Toxicology in Vitro 23 (2009) 580–588

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Cytotoxic activity of sanguinarine and dihydrosanguinarine in human promyelocytic leukemia HL-60 cells

Jiří Vrba [a,*], Petr Doležel [b], Jaroslav Vičar [a], Jitka Ulrichová [a]

[a] Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic
[b] Department of Biology, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic

ARTICLE INFO

Article history:
Received 21 July 2008
Accepted 28 January 2009
Available online 3 February 2009

Keywords:
Apoptosis
Cell cycle
Cytotoxicity
Dihydrosanguinarine
HL-60 cells
Necrosis
Mitochondrial membrane potential
Sanguinarine

ABSTRACT

The benzo[*c*]phenanthridine alkaloid sanguinarine has been studied for its antiproliferative activity in many cell types. Almost nothing however, is known about the cytotoxic effects of dihydrosanguinarine, a metabolite of sanguinarine. We compared the cytotoxicity of sanguinarine and dihydrosanguinarine in human leukemia HL-60 cells. Sanguinarine produced a dose-dependent decline in cell viability with IC_{50} (inhibitor concentration required for 50% inhibition of cell viability) of 0.9 μM as determined by MTT assay after 4 h exposure. Dihydrosanguinarine showed much less cytotoxicity than sanguinarine: at the highest concentration tested (20 μM) and 24 h exposure, dihydrosanguinarine decreased viability only to 52%. Cytotoxic effects of both alkaloids were accompanied by activation of the intrinsic apoptotic pathway since we observed the dissipation of mitochondrial membrane potential, induction of caspase-9 and -3 activities, the appearance of sub-G_1 DNA and loss of plasma membrane asymmetry. This aside, sanguinarine also increased the activity of caspase-8. As shown by flow cytometry using annexin V/propidium iodide staining, 0.5 μM sanguinarine induced apoptosis while 1–4 μM sanguinarine caused necrotic cell death. In contrast, dihydrosanguinarine at concentrations from 5 μM induced primarily necrosis, whereas apoptosis occurred at 10 μM and above. We conclude that both alkaloids may cause, depending on the alkaloid concentration, both necrosis and apoptosis of HL-60 cells.

1. Introduction

The quaternary benzo[*c*]phenanthridine alkaloid sanguinarine (Fig. 1) has been extensively studied for many years. Its ability to interact with proteins and DNA underlies its diverse biological activities (Walterova et al., 1995). Among others, sanguinarine has been reported to suppress activation of transcription factor NF-κB (Chaturvedi et al., 1997) and to modulate the function of various enzymes, such as mitogen-activated protein kinase phosphatase-1 (Vogt et al., 2005), protein kinase C (Gopalakrishna et al., 1995), and phosphoinositide-dependent protein kinase 1 (Vrba et al., 2008a). It also has antimicrobial and antifungal activities (Walterova et al., 1995). This aside, antiproliferative and/or cytotoxic effects of sanguinarine have been demonstrated in normal cells such as human gingival fibroblasts (Malikova et al., 2006a) and rat hepatocytes (Choy et al., 2008), as well as in many cancer cell lines (Malikova et al., 2006b), including human promyelocytic leukemia HL-60 cells (Slaninova et al., 2007). Plants produce sanguinarine in response to stress stimuli and use it as a phytoalexin against fungal and bacterial pathogens. To protect themselves from the alkaloid, the producing cells may enzymatically reduce sanguinarine to the less toxic dihydrosanguinarine (Fig. 1) (Weiss et al., 2006). Similarly, dihydrosanguinarine was identified as the first sanguinarine metabolite in rats too (Dvorak and Simanek, 2007; Psotova et al., 2006). The mechanism of sanguinarine reduction in animals remains unclear, though nonenzymatic conversion of sanguinarine to its dihydroderivative by NAD(P)H has been described *in vitro* (Kovar et al., 1986; Matkar et al., 2008a).

Sanguinarine and dihydrosanguinarine are suspected of being responsible for outbreaks of human poisoning caused by the consumption of edible oils contaminated with alkaloids from *Argemone mexicana* seeds (Das and Khanna, 1997). A pharmacokinetic study in rats administered a single intragastric dose of 10 mg of sanguinarine per kg of body weight showed that the maximum plasma levels of sanguinarine and dihydrosanguinarine reached 192 and 546 ng/ml, i.e. 0.6 and 1.6 μM, respectively (Vecera et al., 2007). *In vivo* toxicity of sanguinarine was observed after an intraperitoneal application in mice (Ansari et al., 2005; Choy et al., 2008). No toxic response was found in animals after an oral administration of dihydrosanguinarine (Vrublova et al., 2008). However, in *Bocconia frutescens* seeds, dihydrosanguinarine is recognized as a defense compound toxic to brine shrimp *Artemia salina*, thus promoting the seed persistence in the soil (Veldman et al., 2007). Moreover, dihydrosanguinarine shows antimicrobial

* Corresponding author. Tel.: +420 585632310; fax: +420 585632302.
E-mail address: vrbambv@seznam.cz (J. Vrba).

0887-2333/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2009.01.016

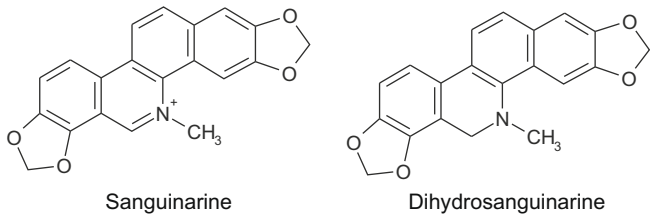

Fig. 1. Chemical structures of tested alkaloids.

activity (Navarro and Delgado, 1999) and cytotoxicity against several cancer cell lines (Chen et al., 1999).

Two major modes of cell death induced by cytotoxic compounds are apoptosis and necrosis. Apoptosis is a regulated process characterized by cell shrinkage, nuclear disintegration, selective degradation of DNA, and formation of apoptotic bodies with a relatively intact plasma membrane. The execution of apoptosis requires energy in the form of ATP and depends on activation of the caspase cascade. Necrosis, in contrast, is considered to be a passive form of cell death resulting from ATP depletion and characterized morphologically by cell swelling and plasma membrane rupture. Induction of necrosis is generally associated with gross injury, which may be caused by an overdose of cytotoxic agent (Darzynkiewicz et al., 1997; Edinger and Thompson, 2004). To date, the apoptogenic activity of sanguinarine has been well-documented. In contrast, the cytotoxicity of dihydrosanguinarine has not been studied in detail. Therefore, the aim of the present work was to examine whether HL-60 cells are sensitive to dihydrosanguinarine, and if so, to compare the cytotoxic effects of dihydrosanguinarine with those of sanguinarine.

2. Materials and methods

2.1. Tested alkaloids

Sanguinarine chloride in 99% purity was isolated from *Macleaya cordata* extract purchased from Camas Technologies (Broomfield, CO, USA). Dihydrosanguinarine in 99% purity was prepared from sanguinarine by reduction with $NaBH_4$ in methanol and did not contain any admixture of sanguinarine (HPLC). All the separations and procedures were performed at the Department of Medical Chemistry and Biochemistry, Palacký University, Olomouc, Czech Republic, and specimens of the preparations are deposited in the alkaloid collection of the Department.

2.2. Cell culture and treatment

The human promyelocytic leukemia cell line HL-60 (ECACC, Salisbury, UK) was cultured at 37 °C in RPMI-1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO_2 and maintained between 1×10^5 and 9×10^5 cells/ml.

Exponentially growing cells, passages 10–30, were used for all experiments performed in the complete medium with 5% FBS. To prevent cell overgrowth, the initial cell density was either 2.5×10^5 cells/ml for 2–24 h treatment or 1×10^5 cells/ml for 48 h treatment. The tested alkaloids were dissolved and diluted freshly before each experiment in dimethyl sulfoxide (DMSO). The final DMSO concentration in all experiments was 0.1% and had negligible effect on the measured parameters. Negative controls were treated with 0.1% DMSO only.

2.3. MTT reduction assay

HL-60 cells were plated into a 96-well plate at 5×10^4 cells/0.2 ml/well and treated for 4 or 24 h at 37 °C with DMSO (control), alkaloids or 1.5% Triton X-100 (positive control). After treatment, the cell viability was assessed by MTT assay as described previously (Vrba et al., 2008b).

2.4. Trypan blue exclusion method

HL-60 cells were plated into a 6-well plate at 2.5×10^5 or 1×10^5 cells/ml and exposed for the appropriate time at 37 °C to DMSO (control) or alkaloids. After this, the cell viability was determined by the trypan blue exclusion technique (Patterson, 1979) using a cell viability analyzer Vi-Cell XR (Beckman Coulter, Fullerton, CA, USA).

2.5. Flow cytometric analysis of cell cycle

HL-60 cells were plated into a 6-well plate at 2.5×10^5 or 1×10^5 cells/ml in a total volume of 4 ml and exposed for the appropriate time at 37 °C to DMSO (control), alkaloids, or camptothecin (positive control). After treatment, cells were collected by centrifugation, fixed in 70% ethanol, and cellular DNA content was analyzed as described (Vrba et al., 2008b). In brief, fixed cells were gently centrifuged and resuspended in 2 ml of PBS containing 0.1% Triton X-100, 20 μg/ml ribonuclease A (Sigma), and 20 μg/ml propidium iodide (PI; Sigma). Samples were incubated for 30 min at room temperature and analyzed by flow cytometry on a Cytomics FC 500 (Beckman Coulter). PI was excited with an argon laser at 488 nm and the fluorescence of 20,000 events per sample was detected on FL3 channel using a 600 nm long-pass filter and a 615 nm short-pass filter. The percentage of cells in G_1, S, and G_2/M phase of cell cycle and the percentage of cells in sub-G_1 peak were calculated using MultiCycle AV software (Phoenix Flow Systems, San Diego, CA, USA), which eliminated the debris effect.

2.6. Measurement of mitochondrial membrane potential by flow cytometry

HL-60 cells were plated into a 6-well plate at 2.5×10^5 or 1×10^5 cells/ml in a total volume of 2 ml and treated for the appropriate time at 37 °C with DMSO (control), alkaloids, or valinomycin (positive control). After treatment, the plate was centrifuged for 3 min at 150*g* and the medium was changed using careful aspiration. To determine the mitochondrial membrane potential, cells were incubated with 0.5 μM tetramethylrhodamine ethyl ester (TMRE; Sigma) for 20 min at 37 °C and immediately analyzed on a Cytomics FC 500 (Beckman Coulter). Cells were excited with a 488 nm argon laser line and TMRE emission was detected through a 575/29 nm band-pass filter on FL2 channel (Jayaraman, 2005), counting 15,000 events per sample. The percentage of cells with decreased mitochondrial potential was calculated.

2.7. Assessment of caspase activity

HL-60 cells were plated into 10-cm Petri dishes at 2.5×10^5 or 1×10^5 cells/ml in a total volume of 12 ml and treated for the appropriate time at 37 °C to DMSO (control), alkaloids, or etoposide (positive control). After treatment, cells were harvested by centrifugation, lysed and fold changes in caspase activities were determined as described previously (Vrba et al., 2008b). In brief, specific fluorogenic substrates, Ac-DEVD-AMC, Z-IETD-AFC (Merck, Darmstadt, Germany), and Ac-LEHD-AFC (Bachem, Bubendorf, Switzerland), and specific inhibitors, Ac-DEVD-al, Ac-IETD-al (Merck), and Ac-LEHD-al (Bachem), were used to assess the activity of caspase-

3, -8, and -9, respectively. The fluorescence of caspase-released AMC or AFC was read on a multifunctional microplate reader Infinite M200 (Tecan, Salzburg, Austria) at excitation and emission wavelengths of 380 and 450 nm for AMC or 400 and 505 nm for AFC. The fluorescence differences in the absence and presence of caspase inhibitors were standardised against the protein content, determined by the coomassie dye binding technique (Bradford, 1976), and used for calculation of fold changes versus control.

2.8. Flow cytometric analysis of apoptosis

HL-60 cells were plated into a 6-well plate at 2.5×10^5 cells/ml in a total volume of 2 ml and exposed for the appropriate time at 37 °C to DMSO (control), alkaloids, or camptothecin (positive control). After treatment, cells were collected by centrifugation for 3 min at 150*g*, washed with PBS, and resuspended in 0.5 ml of HEPES buffer pH 7.4 containing 140 mM NaCl and 2.5 mM $CaCl_2$. The cell suspension was mixed with 5 µl of annexin V-FITC (Molecular Probes, Eugene, OR, USA) and 10 µl of 100 µg/ml PI (Sigma), incubated for 10 min in the dark at room temperature, and immediately analyzed on a Cytomics FC 500 (Beckman Coulter). Using excitation with a 488 nm argon laser line, annexin V-FITC emission was detected through a 525/39 nm band-pass filter on FL1 channel and PI emission was detected through a 600 nm long-pass filter and a 615 nm short-pass filter on FL3 channel, counting 20,000 events per sample. The dual parameter dot plots were used for calculation of the percentage of nonapoptotic live cells in the lower left quadrant (annexin V negative/PI negative), early apoptotic cells in the lower right quadrant (annexin V positive/PI negative), and late apoptotic or necrotic cells in the upper right quadrant (annexin V positive/PI positive) (Darzynkiewicz et al., 1997).

2.9. Statistical analysis

Results were expressed as means ± SD of three independent experiments. The differences in mean values were analyzed by student's *t*-test. A *p* value of less than 0.05 was considered as statistically significant. The IC_{50} values were derived using Microsoft Excel 2000 software (Microsoft Corporation, USA) supplemented with LSW Data Analysis Toolbox software (MDL Information Systems, San Ramon, CA, USA).

3. Results

3.1. Cytotoxicity of sanguinarine and dihydrosanguinarine in HL-60 cells

As shown by the MTT reduction assay, treatment of HL-60 cells with 0.1–4 µM sanguinarine for 4 and 24 h resulted in a dose-dependent decrease in the cell viability with IC_{50} values of 0.92 ± 0.27 and 0.72 ± 0.33 µM, respectively (Fig. 2A). To a lesser extent, HL-60 cells were also found to be sensitive to dihydrosanguinarine, tested with regard to its limited solubility in the culture medium at concentrations 0.1–20 µM. After 24 h, 20 µM dihydrosanguinarine decreased the cell viability to 52% (Fig. 2B), whereas no significant changes in the viability were observed after 4 h of incubation (data not shown). The cytotoxicity of both alkaloids was confirmed by the trypan blue exclusion assay. Untreated HL-60 cells required less than 24 h to double their number (data not shown) and their viability exceeded 98% at the end of all experiments (Fig. 2C and D). A 24 h cell treatment with 0.5 and 1 µM sanguinarine reduced the number of viable cells to 56% and 31%, respectively, compared to untreated cells (data not shown) while the cell viability decreased to 71% and 39%, respectively (Fig. 2C). Cell exposure to 20 µM dihydrosanguinarine for 24 and 48 h diminished the viable cell number respectively to 48% and 4% compared to control cells (data not shown) with the viability decreased to 69% and 22%, respectively (Fig. 2D).

3.2. Effect of sanguinarine and dihydrosanguinarine on cell cycle distribution

To examine whether the cytotoxic effects of sanguinarine and dihydrosanguinarine involved cell cycle alterations, we analyzed the DNA profiles of HL-60 cells by flow cytometry using cell staining with PI. The reliability of the method to detect cell cycle changes and sub-G_1 DNA content, characteristic of apoptotic cells, was confirmed by analysis of cells treated with camptothecin, an

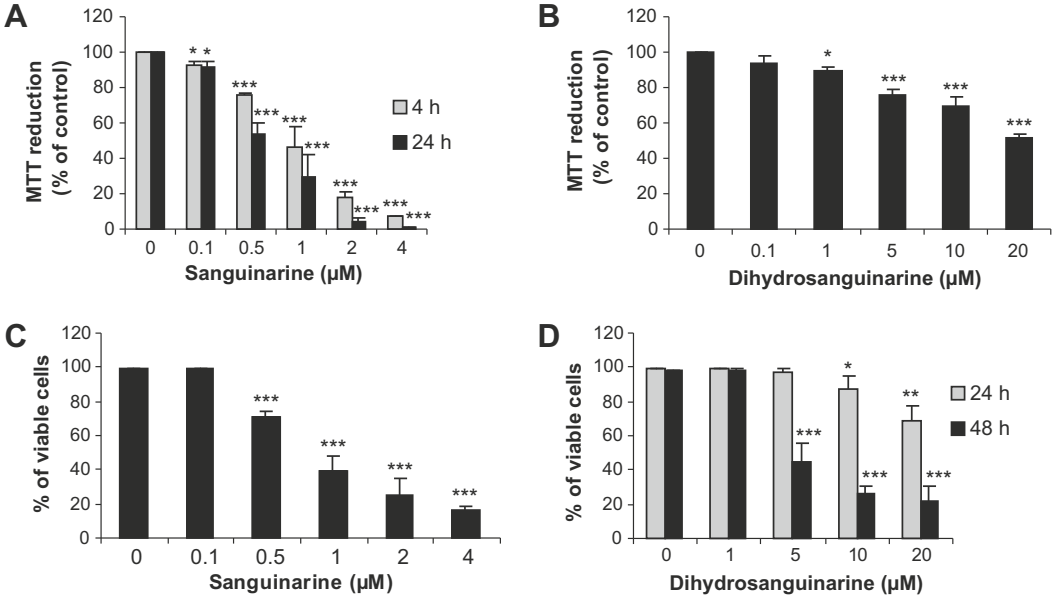

Fig. 2. Cytotoxicity of sanguinarine and dihydrosanguinarine in HL-60 cells. (A and B) Cells were treated with increasing concentrations of (A) sanguinarine for 4 and 24 h or (B) dihydrosanguinarine for 24 h and the viability was determined by the MTT reduction assay. (C and D) Cells were treated with increasing concentrations of (C) sanguinarine for 24 h or (D) dihydrosanguinarine for 24 and 48 h and the percentage of viable cells for each treatment was assessed by the trypan blue exclusion technique. Data are means ± SD of three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, significantly different from control.

inhibitor of topoisomerase I (Del Bino et al., 1990). After 16 h exposure to 1 μM camptothecin, we observed an accumulation of HL-60 cells in the G_1 phase of the cell cycle and an appearance of cells with sub-G_1 DNA (Fig. 3A). In HL-60 cells treated with sanguinarine for 4 h, the cell cycle distribution was significantly affected only at 4 μM concentration where an increase in the G_2/M phase with a parallel decrease in the G_1 phase was found (Fig. 3B and F). This aside, 4 h sanguinarine treatment led to the formation of cells with

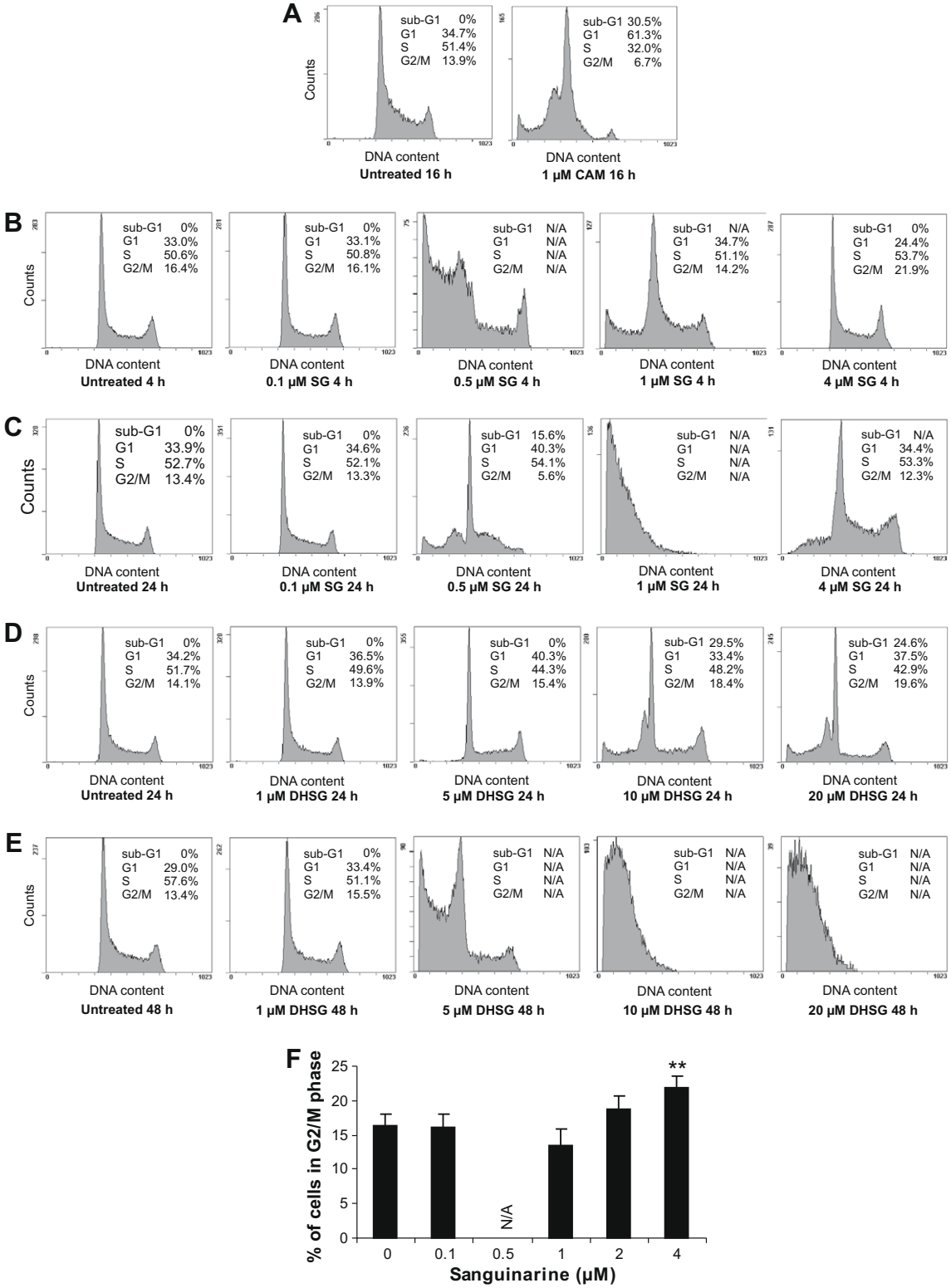

Fig. 3. Effect of sanguinarine (SG) and dihydrosanguinarine (DHSG) on the cell cycle progression in HL-60 cells. Cells were treated with (A) 1 μM camptothecin (CAM) for 16 h or with increasing concentrations of (B and F) sanguinarine for 4 h, (C) sanguinarine for 24 h, (D) dihydrosanguinarine for 24 h, or (E) dihydrosanguinarine for 48 h. Cells were fixed in ethanol, stained with PI, and the percentage of cells in G_1, S, and G_2/M phase of the cell cycle and in the sub-G_1 peak was determined by flow cytometry. Data are means (±SD) and the figures are representative of three (CAM) or five (SG, DHSG) experiments repeated with similar results. N/A, not applicable. $^{**}p < 0.01$, significantly increased versus control.

reduced DNA content. The maximal number of sub-G_1 cells was detected at 0.5 μM sanguinarine concentration but at higher concentrations, a decrease in sub-G_1 cells was found (Fig. 3B). After 24 h exposure, sanguinarine only at a concentration of 0.5 μM significantly decreased the cell population in the G_2/M phase from 13.4 ± 1.1% (control) to 5.6 ± 2.0% ($p < 0.01$; $n = 5$) (Fig. 3C). At concentrations of 0.5–4 μM and 24 h of incubation, sanguinarine also induced the formation of sub-G_1 cells. The number of sub-G_1 cells reached a maximum at 1 μM sanguinarine concentration where the whole cell population showed sub-G_1 DNA but at the concentration of 4 μM, a normal cell cycle profile reappeared (Fig. 3C). Following the treatment of HL-60 cells with dihydrosanguinarine for 24 or 48 h, we detected no significant changes in the cell cycle distribution apart from an appearance of cells with sub-G_1 DNA. After 24 h, the sub-G_1 cell formation was detected at concentrations of 10 and 20 μM (Fig. 3D). After 48 h, dihydrosanguinarine treatment resulted in a large number of sub-G_1 cells at 5 μM concentration followed by a complete loss of the normal cell cycle profile at higher alkaloid concentrations (Fig. 3E).

3.3. Loss of mitochondrial membrane potential induced by sanguinarine and dihydrosanguinarine

Mitochondrial dysfunction produced by toxic stress may lead to both apoptotic and necrotic cell death (Lemasters et al., 1999). Therefore, we examined the effect of sanguinarine and dihydrosanguinarine on the inner mitochondrial membrane potential. Flow cytometric analysis of cells stained with a mitochondria-specific dye TMRE showed that 4–7% of untreated HL-60 cells had low mitochondrial membrane potential (Fig. 4A and B). After 6 h exposure to 2 μM valinomycin, a potassium ionophore used as a positive control (Abdalah et al., 2006), a loss of the mitochondrial potential was found in 39 ± 2% of cells (data not shown). Treatment of HL-60 cells with sanguinarine for 4 or 24 h significantly increased the number of cells with low mitochondrial potential at concentrations from 0.5 μM (Fig. 4A). The dissipation of the mitochondrial potential induced by sanguinarine was generally dose- and time-dependent, with the exception of 1 and 2 μM sanguinarine at 4 h of incubation. At this time point, 0.5 μM sanguinarine raised the cell population with depolarized mitochondria to 32% while 1 and 2 μM sanguinarine reduced the mitochondrial potential in 22% and 24% of cells, respectively. After treatment with 4 μM sanguinarine for 4 h, a complete loss of mitochondrial potential was found (Fig. 4A). These results suggest that at certain concentrations, sanguinarine can stimulate transient stabilization of the mitochondrial potential which may arise, for instance, during early stages of necrosis (Darzynkiewicz et al., 1997). A dose- and time-dependent decline in the mitochondrial potential occurred also after treatment of HL-60 cells with dihydrosanguinarine. A time-response analysis showed that 20 μM dihydrosanguinarine induced a significant increase in the cell population with low mitochondrial potential 12 h after the start of the treatment (data not shown). After 12, 24, and 48 h of incubation with 20 μM dihydrosanguinarine, a loss of mitochondrial potential was detected in 22% (not shown), 46%, and 98% of cells, respectively (Fig. 4B). Dose-response experiments at 24 and 48 h of dihydrosanguinarine treatment showed a significant mitochondrial depolarization at concentrations from 5 μM (Fig. 4B).

3.4. Induction of caspase activities by sanguinarine and dihydrosanguinarine

We assessed whether the cell treatment with sanguinarine and dihydrosanguinarine increased the levels of caspase activities. Chemically induced apoptosis of HL-60 cells may involve activation of two initiator caspases, caspase-8 and caspase-9, both activating effector caspase-3. A topoisomerase II inhibitor etoposide, used as a positive control (Maianski et al., 2004), induced at 100 μM concentration and 6 h exposure (10.3 ± 1.4)-fold, (7.8 ± 0.9)-fold, and (3.1 ± 0.4)-fold increase in the activities of caspase-3, -9, and -8, respectively, compared to untreated cells. Analyzing the time response of HL-60 cells to 4 μM sanguinarine, a dramatic increase in caspase-3 and -9 activities and a modest induction of caspase-8 activity were found after 2 h. The maximal levels of caspase-3, -9, and -8 activities occurred 4 h after the start of the treatment and reached respectively 8.8-fold, 7.2-fold, and 2.8-fold values compared to untreated cells (Fig. 5A). After 4 h of incubation, sanguinarine elevated the caspase-3 and -9 activities in a dose-dependent manner with a significant increase found at concentrations from 0.5 μM. In contrast, the activity of caspase-8 was significantly elevated only at the highest concentration tested (Fig. 5B). Activation of caspases in HL-60 cells by dihydrosanguinarine required higher alkaloid concentration and longer incubation time in comparison with sanguinarine. At the concentration of 20 μM, dihydrosanguinarine appeared to significantly increase the activity of caspase-3 and -9 with a maximum reached at 18 to 24 h of cell treatment, whereas the level of caspase-8 activity remained unaffected. After 24 h of incubation, caspase-3 and -9 activities were elevated by 20 μM dihydrosanguinarine to 4.9-fold and 4.7-fold levels compared to control cells (Fig. 5C). The effect of a 24 h treatment on the caspase-3 and -9 activities was dose-dependent while a significant increase in the activities required at least 5 μM dihydrosanguinarine (Fig. 5D).

3.5. Apoptotic activity of sanguinarine and dihydrosanguinarine

To confirm the apoptotic effect of the tested alkaloids, we analyzed phosphatidylserine externalization and plasma membrane

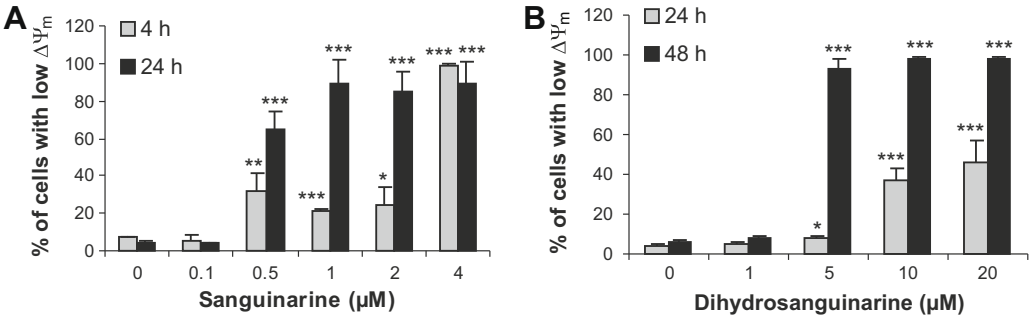

Fig. 4. Dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) induced by sanguinarine and dihydrosanguinarine in HL-60 cells. Cells were treated (A) with 0.1–4 μM sanguinarine for 4 and 24 h or (B) with 1–20 μM dihydrosanguinarine for 24 and 48 h. Cells were stained with TMRE and the percentage of cells with decreased $\Delta\Psi_m$ was evaluated by flow cytometry. Data are means ± SD of three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, significantly different from control.

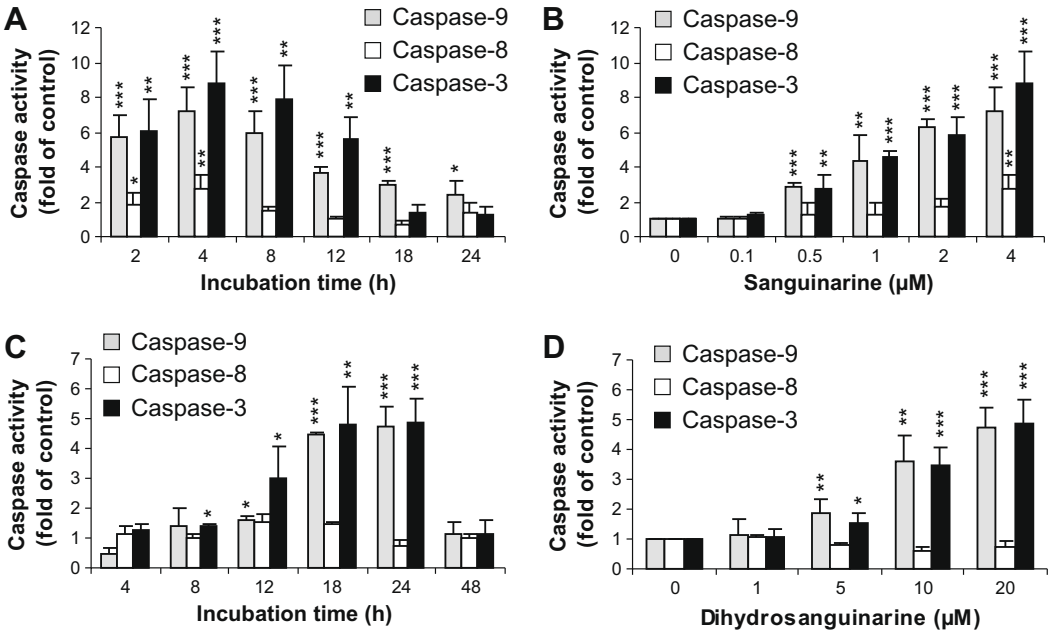

Fig. 5. Effect of sanguinarine and dihydrosanguinarine on activation of caspase-3, -8, and -9 in HL-60 cells. Cells were treated (A) with 4 μM sanguinarine for 2–24 h, (B) with 0.1–4 μM sanguinarine for 4 h, (C) with 20 μM dihydrosanguinarine for 4 to 48 h, or (D) with 1–20 μM dihydrosanguinarine for 24 h. The levels of caspase-3, -8, and -9 activities were evaluated using specific fluorogenic substrates. Data are means ± SD of three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, significantly different from control.

integrity by flow cytometry using the annexin V/PI dual cell staining. This method showed that 1 μM camptothecin, which induced apoptotic DNA fragmentation after 16 h exposure (Fig. 3A), under the same conditions increased the proportion of early apoptotic cells (annexin V-positive/PI-negative) from 15 ± 2% (control) to 36 ± 2% and the proportion of late apoptotic or necrotic cells (annexin V-positive/PI-positive) from 2 ± 1% (control) to 16 ± 1% (data not shown). The effects of sanguinarine and dihydrosanguinarine were tested after 4 and 24 h, respectively, i.e. the time of the maximal caspase activation (Fig. 5A and C). After 4 h of incubation, untreated HL-60 cells showed 18% of early apoptotic cells and 3% of late apoptotic or necrotic cells (Fig. 6A and C). Cell treatment with 0.5 μM sanguinarine for 4 h solely elevated the population of early apoptotic cells. In contrast, 1 μM sanguinarine increased primarily the proportion of late apoptotic or necrotic cells and at higher sanguinarine concentrations almost the whole cell population was late apoptotic or necrotic. In cells exposed to 0.5, 1, and 2 μM sanguinarine, we observed 61%, 27%, and 4% of annexin V-positive/PI-negative cells together with 4%, 65%, and 94% of annexin V/PI double positive cells, respectively (Fig. 6A and C). After 24 h, 15% of untreated HL-60 cells were early apoptotic and 3% of the cells were late apoptotic or necrotic (Fig. 6B and D). When the cells were treated with dihydrosanguinarine for 24 h, we observed a significant dose-dependent increase in late apoptotic/necrotic cells at concentrations from 5 μM, whereas the increase in the proportion of early apoptotic cells required at least 10 μM concentration of dihydrosanguinarine. After the cell exposure to 5, 10, and 20 μM dihydrosanguinarine, we detected 14%, 22%, and 25% of annexin V-positive/PI-negative cells along with 11%, 30%, and 58% of annexin V/PI double positive cells, respectively (Fig. 6B and D).

4. Discussion

This study was designed to compare the cytotoxic effects of two benzo[*c*]phenanthridine alkaloids, sanguinarine and its metabolite dihydrosanguinarine. Cell exposure to sanguinarine produces a number of alterations arising from the alkaloid's ability to intercalate DNA (Kaminskyy et al., 2006) and nonspecific protein interactions through the addition of thiol groups to the iminium bond or by ionic interactions (Walterova et al., 1995). In various cell models, sanguinarine was found to cause DNA damage (Kaminskyy et al., 2008a; Matkar et al., 2008b; Philchenkov et al., 2008) and to modulate expression of proteins involved in regulating the cell cycle (Adhami et al., 2004; Lee et al., 2008) and apoptosis (Ahsan et al., 2007; Kim et al., 2008; Weerasinghe et al., 2006). However, sanguinarine may induce apoptosis independently of the tumour suppressor p53 (Matkar et al., 2008b), a transcriptional factor mediating cell response to DNA damage and other kinds of cellular stress (Yu and Zhang, 2005). Further, the rapid apoptotic cell death induced by sanguinarine appears to be a consequence of mitochondrial impairment that may occur without parallel changes in expression of the Bcl-2 family proteins (Kaminskyy et al., 2008b).

In this study we chose p53-null human promyelocytic leukemia HL-60 cells (Wolf and Rotter, 1985), which are known to be sensitive to sanguinarine treatment (Slaninova et al., 2007). Our results confirm the cytotoxicity of sanguinarine against HL-60 cells with the IC_{50} value of 0.9 μM determined after 4 h exposure by MTT reduction assay. The cytotoxic effect of sanguinarine was associated with dissipation of the inner mitochondrial membrane potential. Mitochondrial depolarization by sanguinarine, also demonstrated in human KB cancer cells (Chang et al., 2007), human CEM T-leukemia cells (Kaminskyy et al., 2008b) and rat hepatocytes (Choy et al., 2008), may be produced by several mechanisms. Sanguinarine is known to block the respiratory chain by inhibiting succinate dehydrogenase and NADH dehydrogenase (Barreto et al., 2003). In addition, sanguinarine, similarly as other hydrophobic cations, may neutralize the negative charge of the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation (Faddeeva and Beliaeva, 1997). The loss of the mitochondrial potential facilitates the release of cytochrome *c* from mitochondria, a key event in initiating the intrinsic/mitochondrial apoptotic pathway (Gottlieb et al., 2003). In the cytosol, cytochrome *c* triggers the formation of the apoptosome complex leading to activation of caspase-9 which in turn activates effector

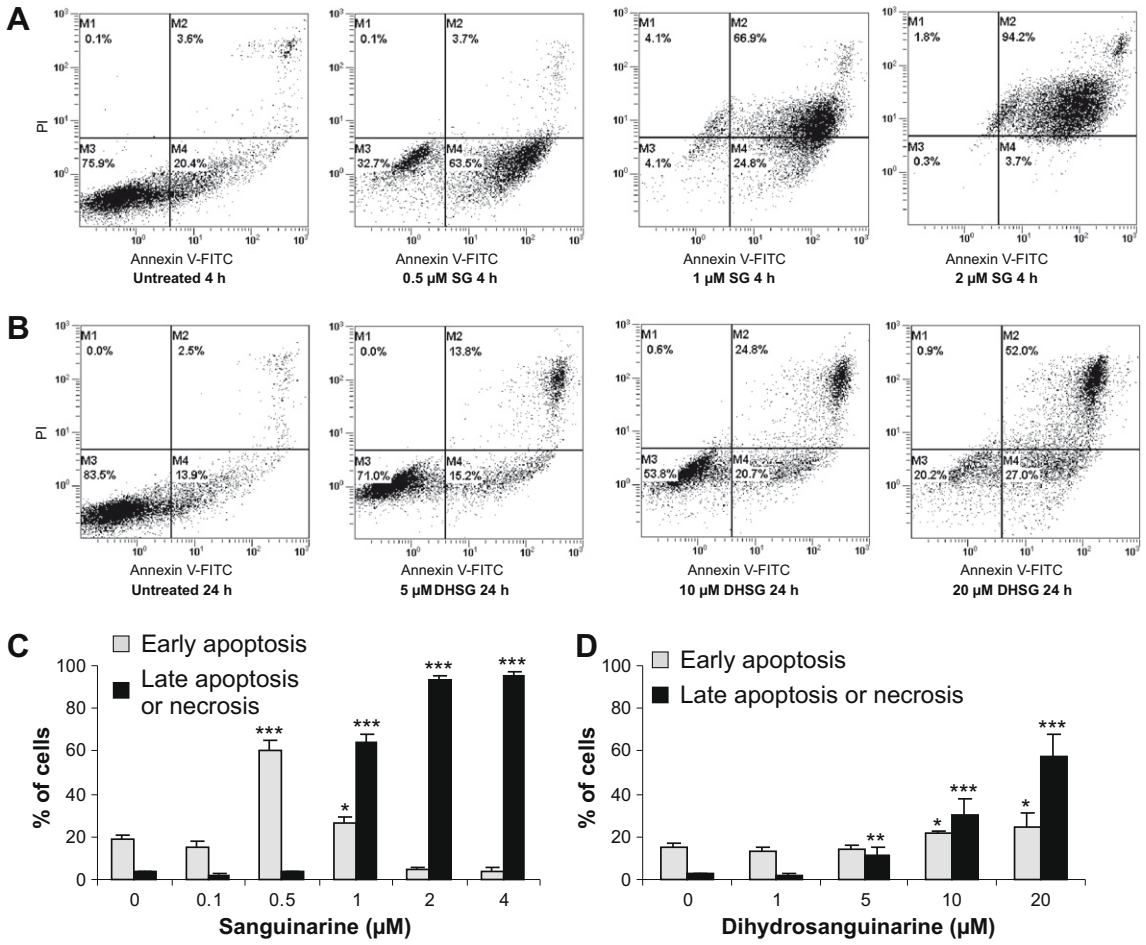

Fig. 6. Effect of sanguinarine (SG) and dihydrosanguinarine (DHSG) on phosphatidylserine externalization (annexin V binding) and cell membrane integrity (PI staining) in HL-60 cells. Cells were treated with increasing concentrations of (A and C) sanguinarine for 4 h or (B and D) dihydrosanguinarine for 24 h. Cells were incubated with annexin V-FITC and PI, and analyzed by flow cytometry. (A and B) The dual parametric dot plots show nonapoptotic live cells in the lower left quadrant (annexin V^-/PI^-), early apoptotic cells in the lower right quadrant (annexin V^+/PI^-), and late apoptotic or necrotic cells in the upper right quadrant (annexin V^+/PI^+). The figures are representative of three experiments repeated with similar results. (C and D) Data are means ± SD of three experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, significantly increased versus control.

caspases including caspase-3 (Orrenius, 2004). Consistently, after 4 h treatment of HL-60 cells with 0.5 μM sanguinarine, the decrease in the mitochondrial potential is accompanied by the induction of caspase-9 and -3 activities, the appearance of sub-G_1 DNA, and the loss of plasma membrane asymmetry detected as externalization of phosphatidylserine on the cell surface. Since the treated cells underwent these apoptotic changes while maintaining the plasma membrane integrity intact, 0.5 μM sanguinarine solely induced apoptosis of HL-60 cells. At higher concentrations ($\geqslant$1 μM), sanguinarine appeared to cause necrotic cell death as indicated by the loss of the plasma membrane integrity. It should be mentioned that sanguinarine was also found to increase the level of caspase-8 activity and to induce modest cell accumulation in the G_2/M phase. However, these events were found only at 4 μM concentration of the alkaloid, and thus they play a negligible role in mediating cell death.

In comparison with sanguinarine, little is known about the cytotoxic effects of dihydrosanguinarine. The absence of positive charge and nonplanarity due to lack of conjugation in the dihydrosanguinarine molecule seem to be linked with markedly decreased biological activity. For instance, dihydrosanguinarine, unlike sanguinarine, does not inhibit protein kinase C (Vrba et al., 2008a) and is not cytotoxic to rat hepatocytes (Vavreckova et al., 1994). On the other hand, the cytotoxicity of dihydrosanguinarine in four mammalian cancer cell lines has been reported with murine lymphocytic leukemia P388 cells found to be the most sensitive (Chen et al., 1999). In HL-60 cells, dihydrosanguinarine showed cytotoxic effect after 24 h exposure. At 20 μM concentration, i.e. the highest concentration tested, dihydrosanguinarine reduced the cell viability to 52% as shown by the MTT assay. Cell treatment with the alkaloid caused no significant changes in the cell cycle. However, treatment of HL-60 cells with 5 to 20 μM dihydrosanguinarine resulted in dissipation of the mitochondrial potential and increase in the levels of caspase-9 and -3 but not caspase-8 activity. At 10 to 20 μM concentrations of dihydrosanguinarine, these events were accompanied by the formation of apoptotic sub-G_1 peak, suggesting involvement of the intrinsic apoptotic pathway in the cell response to dihydrosanguinarine. Apart from these results, 5 to 20 μM dihydrosanguinarine induced primarily necrosis of HL-60 cells while the induction of apoptotic cell death required at least 10 μM concentration of the alkaloid as shown by the annexin V/PI dual cell staining.

In summary, we have shown that dihydrosanguinarine is less cytotoxic than sanguinarine. Nevertheless, dihydrosanguinarine as well as sanguinarine may cause, depending on the alkaloid concentration, both necrosis and apoptosis of HL-60 cells with the latter mode of cell death linked to activation of the intrinsic apoptotic pathway. Since sanguinarine and dihydrosanguinarine induce respectively apoptosis and necrosis at low concentrations, we may presume that the cytotoxic effect of sanguinarine is not

caused by its conversion to dihydrosanguinarine or vice versa. The metabolic fate of the tested alkaloids in different types of animal cells remains to be elucidated.

Conflicts of interest statement

None declared.

Acknowledgments

This work was supported by the Grant No. 303/06/P193 from the Czech Science Foundation and by the Grant No. MSM 6198959216 from the Ministry of Education, Youth and Sports of the Czech Republic.

References

Abdalah, R., Wei, L., Francis, K., Yu, S.P., 2006. Valinomycin-induced apoptosis in Chinese hamster ovary cells. Neuroscience Letters 405, 68–73.

Adhami, V.M., Aziz, M.H., Reagan-Shaw, S.R., Nihal, M., Mukhtar, H., Ahmad, N., 2004. Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin–cyclin-dependent kinase machinery. Molecular Cancer Therapeutics 3, 933–940.

Ahsan, H., Reagan-Shaw, S., Breur, J., Ahmad, N., 2007. Sanguinarine induces apoptosis of human pancreatic carcinoma AsPC-1 and BxPC-3 cells via modulations in Bcl-2 family proteins. Cancer Letters 249, 198–208.

Ansari, K.M., Dhawan, A., Khanna, S.K., Das, M., 2005. In vivo DNA damaging potential of sanguinarine alkaloid, isolated from *Argemone* oil, using alkaline Comet assay in mice. Food and Chemical Toxicology 43, 147–153.

Barreto, M.C., Pinto, R.E., Arrabaca, J.D., Pavao, M.L., 2003. Inhibition of mouse liver respiration by *Chelidonium majus* isoquinoline alkaloids. Toxicology Letters 146, 37–47.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Chang, M.C., Chan, C.P., Wang, Y.J., Lee, P.H., Chen, L.I., Tsai, Y.L., Lin, B.R., Wang, Y.L., Jeng, J.H., 2007. Induction of necrosis and apoptosis to KB cancer cells by sanguinarine associated with reactive oxygen species production and mitochondrial membrane depolarization. Toxicology and Applied Pharmacology 218, 143–151.

Chaturvedi, M.M., Kumar, A., Darnay, B.G., Chainy, G.B.N., Agarwal, S., Aggarwal, B.B., 1997. Sanguinarine (pseudochelerythrine) is a potent inhibitor of NF-κB activation, IκBα phosphorylation, and degradation. Journal of Biological Chemistry 272, 30129–30134.

Chen, J.J., Duh, C.Y., Chen, I.S., 1999. New tetrahydroprotoberberine N-oxide alkaloids and cytotoxic constituents of *Corydalis tashiroi*. Planta Medica 65, 643–647.

Choy, C.S., Cheah, K.P., Chiou, H.Y., Li, J.S., Liu, Y.H., Yong, S.F., Chiu, W.T., Liao, J.W., Hu, C.M., 2008. Induction of hepatotoxicity by sanguinarine is associated with oxidation of protein thiols and disturbance of mitochondrial respiration. Journal of Applied Toxicology 28, 945–956.

Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T., Traganos, F., 1997. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 27, 1–20.

Das, M., Khanna, S.K., 1997. Clinicoepidemiological, toxicological, and safety evaluation studies on *Argemone* oil. Critical Reviews in Toxicology 27, 273–297.

Del Bino, G., Skierski, J.S., Darzynkiewicz, Z., 1990. Diverse effects of camptothecin, an inhibitor of topoisomerase I, on the cell cycle of lymphocytic (L1210, MOLT-4) and myelogenous (HL-60, KG1) leukemic cells. Cancer Research 50, 5746–5750.

Dvorak, Z., Simanek, V., 2007. Metabolism of sanguinarine: the facts and the myths. Current Drug Metabolism 8, 173–176.

Edinger, A.L., Thompson, C.B., 2004. Death by design: apoptosis, necrosis and autophagy. Current Opinion in Cell Biology 16, 663–669.

Faddeeva, M.D., Beliaeva, T.N., 1997. Sanguinarine and ellipticine cytotoxic alkaloids isolated from well-known antitumor plants. Intracellular targets of their action. Tsitologiia 39, 181–208.

Gopalakrishna, R., Chen, Z.H., Gundimeda, U., 1995. Modifications of cysteine-rich regions in protein kinase C induced by oxidant tumor promoters and enzyme-specific inhibitors. Methods in Enzymology 252, 132–146.

Gottlieb, E., Armour, S.M., Harris, M.H., Thompson, C.B., 2003. Mitochondrial membrane potential regulates matrix configuration and cytochrome *c* release during apoptosis. Cell Death and Differentiation 10, 709–717.

Jayaraman, S., 2005. Flow cytometric determination of mitochondrial membrane potential changes during apoptosis of T lymphocytic and pancreatic beta cell lines: comparison of tetramethylrhodamineethylester (TMRE), chloromethyl-X-rosamine (H2-CMX-Ros) and MitoTracker Red 580 (MTR580). Journal of Immunological Methods 306, 68–79.

Kaminskyy, V.O., Lootsik, M.D., Stoika, R.S., 2006. Correlation of the cytotoxic activity of four different alkaloids, from *Chelidonium majus* (greater celandine), with their DNA intercalating properties and ability to induce breaks in the DNA of NK/Ly murine lymphoma cells. Central European Journal of Biology 1, 2–15.

Kaminskyy, V., Lin, K.W., Filyak, Y., Stoika, R., 2008a. Differential effect of sanguinarine, chelerythrine and chelidonine on DNA damage and cell viability in primary mouse spleen cells and mouse leukemic cells. Cell Biology International 32, 271–277.

Kaminskyy, V., Kulachkovskyy, O., Stoika, R., 2008b. A decisive role of mitochondria in defining rate and intensity of apoptosis induction by different alkaloids. Toxicology Letters 177, 168–181.

Kim, S., Lee, T.J., Leem, J., Choi, K.S., Park, J.W., Kwon, T.K., 2008. Sanguinarine-induced apoptosis: generation of ROS, down-regulation of Bcl-2, c-FLIP, and synergy with TRAIL. Journal of Cellular Biochemistry 104, 895–907.

Kovar, J., Stejskal, J., Paulova, H., Slavik, J., 1986. Reduction of quaternary benzophenanthridine alkaloids by NADH and NADPH. Collection of Czechoslovak Chemical Communications 51, 2626–2634.

Lee, B.Y., Lee, S.J., Park, S.S., Kim, S.K., Kim, S.R., Jung, J.H., Kim, W.J., Moon, S.K., 2008. Sanguinarine-induced G1-phase arrest of the cell cycle results from increased p27KIP1 expression mediated via activation of the Ras/ERK signaling pathway in vascular smooth muscle cells. Archives of Biochemistry and Biophysics 471, 224–231.

Lemasters, J.J., Qian, T., Bradham, C.A., Brenner, D.A., Cascio, W.E., Trost, L.C., Nishimura, Y., Nieminen, A.L., Herman, B., 1999. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. Journal of Bioenergetics and Biomembranes 31, 305–319.

Maianski, N.A., Geissler, J., Srinivasula, S.M., Alnemri, E.S., Roos, D., Kuijpers, T.W., 2004. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. Cell Death and Differentiation 11, 143–153.

Malikova, J., Zdarilova, A., Hlobilkova, A., Ulrichova, J., 2006a. The effect of chelerythrine on cell growth, apoptosis, and cell cycle in human normal and cancer cells in comparison with sanguinarine. Cell Biology and Toxicology 22, 439–453.

Malikova, J., Zdarilova, A., Hlobilkova, A., 2006b. Effects of sanguinarine and chelerythrine on the cell cycle and apoptosis. Biomedical Papers 150, 5–12.

Matkar, S.S., Wrischnik, L.A., Hellmann-Blumberg, U., 2008a. Production of hydrogen peroxide and redox cycling can explain how sanguinarine and chelerythrine induce rapid apoptosis. Archives of Biochemistry and Biophysics 477, 43–52.

Matkar, S.S., Wrischnik, L.A., Hellmann-Blumberg, U., 2008b. Sanguinarine causes DNA damage and p53-independent cell death in human colon cancer cell lines. Chemico-Biological Interactions 172, 63–71.

Navarro, V., Delgado, G., 1999. Two antimicrobial alkaloids from *Bocconia arborea*. Journal of Ethnopharmacology 66, 223–226.

Orrenius, S., 2004. Mitochondrial regulation of apoptotic cell death. Toxicology Letters 149, 19–23.

Patterson Jr., M.K., 1979. Measurement of growth and viability of cells in culture. Methods in Enzymology 58, 141–152.

Philchenkov, A., Kaminskyy, V., Zavelevich, M., Stoika, R., 2008. Apoptogenic activity of two benzophenanthridine alkaloids from *Chelidonium majus* L. does not correlate with their DNA damaging effects. Toxicology in Vitro 22, 287–295.

Psotova, J., Klejdus, B., Vecera, R., Kosina, P., Kuban, V., Vicar, J., Simanek, V., Ulrichova, J., 2006. A liquid chromatographic–mass spectrometric evidence of dihydrosanguinarine as a first metabolite of sanguinarine transformation in rat. Journal of Chromatography B – Analytical Technologies in the Biomedical and Life Sciences 830, 165–172.

Slaninova, I., Slunska, Z., Sinkora, J., Vlkova, M., Taborska, E., 2007. Screening of minor benzo(c)phenanthridine alkaloids for antiproliferative and apoptotic activities. Pharmaceutical Biology 45, 131–139.

Vavreckova, C., Ulrichova, J., Hajduch, M., Grambal, F., Weigl, E., Simanek, V., 1994. Effect of quaternary benzo[*c*]phenanthridine alkaloids sanguinarine, chelerythrine and fagaronine on some mammalian cells. Acta Universitatis Palackianae Olomucensis Facultatis Medicae 138, 7–10.

Vecera, R., Klejdus, B., Kosina, P., Orolin, J., Stiborova, M., Smrcek, S., Vicar, J., Dvorak, Z., Ulrichova, J., Kuba, V., Anzenbacher, P., Simanek, V., 2007. Disposition of sanguinarine in the rat. Xenobiotica 37, 549–558.

Veldman, J.W., Murray, K.G., Hull, A.L., Garcia, J.M., Mungall, W.S., Rotman, G.B., Plosz, M.P., McNamara, L.K., 2007. Chemical defense and the persistence of pioneer plant seeds in the soil of a tropical cloud forest. Biotropica 39, 87–93.

Vogt, A., Tamewitz, A., Skoko, J., Sikorski, R.P., Giuliano, K.A., Lazo, J.S., 2005. The benzo[*c*]phenanthridine alkaloid, sanguinarine, is a selective, cell-active inhibitor of mitogen-activated protein kinase phosphatase-1. Journal of Biological Chemistry 280, 19078–19086.

Vrba, J., Dvorak, Z., Ulrichova, J., Modriansky, M., 2008a. Conventional protein kinase C isoenzymes undergo dephosphorylation in neutrophil-like HL-60 cells treated by chelerythrine or sanguinarine. Cell Biology and Toxicology 24, 39–53.

Vrba, J., Dolezel, P., Vicar, J., Modriansky, M., Ulrichova, J., 2008b. Chelerythrine and dihydrochelerythrine induce G1 phase arrest and bimodal cell death in human leukemia HL-60 cells. Toxicology in Vitro 22, 1008–1017.

Vrublova, E., Vostalova, J., Vecera, R., Klejdus, B., Stejskaj, D., Kosina, P., Zdarilova, A., Svobodova, A., Lichnovsky, V., Anzenbacher, P., Dvorak, Z., Vicar, J., Simanek, V., Ulrichova, J., 2008. The toxicity and pharmacokinetics of dihydrosanguinarine in rat: a pilot study. Food and Chemical Toxicology 46, 2546–2553.

Walterova, D., Ulrichova, J., Valka, I., Vicar, J., Vavreckova, C., Taborska, E., Harkrader, R.J., Meyer, D.L., Cerna, H., Simanek, V., 1995. Benzo[*c*]phenanthridine alkaloids sanguinarine and chelerythrine: biological activities and dental care applications. Acta Universitatis Palackianae Olomucensis Facultatis Medicae 139, 7–16.

Weerasinghe, P., Hallock, S., Tang, S.C., Trump, B., Liepins, A., 2006. Sanguinarine overcomes P-glycoprotein-mediated multidrug-resistance via induction of apoptosis and oncosis in CEM-VLB 1000 cells. Experimental and Toxicologic Pathology 58, 21–30.

Weiss, D., Baumert, A., Vogel, M., Roos, W., 2006. Sanguinarine reductase, a key enzyme of benzophenanthridine detoxification. Plant Cell and Environment 29, 291–302.

Wolf, D., Rotter, V., 1985. Major deletions in the gene encoding the p53 tumor-antigen cause lack of p53 expression in HL-60 cells. Proceedings of the National Academy of Sciences of the United States of America 82, 790–794.

Yu, J., Zhang, L., 2005. The transcriptional targets of p53 in apoptosis control. Biochemical and Biophysical Research Communications 331, 851–858.