determine if sanguinarine-induced apoptosis is associated with the activation of caspases, the expression and activity of caspases such as capase-3, caspase-8 and caspase-9 in the sanguinarine-treated C6 cells were examined by Western blotting analysis and an in-vitro activity assay. As shown in Fig. 4a and b, the sanguinarine treatment increased the expression levels of the active subunits and the in-vitro activity of caspase-3, caspase-8 and caspase-9. Western blot analysis showed the progressive proteolytic cleavage of PARP and PLC-γ1 protein, which are downstream targets of the activated caspase-3 [15,16], in C6 cells after the sanguinarine treatment in a concentration-dependent manner (Fig. 4c).

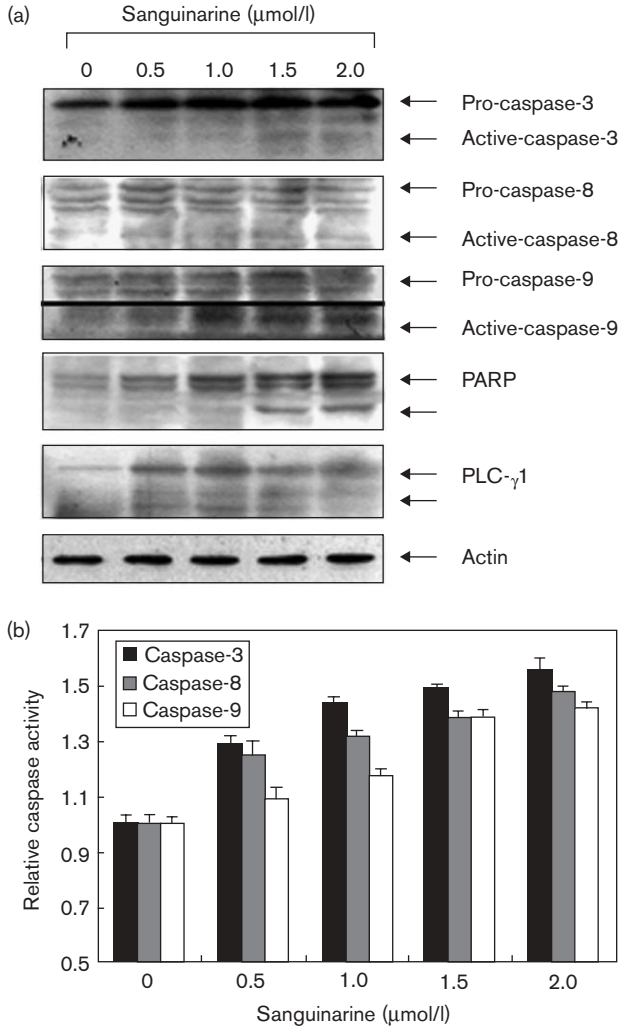
Induction of DNA fragmentation by sanguinarine

Apart from the key role of caspases and other proteases, cell death triggers DNA fragmentation so that DNases play an active role in apoptotic cell death [17]. The best-characterized apoptotic DNase is a caspase-activated DNase/DNA fragmentation factor (CAD/DFF40). The activity of CAD/DFF40 is regulated by its inhibitor, the inhibitor of the CAD/DNA fragmentation factor (ICAD/DFF45), which is cleaved by caspases [18,19]. This study, therefore, examined the effects of sanguinarine on the levels of CAD/DFF40 and ICAD/DFF45 expression, and measured the level of DNA fragmentation to determine if it was induced by sanguinarine in C6 cells. As shown in Fig. 4a, sanguinarine treatment induced the cleavage of ICAD/DFF45 proteins in a concentration-dependent manner; however, the CAD/DFF40 levels remained unchanged. Agarose gel electrophoresis of the cells treated with sanguinarine showed a typical ladder pattern of internucleosomal fragmentation (Fig. 4b), suggesting that the activation of caspase is a key step in the sanguinarine-induced apoptotic pathway in C6 cells. The sanguinarine treatment also caused the proteolytic activation of caspases such as caspase-3, caspase-8 and caspase-9 in a concentration-dependent manner (Fig. 5).

Inhibition of sanguinarine-induced apoptosis by caspase-3 inhibitor

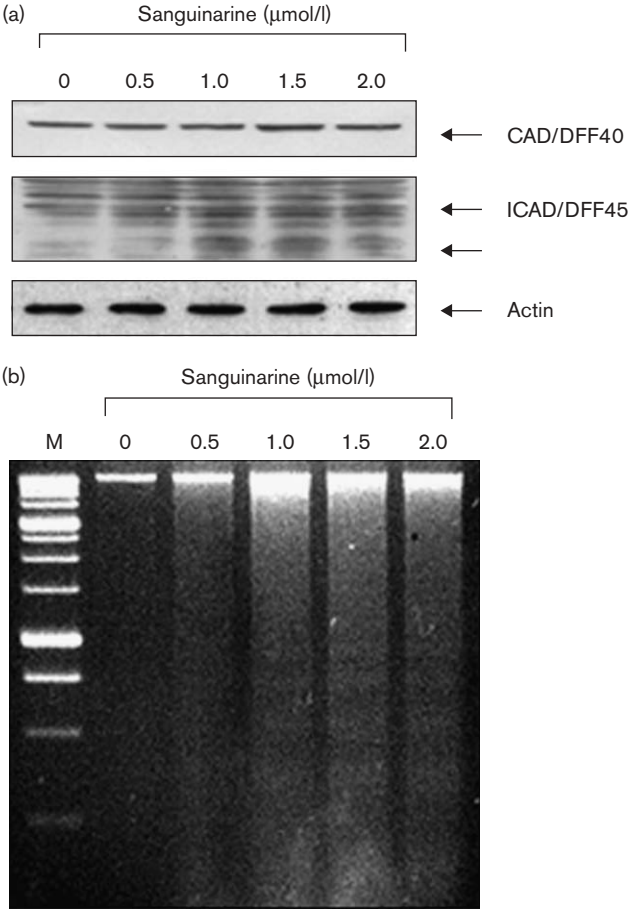
The C6 cells were pretreated with z-DEVD-fmk (50 μmol/l), a cell-permeable caspase-3 inhibitor, for 2 h, followed by a treatment with 1.5 μmol/l sanguinarine for 24 h to confirm that the activation of an initiator caspase such as caspase-3 is a key step in the sanguinarine-induced apoptotic pathway. The sanguinarine-induced

Fig. 4



Activation of caspases and the degradation of the poly(ADP ribose) polymerase (PARP) and phospholipase C (PLC)-γ1 protein by the sanguinarine treatment in C6 cells. (a) After 24-h incubation with sanguinarine, the cells were lysed. The cellular proteins were separated by sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anticaspase-3, anticaspase-8, anticaspase-9, anti-PARP and anti-PLC-γ1 antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. Actin was used as the internal control. (b) The cell lysates from the cells treated with sanguinarine for 24 h were assayed for the in-vitro caspase-3, caspase-8 and caspase-9 activity using Asp-Glu-Val-Asp (DEVD)-*p*-nitroaniline (pNA), Ile-Glu-Thr-Asp (IETD)-pNA, and Leu-Glu-His-Asp (LEHD)-pNA, respectively, as substrates. The concentrations of the fluorescent products released were measured. The results are expressed as the mean ± SD of three independent experiments.

Fig. 5



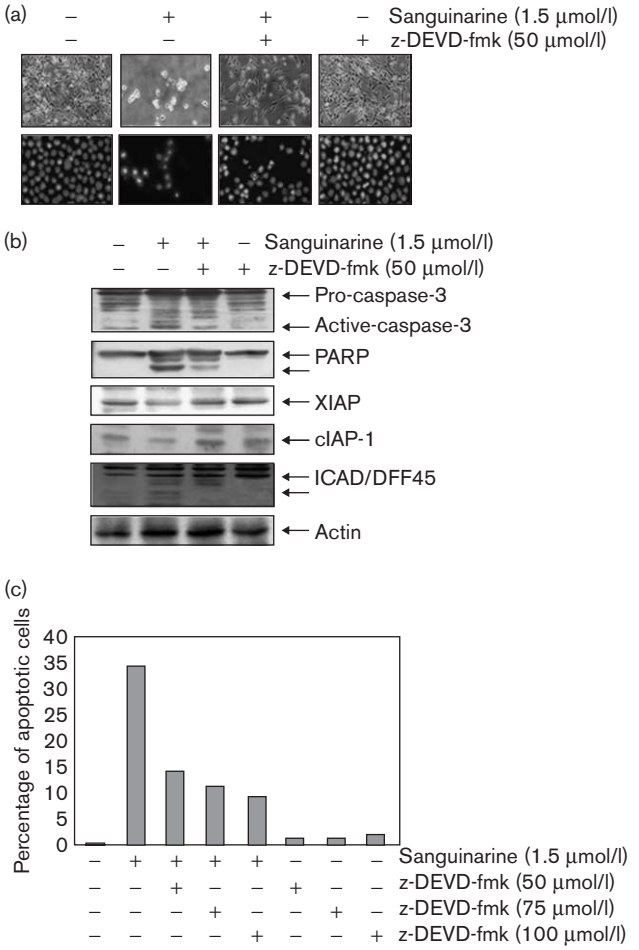
Inhibition of caspase-activated DNase/DNA fragmentation factor (ICAD/DFF45) expression and the induction of DNA fragmentation by sanguinarine treatment in C6 cells. (a) After 24-h incubation with sanguinarine, the cells were lysed, and the cellular proteins were separated by sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anticaspase-activated DNase/DNA fragmentation factor (CAD/DFF40) and anti-ICAD/DFF45 antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. Actin was used as the internal control. (b) For the analysis of DNA fragmentation, C6 cells were treated with sanguinarine for 24 h. The genomic DNA was extracted, and separated by 1% agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide (EtBr). M indicates a size marker of the DNA ladder.

morphological changes, chromatin condensation and increase in the number of apoptotic cells were prevented by a blockade of caspase-3 by a z-DEVD-fmk pretreatment in a concentration-dependent manner (Fig. 6a and c). Furthermore, z-DEVD-fmk markedly blocked the cleavage and/or degradation of caspase-3, PARP, XIAP, cIAP-1 and ICAD/DFF45 in the sanguinarine-treated C6 cells (Fig. 6b). This shows that sanguinarine-induced apoptosis is associated with caspase-3 activation.

Extracellular signal-regulated kinase inhibitor PD98059 sensitizes sanguinarine-induced apoptosis

As the activation of the mitogen-activated protein kinase (MAPK) signaling pathway plays important roles in the regulation of cell growth and cell death [20], this study next examined whether or not members of the MAPK family proteins were activated during sanguinarine-induced apoptosis. As shown in Fig. 7a, the sanguinarine

Fig. 6



Inhibition of sanguinarine-induced apoptosis by the caspase-3 inhibitor in C6 cells. The cells were treated with DNA fragmentation. z-DEVD-fmk (50 μmol/l) for 2 h before being challenged with 1.5 μmol/l sanguinarine for 24 h. (a) The cells were sampled and examined by optical microscopy or were stained with 4,6-diamidino-2-phenylindole for 10 min and photographed with a fluorescence microscope using a blue filter. (b) Equal amounts of the cell lysates (30–50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the anticaspase-3 and antipoly(ADP ribose) polymerase antibodies. Actin was used as an internal control. (c) The cells grown under the same conditions as (a) were collected and stained with fluorescein isothiocyanate-conjugated annexin-V and propidium iodide (PI) for flow-cytometry analysis. The apoptotic cells were determined by counting the % of annexin V^+, PI^- cells and the % of annexin V^+, PI^+ cells. Results are expressed as the mean ± SD of three independent experiments.

treatment significantly inhibited the phosphorylation of ERK in a concentration-dependent manner. The state of p38 MAPK and JNK activation was, however, not changed in the sanguinarine-treated cells. Therefore, specific inhibitors, PD98059, that blocks the activation of MAPK kinase 1 (the direct activator of ERK), SB203580, that is a specific inhibitor of p38 MAPK, and SP600125, that is a specific inhibitor of JNK, were used to examine the role of the MAPK proteins. As shown in Fig. 8b and c, the PD98059 treatment significantly increased the sanguinarine-induced PARP cleavage and apoptosis, whereas SB203580 and SP600125 did not increase the level of sanguinarine-induced cell death. This suggests that low levels of ERK induced by sanguinarine treatment may decrease the cell survival and compromise the efficacy of sanguinarine. Moreover, the results suggest that sanguinarine-induced apoptosis is independent of the p38 and JNK pathways.

Phosphatidylinositol 3′-kinase/Akt inhibitors sensitize sanguinarine-induced apoptosis

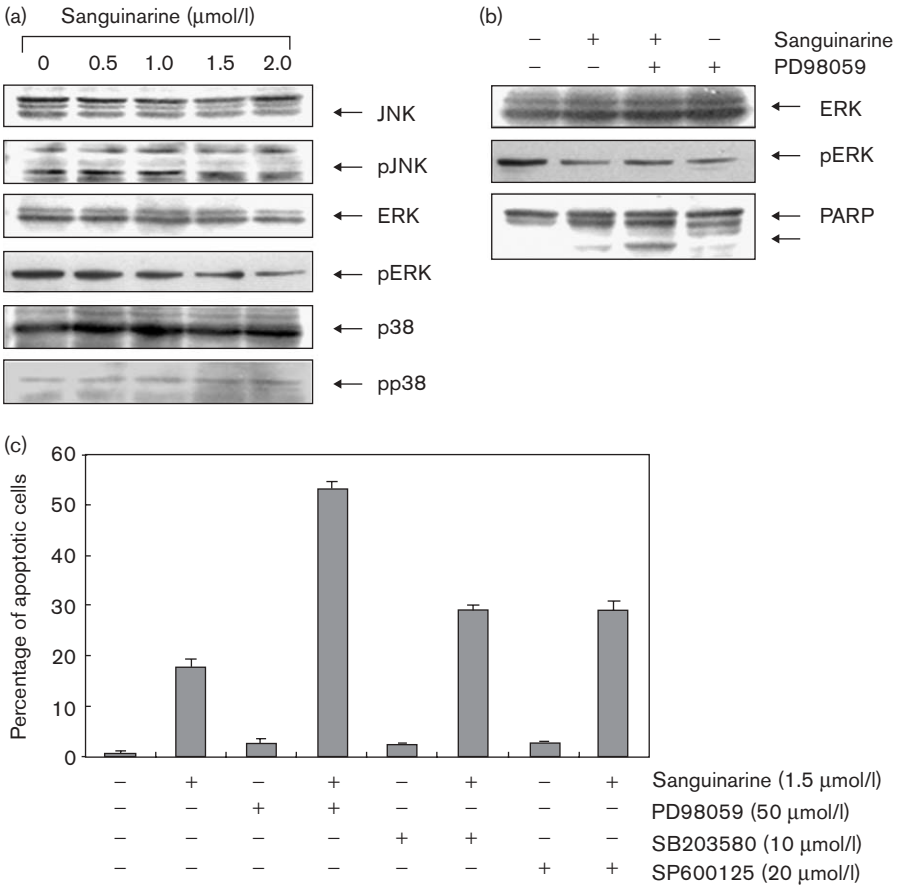
The phosphorylation state of the Akt protein in C6 cells after sanguinarine treatment for 24 h was examined to determine if sanguinarine-induced apoptosis is closely related to the Akt signal, which is a downstream effector of PI3K for survival signaling [21]. As shown in Fig. 8a, the levels of the total Akt protein remained unchanged during sanguinarine-induced apoptosis., Its phosphorylation levels were, however, markedly decreased in a dose-dependent manner. The involvement of Akt signal pathways in sanguinarine-induced apoptosis was examined using the PI3K/Akt inhibitors, LY294002 and wortmanin, to determine if the inhibition of Akt phosphorylation was responsible for the induction of apoptosis. As shown in Fig. 8, the combined treatment with sanguinarine and LY294002 or wortmanin significantly increased the number of apoptotic cells and the level of PARP cleavage. This indicates that sanguinarine-induced apoptosis is associated with the downregulation of the PI3K/Akt signaling pathway.

Discussion

Several studies have reported that sanguinarine treatment causes the accumulation of cells in the G_1 phase of the cell cycle and apoptosis. This suggests that the growth-inhibitory effect of sanguinarine occurs through the blockage of the G_1 phase and that these cells do not enter the S phase [10–14,22,23]. Although the cell-killing mechanism of sanguinarine has been suggested, little is known of the effects of this compound on the growth of cancer cells. This study, therefore, examined whether or not sanguinarine induced apoptosis and the mechanisms related to cell death, using a C6 rat glioblastoma cell model.

It was found that sanguinarine dose-dependently inhibited the cell viability and induced apoptosis in C6 cells. The induction of apoptosis by sanguinarine was confirmed by the characteristic morphological changes, that is chromatin condensation, agarose gel electrophoresis and annexin-V-based assay. Further experiments showed that the sanguinarine treatment significantly decreased the level of antiapoptotic Bcl-2 protein expression and increased the level of proapoptotic Bax or Bad protein expression, thus shifting the Bax/Bcl-2 or Bad/Bcl-2 ratio in favor of apoptosis (Fig. 3). These observations show

Fig. 7



Involvement of the extracellular signal-regulated kinase (ERK) pathway in sanguinarine-induced apoptosis in C6 cells. (a) After 24-h incubation with sanguinarine, the cells were lysed, and the cellular proteins were separated by sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. (b) The C6 cells were treated with PD98059 (50 μmol/l) for 2 h before being challenged with 1.5 μmol/l sanguinarine for 24 h. The cells were lysed, and the cellular proteins were separated by sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-ERK, anti-pERK and anti-poly(ADP ribose) polymerase (PARP) antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. (c) The cells were treated with PD98059 (50 μmol/l), SB203589 (10 μmol/l) and SP600125 (20 μmol/l) for 2 h before being challenged with 1.5 μmol/l sanguinarine for 24 h. The cells were collected and stained with fluorescein isothiocyanate conjugated annexin-V and propidium iodide (PI) for flow-cytometry analysis. The apoptotic cells were determined by counting the % of annexin V^+, PI^- cells and the % of annexin V^+, PI^+ cells. The results are expressed as the means ± SD of three independent experiments.

that the sanguinarine-induced apoptosis in C6 cells was triggered by the downregulation of Bcl-2, and the upregulation of Bax and Bad. The sanguinarine treatment also caused the proteolytic activation of caspases such as caspase-3, caspase-8 and caspase-9 in a concentration-dependent manner (Fig. 5). Activated caspases induce limited proteolysis in a number of cellular proteins, which are degraded by the caspase family as a result of apoptosis and have been used as markers of chemotherapy-induced apoptosis. This study examined whether or not PARP and the PLC-γ1 protein, which are substrates of caspase-3 [15,16], are cleaved in the sanguinarine-treated cells. As expected, both proteins were clearly degraded in a dose-dependent manner, which correlates with the activation of caspase-3 during the apoptosis caused by sanguinarine as seen in HaCaT cells [10,11]. Under the same conditions, z-DEVD-fmk, a specific caspase-3 inhibitor, prevented the sanguinarine-induced apoptosis by blocking not only the morphological changes but also degradation of PARP, XIAP, cIAP-1 and ICAD/DFF45 (Fig. 6). This indicates that caspase-3 plays an important role in sanguinarine-induced apoptosis in C6 cells.

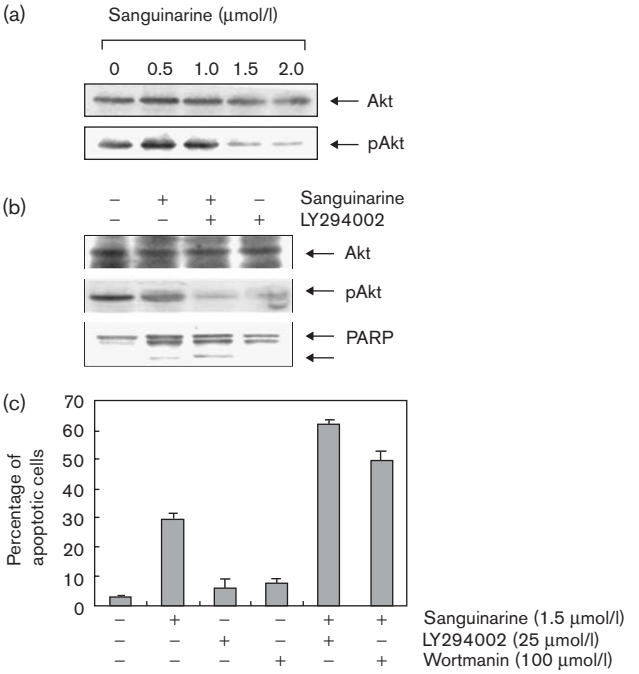
The IAP family, such as XIAP, cIAP-1 and cIAP-2, has been shown to regulate apoptosis. In particular, they have been reported to directly inhibit members of the caspase family including caspase-3 or caspase-9 [24,25]. In the final execution of apoptosis, the activated caspases induce DNA fragmentation through the activation of CAD/DFF40 [18,26]. In growing cells, CAD/DFF40

remains inactive and forms an inactive cytoplasmic heterodimer with ICAD/DFF45. During apoptosis, caspase-3 cleaves and releases ICAD/DFF45 from CAD/DFF40. CAD/DFF40 then forms an active homodimeric endonuclease in the nucleus for DNA fragmentation [26,27]. In this study, the sanguinarine treatment did not alter the levels of cIAP-2 expression in the C6 cells but downregulated the levels of XIAP and cIAP-1 (Fig. 3). Moreover, the induction of DNA fragmentation in the sanguinarine-treated cells was associated with the concentration-dependent cleavage of ICAD/DFF45 (Fig. 5). Although it is unclear how sanguinarine decreases the XIAP and cIAP-1 contents and cleaves the ICAD/DFF45 protein in C6 cells, sanguinarine activates the downstream caspase-3. As the IAP family and ICAD/DFF45 proteins are substrates of caspase-3 [27,28], the observed decrease in XIAP and cIAP-1 expression, and the cleavage of ICAD/DFF45 might be consequences of caspase-3-mediated processing after sanguinarine treatment.

The MAPKs and PI3K/Akt play key roles in cell survival and death in many physiological and pathological settings. The activation of the p38 MAPK and JNK pathways leads to the phosphorylation of a variety of proapoptotic downstream effectors, whereas the ERK and PI3K/Akt pathway is more often associated with cell survival [20,29]. This study identified the functional relationship between the MAPK or PI3K/Akt pathways and apoptosis in the sanguinarine-treated C6 cells. As shown in Figs 7 and 8, sanguinarine treatment caused the downregulation of ERK and Akt activation, and the combined exposure with PD98059 (a specific ERK inhibitor) or LY294002 and wortmanin (PI3K/Akt inhibitors) made the cells more sensitive to sanguinarine-induced apoptosis. These results suggest that the suppression of ERK, but not of p38 and JNK, is essential for sanguinarine-induced apoptosis. In addition, Akt might have a survival role in response to sanguinarine-induced apoptosis in C6 cells.

In summary, in sanguinarine-induced apoptosis, the imbalance of Bax/Bcl-2 may be an upstream event in the mitochondrial pathway and the later activation of caspases may lead to apoptosis. Moreover, a caspase-3 inhibitor markedly prevented the sanguinarine-induced apoptosis, suggesting that caspase-3 plays an important role in the sanguinarine-induced apoptosis of C6 cells. The downregulation of ERK and Akt may play an important role in sanguinarine-induced apoptosis. These results provide new information on the possible mechanisms for the anticancer activity of sanguinarine. It is however, still unclear if sanguinarine can induce apoptosis through other pathways, such as the death receptors pathway or the endoplasmic reticulum pathway. More study will, therefore, be needed to examine the mechanisms for the phosphorylation and activation of multiple apoptosis-related proteins in sanguinarine-induced apoptosis.

Fig. 8



Increase in sanguinarine-induced apoptosis by the inhibition of the phosphatidylinositol 3′-kinase/Akt signal pathway in C6 cells. (a) After 24 h incubation with sanguinarine, the cells were lysed and the cellular proteins were separated by sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-Akt and anti-pAkt antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. (b) The C6 cells were treated with LY294002 (25 μmol/l) for 2 h before being challenged with 1.5 μmol/l sanguinarine for 24 h. Equal amounts of the cell lysates (30 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose and probed with the anti-Akt, anti-pAkt and anti-poly(ADP ribose) polymerase (PARP) antibodies. The proteins were visualized using an enhanced chemiluminescence detection system. (c) The cells grown under the same conditions as (b) were collected and stained with fluorescein isothiocyanate-conjugated annexin-V and propidium iodide (PI) for flow cytometry analysis. The apoptotic cells were determined by counting the % of annexin V^+, PI^- cells and the % of annexin V^+, PI^+ cells. The results are expressed as the mean ± SD of three independent experiments.

References

1 Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; **168**:251–306.
2 Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. *Cell* 1997; **88**:347–354.
3 Deigner HP, Kinscherf R. Modulating apoptosis: current application and prospects for future drug development. *Curr Med Chem* 1999; **6**:399–414.
4 Sporn MB, Suh N. Chemoprevention of cancer. *Carcinogenesis* 2000; **21**:525–530.
5 Walterova D, Ulrichova J, Valka I, Vicar J, Vavreckova C, Taborska E, *et al.* Benzo[c]phenanthridine alkaloids sanguinarine and chelerythrine: biological activities and dental care applications. *Acta Univ Palacki Olomuc Fac Med* 1995; **139**:7–16.
6 Mandel ID. Chemotherapeutic agents for controlling plaque and gingivitis. *J Clin Periodontol* 1998; **15**:488–498.
7 Mitscher LA, Park YH, Clark D, Clark GW III, Hammesfahr PD, Wu WN, *et al.* Antimicrobial agents from higher plants: an investigation of *Hunnemannia fumariaefolia* pseudoalcoholates of sanguinarine and chelerythrine. *Lloydia* 1978; **41**:145–150.

8 Lenfeld J, Kroutil M, Marsalek E, Slavik J, Preininger V, Simanek V. Antiinflammatory activity of quaternary benzophenanthridine alkaloids from *Chelidonium majus*. *Planta Med* 1981; **43**:161–165.

9 Vavreckova C, Ulrichova J, Hajduch M, Grambal F, Weigl E, Simanek V. Effect of quaternary benzo[*c*]phenanthridine alkaloids sanguinarine, chelerythrine and fagaronine on some mammalian cells. *Acta Univ Palacki Olomuc Fac Med* 1994; **138**:7–10.

10 Adhami VM, Aziz MH, Mukhtar H, Ahmad N. Activation of prodeath Bcl-2 family proteins and mitochondrial apoptosis pathway by sanguinarine in immortalized human HaCaT keratinocytes. *Clin Cancer Res* 2003; **9**:3176–3182.

11 Adhami VM, Aziz MH, Reagan-Shaw SR, Nihal M, Mukhtar H, Ahmad N. Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor–cyclin–cyclin-dependent kinase machinery. *Mol Cancer Ther* 2004; **3**:933–940.

12 Ahmad N, Gupta S, Husain MM, Heiskanen KM, Mukhtar H. Differential antiproliferative and apoptotic response of sanguinarine for cancer cells vs. normal cells. *Clin Cancer Res* 2000; **6**:1524–1528.

13 Debiton E, Madelmont JC, Legault J, Barthomeuf C. Sanguinarine-induced apoptosis is associated with an early and severe cellular glutathione depletion. *Cancer Chemother Pharmacol* 2003; **51**:474–482.

14 Ding Z, Tang SC, Weerasinghe P, Yang X, Pater A, Liepins A. The alkaloid sanguinarine is effective against multidrug resistance in human cervical cells via bimodal cell death. *Biochem Pharmacol* 2002; **63**: 1415–1421.

15 Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 1994; **371**:346–347.

16 Bae SS, Perry DK, Oh YS, Choi JH, Galadari SH, Ghayur T, *et al*. Proteolytic cleavage of phospholipase C-γ1 during apoptosis in Molt-4 cells. *FASEB J* 2000; **14**:1083–1092.

17 Widlak P. The DFF40/CAD endonuclease and its role in apoptosis. *Acta Biochim Pol* 2000; **47**:1037–1044.

18 Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 1998; **391**:43–50.

19 Liu X, Zou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to tigger DNA fragmentation during apoptosis. *Cell* 1997; **89**:175–184.

20 Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Oppossing effect of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995; **270**:1326–1331.

21 Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, *et al*. The PI3-kinasel/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev* 1997; **11**:701–713.

22 Weerasinghe P, Hallock S, Liepins A. Bax, Bcl-2, and NF-κB expression in sanguinarine induced bimodal cell death. *Exp Mol Pathol* 2001; **71**:89–98.

23 Weerasinghe P, Hallock S, Tang SC, Liepins A. Role of Bcl-2 family proteins and caspase-3 in sanguinarine-induced bimodal cell death. *Cell Biol Toxicol* 2001; **17**:371–381.

24 Deveraux QL, Reed JC. IAP family protein-suppressors of apoptosis. *Gene* 1999; **9**:1799–1805.

25 Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, *et al*. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. *Oncogene* 1994; **9**:1799–1805.

26 Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 1998; **391**:96–99.

27 Lechardeur D, Drzymala L, Sharma M, Zylka D, Kinach R, Pacia J, *et al*. Determinants of the nuclear localization of the heterodimeric DNA fragmentation factor (ICAD/CAD). *J Cell Biol* 2000; **150**:321–334.

28 Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 protein are direct inhibitors of specific caspases. *EMBO J* 1997; **16**:6914–6925.

29 Kim D, Cheng GZ, Lindsley CW, Yang H, Cheng JQ. Targeting the phosphatidylinositol-3 kinase/Akt pathway for the treatment of cancer. *Curr Opin Investig Drugs* 2005; **6**:1250–1258.