

Sanguinarine-Dependent Induction of Apoptosis in Primary Effusion Lymphoma Cells

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Abstract

Primary effusion lymphoma (PEL) is an incurable, aggressive B-cell malignancy that develops rapid resistance to conventional chemotherapy. In efforts to identify novel approaches to block proliferation of PEL cells, we found that sanguinarine, a natural compound isolated from the root plant *Sanguinaria canadensis*, inhibits cell proliferation and induces apoptosis in a dose-dependent manner in several PEL cell lines. Our data show that sanguinarine treatment of PEL cells results in up-regulation of death receptor 5 (DR5) expression via generation of reactive oxygen species (ROS) and causes activation of caspase-8 and truncation of Bid (tBid). Subsequently, tBid translocates to the mitochondria causing conformational changes in Bax, leading to loss of mitochondrial membrane potential and release of cytochrome *c* to the cytosol. Sanguinarine-induced release of cytochrome *c* results in activation of caspase-9 and caspase-3 and poly(ADP-ribose) polymerase (PARP) cleavage, leading to induction of caspase-dependent apoptosis. In addition, we show that pretreatment of PEL cells with carbobenzoxy-Val-Ala-Asp-fluoromethylketone, a universal inhibitor of caspases, abrogates caspase and PARP activation and prevents cell death induced by sanguinarine. Moreover, treatment of PEL cells with sanguinarine down-regulates expression of inhibitor of apoptosis proteins (IAP). Finally, *N*-acetylcysteine, an inhibitor of ROS, inhibits sanguinarine-induced generation of ROS, up-regulation of DR5, Bax conformational changes, activation of caspase-3, and down-regulation of IAPs. Taken together, our findings suggest that sanguinarine is a potent inducer of apoptosis of PEL cells via up-regulation of DR5 and raise the possibility that this agent may be of value in the development of novel therapeutic approaches for the treatment of PEL. [Cancer Res 2007;67(8):3888–97]

Introduction

Primary effusion lymphoma (PEL) is a subtype of non-Hodgkin's B-cell lymphoma that mainly presents in patients with advanced AIDS but is sometimes also found in HIV-negative individuals (1, 2).

PEL cells grow as a lymphomatous effusion in body cavities and are infected with Kaposi's sarcoma-associated herpes virus [human herpesvirus (HHV)-8]. Most cases show dual infection with EBV (HHV-4; ref. 3). Pleural and abdominal effusions from patients with AIDS-PEL contain a number of cytokines, which serve as autocrine growth factors (4, 5). For example, interleukin (IL)-10 has been reported to serve as an autocrine growth factor for AIDS-related B-cell lymphoma (6). Recently, it has also been shown that PEL cells use viral IL-6 and IL-10 in an autocrine fashion for their survival and proliferation (4).

Programmed cell death or apoptosis is a genetically regulated process, which plays an essential role in the development and homeostasis of higher organisms (7). Defective apoptosis contributes to the development of many different types of malignancies. Cell death may be triggered by a wide variety of death signals. There are two major pathways involved by which apoptosis occurs: (a) the mitochondrion-initiated pathway, also defined as the intrinsic pathway, and (b) the cell-surface death receptor pathway, also defined as the extrinsic pathway (8). In the mitochondrial pathway, cytochrome *c* is released from the intramembrane space to the cytosol. Once released, cytochrome *c* and dATP bind to apoptotic protease-activating factor 1 (Apaf-1), and this complex, along with adenine nucleotides, promotes procaspase-9 autoactivation, which in turn activates caspase-3, ultimately resulting in PARP cleavage (9). In the death receptor-mediated pathway (e.g., TRAIL/TRAIL ligand interaction and cell death), caspase-8 is an initiator caspase that can activate downstream caspases, including caspase-3. Active caspase-8 also cleaves a proapoptotic Bcl-2 family member, Bid, and the truncated Bid (tBid) induces mitochondrial cytochrome *c* release (10). Cross talk between the two pathways is mediated by the tBid (11). In addition, a third pathway has also been identified in which Bid is cleaved downstream of the point of Bcl-2 action, catalyzed by caspase-3, upstream of caspase-8 activation, and acts as a potential feedback loop for amplification of apoptosis-associated release of cytochrome *c* from the mitochondria (12).

Sanguinarine (13-methylbenzodi-oxolo[5,6-]-1,3-dioxolo[4,5-1]phenanthridinium) is a benzophenanthridin alkaloid that has significant structural homology to chelethrine, derived from the root of *Sanguinaria canadensis* and other poppy fumaria species (13, 14). Sanguinarine has been shown to possess antimicrobial, antioxidant, and antiproliferative properties (13, 15, 16). Recently, one study suggested that sanguinarine suppresses the growth and survival of human epidermoid carcinoma cells. In addition, this agent has been shown to inhibit the activation of nuclear factor κ B (NF- κ B; ref. 13), a transcription factor of critical importance in the regulation of cell growth and survival. It should also be pointed out that recent studies have implicated sanguinarine in the induction

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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of apoptosis of prostate cancer cells via formation of nitric oxide and generation of superoxide radicals (17). Interestingly, sanguinarine does not exert an apoptotic effect on normal cells and therefore can be developed as an anticancer drug (15).

In the present study, we investigated the antitumor activity of sanguinarine against human PEL cell lines. Our data provide the first evidence that sanguinarine induces apoptosis of PEL cells via a mechanism involving up-regulation of death receptor 5 (DR5) expression and generation of reactive oxygen species (ROS). Our findings establish that up-regulation of DR5 results in activation of caspase-8 and truncation of Bid (tBid), which in turn translocates to the mitochondria and causes conformational changes as well as oligomerization of the Bax protein, leading to loss of mitochondrial membrane potential and subsequent release of cytochrome *c*. Altogether, these studies establish that sanguinarine is a potent inducer of apoptosis in PEL cells and identify the mechanisms by which such apoptosis is induced.

Materials and Methods

Cell Culture and Treatment

The human PEL cell lines BC1, BC3, BCBL1, and HBL6 were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, 100 units/mL streptomycin at 37°C in an humidified atmosphere containing 5% CO₂. BC1 and HBL6 cell lines are co-infected with EBV and HHV-8 whereas BC3 and BCBL1 cell lines are HHV-8 positive only. The cells were treated with sanguinarine dissolved in DMSO at concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 μmol/L for different time periods in RPMI culture media supplemented with 5% (v/v) FBS. The control cells were incubated with maximum used amount of DMSO only.

Reagents and Antibodies

Sanguinarine, *N*-acetylcysteine (NAC), DMSO, and Bax 6A7 monoclonal antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Carbobenzoxy-Val-Ala-Asp-fluoromethylketone (*z*-VAD-fmk) was purchased from Calbiochem (San Diego, CA). Antibodies against cleaved caspase-3 and Bid were purchased from Cell Signaling Technologies (Beverly, MA). Cytochrome *c*, β-actin, caspase-3, and PARP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein (cIAP)-1, cIAP2, DR4, and caspase-8 antibodies were purchased from R&D Systems (Minneapolis, MN). DR5 antibody was purchased from Cayman (Ann Arbor, MI). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit was obtained from MBL (Watertown, MA). Annexin V was purchased from Molecular Probes (Eugene, OR). Apoptotic DNA ladder kit was obtained from Roche (Penzberg, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays

Cells (10⁴) were incubated in triplicate in a 96-well plate in the presence or absence of indicated test doses of sanguinarine in a final volume of 0.20 mL for 24 h. The ability of sanguinarine to suppress cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays as previously described (18).

Cell cycle analysis. Cell lines were either treated with and without 1 and 2 μmol/L sanguinarine for 24 h and the cells were washed once with PBS, resuspended in 500 μL of hypotonic staining buffer (250 mg sodium citrate, 0.75 mL Triton X-100, 25 μg propidium iodide, 5 μg RNase A, and 250 mL water), and analyzed by flow cytometry as previously described (19).

TUNEL Assay

PEL cells were treated with various concentrations of sanguinarine for 24 h, washed twice with PBS containing 0.2% bovine serum albumin, fixed

with paraformaldehyde, and permeabilized in 70% ethanol. The cells were subsequently stained with FITC-dUTP and stained cells were analyzed using FACSscan flow cytometry equipped with a Cell Quest data analysis program (Becton Dickinson, San Diego, CA) as described earlier (20).

Annexin V/Propidium Iodide Dual Staining

PEL cell lines were treated with the indicated concentrations of sanguinarine. The cells were harvested and the percentage of cells undergoing apoptosis was measured by flow cytometry after staining with fluorescein-conjugated Annexin V and propidium iodide (Molecular Probes) as previously described (21).

Measurement of ROS. ROS production was detected using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), a cell-permeable fluorescent probe. Exponentially growing cells were loaded with 10 μmol/L H₂DCFDA for 45 min before treatment at 37°C and were washed with PBS. The cells were allowed to recover for 15 min at 37°C in RPMI media and then treated with 2 μmol/L sanguinarine alone or in the presence of 10 mmol/L NAC for the indicated time periods. Following treatment, the cells were washed in PBS and the green fluorescence intensity in the cells was examined by FACS analysis. Propidium iodide was added 1 min before flow cytometry to differentiate apoptosis from necrosis.

Reverse transcription-PCR assays. Total RNA was extracted following treatment with 2 μmol/L sanguinarine for the indicated time periods using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed with random hexamers. Reverse transcription-PCR (RT-PCR) amplifications were done using the following primers: *DR5* forward, GGAAGCCGCTCATGAG-GAAGTTGG; *DR5* reverse, GGCAAGTCTCTCTCCAGCGTCTC for 35 cycles (60°C annealing temperature) to yield an 181-bp product. Amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control to test the integrity of all cDNA and to provide a measure of relative expression. All PCR products were analyzed by 4% agarose gel electrophoresis and visualized with ethidium bromide under UV light using an Alpha Imager (Alpha Innotech, San Leandro, CA).

Quantification Analysis by Real-time RT-PCR

All reactions were done in glass capillaries (Roche) with a final reaction volume of 10 μL of 1× LightCycler FastStart DNA Master SYBR Green I reaction mixture (Roche, Mannheim, Germany) containing FastStart Taq, reaction buffer, and deoxynucleotide triphosphate, 1 mmol/L MgCl₂, and final concentrations of 0.5 μmol/L for each primer. Thermocycling and detection were done on the LightCycler (Roche). Real-time PCR efficiencies were calculated from the given slopes in LightCycler software using the following equation:

$$E = 10^{[-1/\text{slope}]}$$

Pfaffl et al.'s (22) method for relative quantification was used to calculate fold changes for *DR5* gene after sanguinarine treatment. The relative expression ratio of a target gene is calculated based on efficiency (*E*) and crossing point (CP) deviation of unknown samples (treated samples) versus control (nontreated samples) and expressed in comparison with a reference gene (*GAPDH*): Ratio = ($E_{\text{target}}^{\text{CP (control-sample)}}$) / ($E_{\text{ref}}^{\text{CP (control-sample)}}$).

Cell Lysis and Immunoblotting

Cells were treated with sanguinarine as described in the legends and lysed as previously described (23). Proteins (15–20 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Billerica, MA). Immunoblotting was done with different antibodies and visualized by the enhanced chemiluminescence (Amersham, Piscataway, NJ) method.

Detection of Bax Conformational Changes

This assay was done as previously described (24). Briefly, following treatment with indicated reagents for indicated time points, cells were harvested and washed with PBS, after which they were lysed with Chaps lysis buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Chaps] containing protease inhibitors as described. Protein concentrations were

assessed by Bradford assay and 500 µg of total protein were incubated with 2 µg of anti-Bax 6A7 monoclonal antibody for 2 h at 4°C. Following incubation, 25 µL of protein G-beads were added into the reaction and incubated at 4°C overnight on a shaker with gentle agitation. Following washes in lysis buffer, samples were separated by SDS-PAGE, transferred, and immunoblotted with N20 Bax polyclonal antibody.

Cross-Linking of Bax Protein

Following treatment with 2 µmol/L sanguinarine for indicated time periods, cells were washed twice with PBS and once with conjugating buffer containing 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.2), 1.5 mmol/L MgCl₂, and 10 mmol/L glucose. Disuccinimidyl suberate in DMSO was added at a final concentration of 2 mmol/L and incubated at room temperature for 30 min. After incubation, the cross-linker was quenched by the addition of 1 mol/L Tris-HCl (pH 7.5) to a final concentration of 20 mmol/L for 15 min at room temperature. Samples were then solubilized in 0.5% NP40 lysis buffer and centrifuged at 14,000 rpm for 10 min. Bax was immunoprecipitated from the supernatant with Bax monoclonal antibody and then immunoblotted with Bax polyclonal antibody. Samples incubated with DMSO without disuccinimidyl suberate were used as negative controls.

Assays for Cytochrome c Release

The release of cytochrome c from mitochondria was assayed as described earlier (25). Briefly, cells were treated with and without sanguinarine as described in figure legends, harvested, and resuspended in hypotonic buffer. Cells were homogenized and cytosolic as well as mitochondrial fractions were isolated by differential centrifugation as

described before (25). Proteins (20–25 µg) from cytosolic and mitochondrial fractions of each sample were analyzed by immunoblotting with anti-cytochrome c antibody.

Measurement of mitochondrial membrane potential. Cells were treated with and without sanguinarine for 24 h, washed twice, and resuspended in mitochondrial incubation buffer. JC1 was added to a final concentration of 10 µmol/L and cells were incubated at 37°C in dark for 15 min. Cells were washed and resuspended in mitochondrial incubation buffer and mitochondrial membrane potential (percent of green and red aggregates) was determined by flow cytometry as previously described (26).

Statistical analysis. Comparisons between groups were made using paired Student's *t* test. *P* < 0.05 was considered statistically significant.

Results

Sanguinarine causes a dose-dependent inhibition of proliferation and apoptosis of PEL cells. We initially sought to determine whether sanguinarine treatment leads to the inhibition of PEL cell proliferation. BC1, BC3, BCBL, and HBL6 cells were cultured in the presence of 0.25, 1.0, 2.0, and 4 µmol/L sanguinarine for 24 h and proliferation was analyzed by MTT assays. Figure 1A shows that as the dose of sanguinarine increased from 0.25 to 4.0 µmol/L, cell growth inhibition increased in a dose-dependent fashion in all the PEL cell lines. The growth inhibition induced by sanguinarine treatment was found to be statistically significant

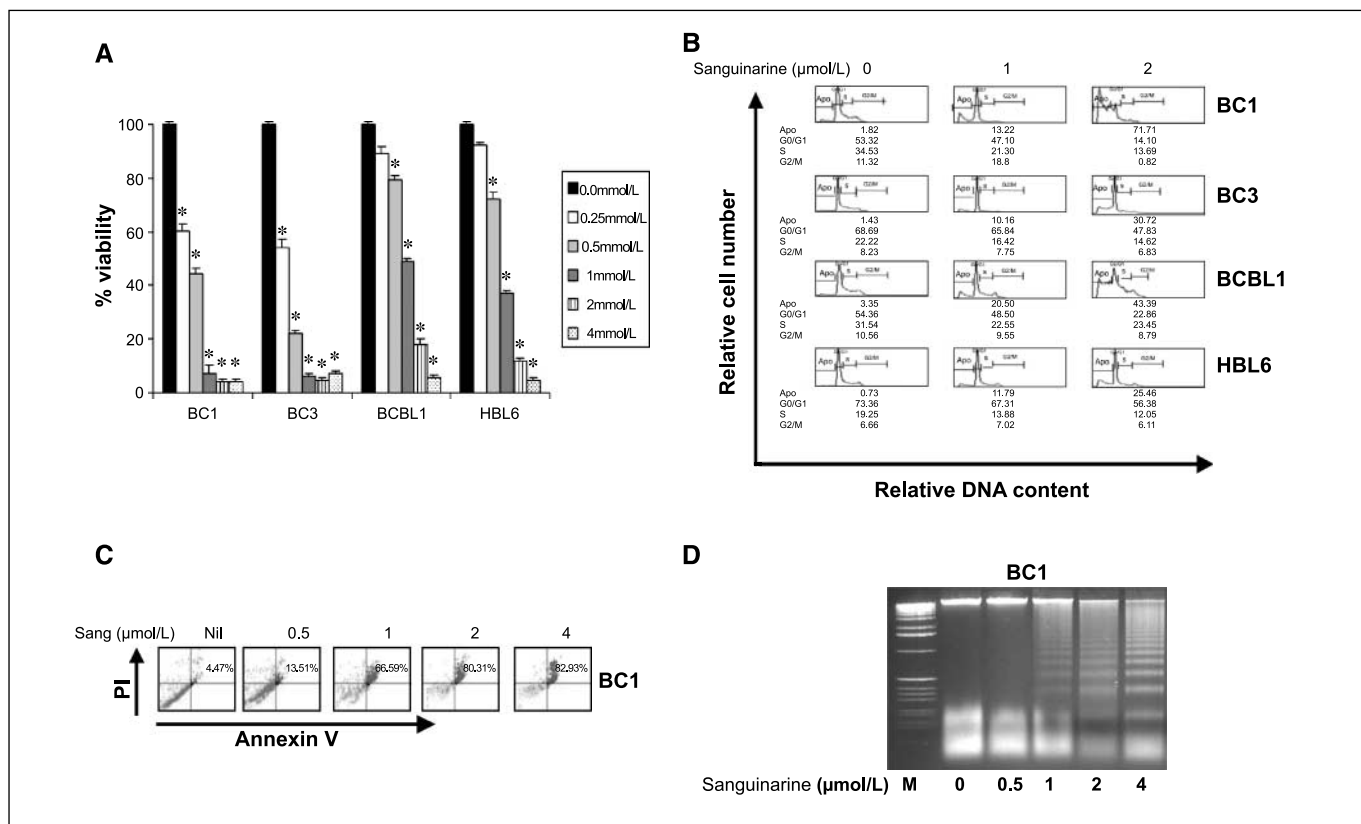


Figure 1. A, sanguinarine inhibits the proliferation of PEL cells. BC1, BC3, BCBL1, and HBL6 cells were incubated with 0.25, 0.5, 1.0, 2.0, and 4.0 µmol/L sanguinarine for 24 h. Cell proliferation assays were done using MTT as described in Materials and Methods. Columns, mean of three independent experiments with replicates of three wells for all the doses and vehicle control for each experiment; bars, SD. * *P* < 0.001, Student's *t* test. B, sanguinarine treatment increases sub-G₁ (*Apo*) populations in PEL cells. BC1, BC3, BCBL1, and HBL6 cells were treated with 1.0 and 2.0 µmol/L sanguinarine for 24 h. Thereafter, the cells were washed, fixed, and stained with propidium iodide and then analyzed for DNA content by flow cytometry as described in Materials and Methods. C, sanguinarine-induced apoptosis detected by Annexin V/propidium iodide dual staining. BC1 cells were treated with various doses of sanguinarine (as indicated) for 24 h and cells were subsequently stained with fluorescein-conjugated Annexin V and propidium iodide (*PI*) and analyzed by flow cytometry. D, BC1 cells were treated with various doses of sanguinarine as indicated for 24 h and DNA was extracted and separated by electrophoresis on 1.5% agarose gel.

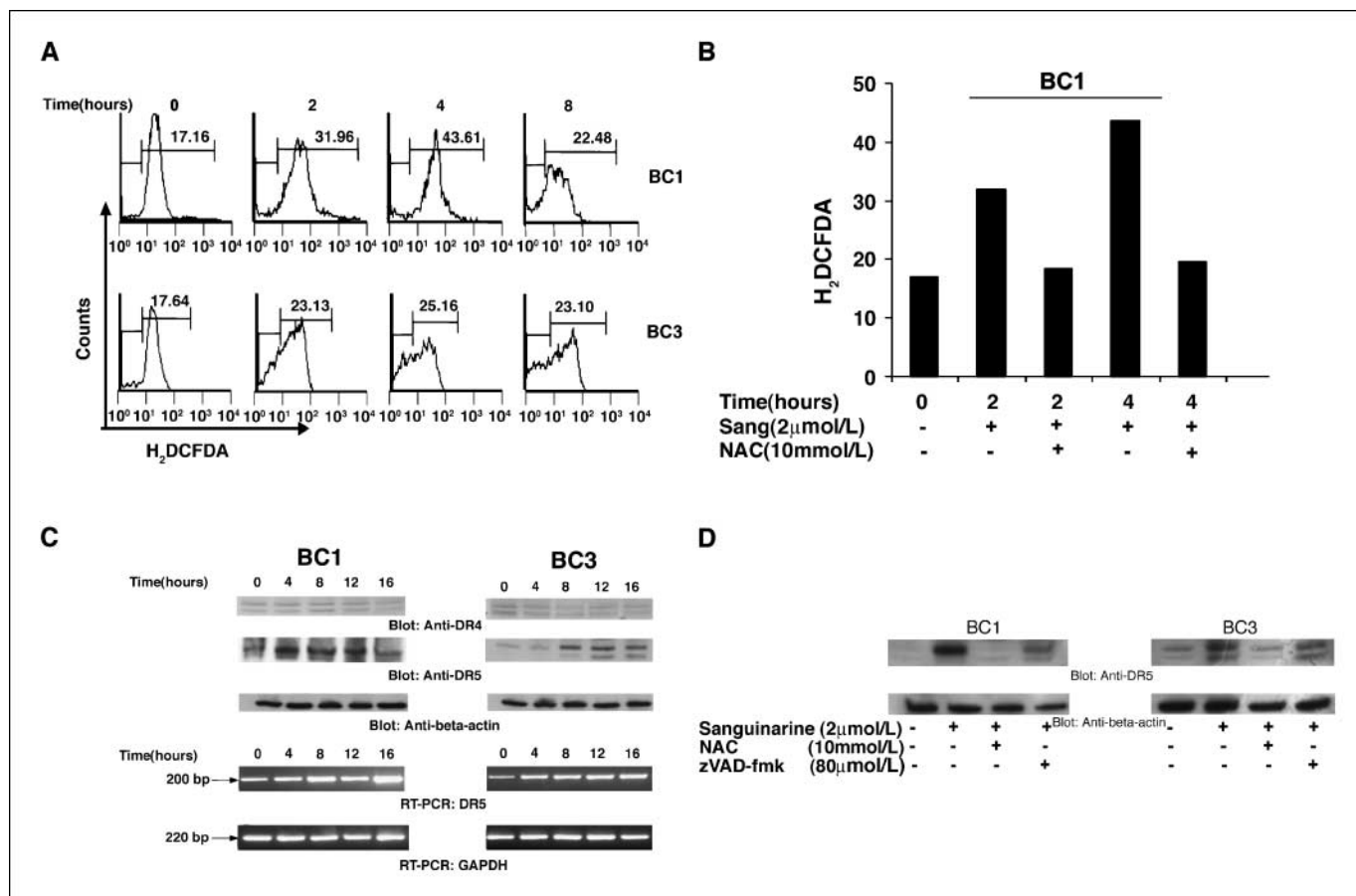


Figure 2. Sanguinarine increases ROS generation and up-regulation of DR5 in PEL cells. *A*, sanguinarine increases ROS generation in PEL cells. BC1 and BC3 cells were loaded with 10 μmol/L H₂DCFDA for 45 min and then were incubated in the absence or presence of 2.0 μmol/L sanguinarine for indicated time periods, or pretreated with 10 mmol/L NAC for 1 h followed by treatment with 2.0 μmol/L sanguinarine for indicated time periods (*B*). After washing with PBS, cells were resuspended in PBS and immediately analyzed using flow cytometry for intracellular accumulation of ROS. *C*, sanguinarine induces up-regulation of DR5 expression. BC1 and BC3 cells were treated with 2.0 μmol/L sanguinarine for indicated time periods. After cell lysis, equal amounts of proteins were separated by SDS-PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against DR4, DR5, and β-actin. For mRNA analysis, BC1 and BC3 cells were treated with 2.0 μmol/L sanguinarine for indicated time periods and total RNA was isolated, reverse transcribed, and RT-PCR analyses for *DR5* and *GAPDH* were done. *D*, effect of NAC and z-VAD-fmk on sanguinarine-induced DR5 up-regulation in PEL cells. BC1 and BC3 cells were pretreated with either 10 mmol/L NAC or 80 μmol/L z-VAD-fmk for 60 min and subsequently treated with 2.0 μmol/L sanguinarine for 16 h. After cell lysis, equal amounts of proteins were separated by SDS-PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against DR5 and β-actin.

($P < 0.001$, Student's t test) at most of the doses tested in all cell lines.

In subsequent experiments, we determined whether the observed suppressive effects of sanguinarine in MTT assays are due to induction of cell cycle arrest or apoptosis. Different PEL cell lines were treated with 1 and 2 μmol/L sanguinarine for 24 h or vehicle alone. The cells were stained and cell cycle fractions were determined by flow cytometry. As shown in Fig. 1*B*, the sub-G₁ population of cells increased from 1.82% in control to 13.22% and 71.71% with 1.0 and 2.0 μmol/L sanguinarine, respectively, in BC1 cells. Similar results were obtained in BC3, 1.43% to 10.16% and 30.72%; in BCBL1, 3.35% to 20.50% and 43.39%; and in HBL6, 0.73% to 11.79% and 25%. This increase in sub-G₁ population was accompanied by loss in G₀-G₁, S, and G₂-M phase in sanguinarine-treated PEL cells. It has been reported that cells with these features are those dying of apoptosis (27). We also used Annexin V/propidium iodide dual staining, TUNEL assay, and DNA laddering detection methods for further confirmation of sanguinarine-induced apoptosis in PEL cells. BC1 cells were treated with different doses of sanguinarine for 24 h and apoptosis was measured by

Annexin V/propidium iodide dual staining. As shown in Fig. 1*C*, sanguinarine induced apoptosis in a dose-dependent manner. As shown in Supplementary Fig. S1*B*, 2 μmol/L sanguinarine treatment for 24 h causes significant apoptosis in all the cell lines tested ($P < 0.001$). TUNEL assay was done on cells treated with 2.0 μmol/L sanguinarine for 24 h. As shown in Supplementary Fig. S1, sanguinarine treatment resulted in apoptosis in all the cell lines studied. Finally, we analyzed DNA fragmentation, which is another hallmark of apoptosis. BC1 cells were treated with DMSO and 0.5, 1.0, 2.0, and 4.0 μmol/L sanguinarine for 24 h; DNA was isolated using apoptotic DNA laddering kit from Roche. As shown in Fig. 1*D*, sanguinarine caused a dose-dependent fragmentation formation of DNA, a characteristic of apoptotic cell death. These results suggest that suppression of growth by sanguinarine treatment in PEL cells is via induction of apoptosis.

Sanguinarine-induced ROS generation regulates up-regulation of DR5. A number of compounds used in chemotherapy induce cell death through the generation of ROS (28). We examined whether ROS were also generated in PEL cells treated with 2 μmol/L sanguinarine for various time periods, and if so,

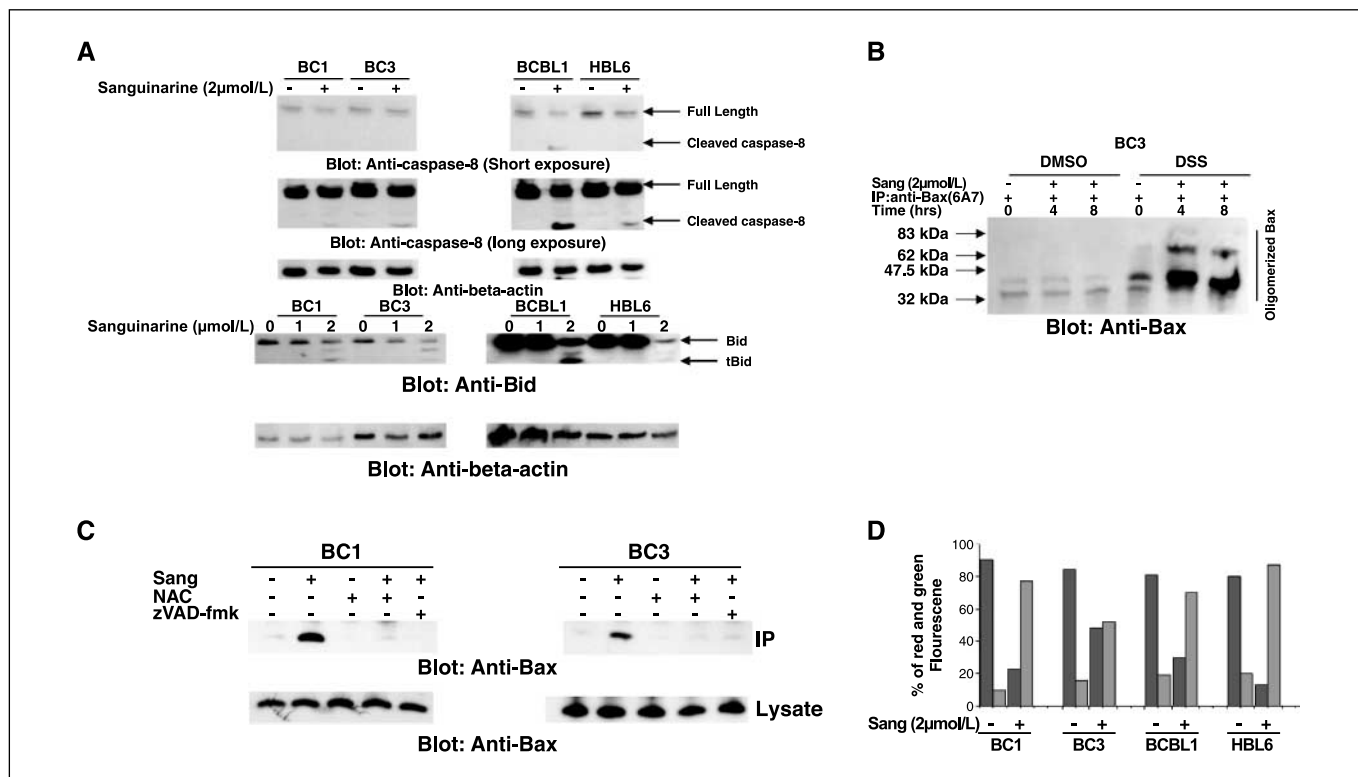


Figure 3. Sanguinarine-induced activation of caspase-8 and mitochondrial apoptotic pathway. **A**, sanguinarine-induced activation of caspase-8 and Bid cleavage. BC1, BC3, BCBL, and HBL6 cells were treated with 2.0 $\mu\text{mol/L}$ sanguinarine for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against caspase-8 and β -actin. BC1, BC3, BCBL, and HBL6 cells were treated with 1 and 2.0 $\mu\text{mol/L}$ sanguinarine for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to Immobilon membrane, and immunoblotted with antibodies against Bid and β -actin. **B**, sanguinarine induces Bax oligomerization in PEL cells. BC3 cells were treated with 2.0 $\mu\text{mol/L}$ sanguinarine for 24 h. The oligomerization of Bax was assessed by cross-linking with disuccinimidyl suberate (DSS) followed by immunoblot analysis with Bax rabbit polyclonal antibody. DMSO was used as the vehicle control. *Arrows*, protein markers used. **C**, effect of NAC and z-VAD-fmk on sanguinarine-induced Bax activation in PEL cells. BC1 and BC3 cells were pretreated with either 10 mmol/L NAC or 80 $\mu\text{mol/L}$ z-VAD-fmk for 1 h and subsequently treated with 2.0 $\mu\text{mol/L}$ sanguinarine for 24 h. Cells were lysed with 1% Chaps lysis buffer and the lysates were immunoprecipitated with anti-Bax 6A7 antibody and proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with Bax rabbit polyclonal antibody. **D**, loss of mitochondrial membrane potential by sanguinarine treatment in PEL cells. BC1, BC3, BCBL1, and HBL6 cells were treated with and without 2.0 $\mu\text{mol/L}$ sanguinarine for 24 h. Live cells with intact mitochondrial membrane potential (*dark column*) and dead cells with lost mitochondrial membrane potential (*light column*) were measured by JC1 staining and analyzed by flow cytometry as described in Materials and Methods.

whether this is a mechanism for the induction of sanguinarine-induced apoptosis. H_2DCFDA -based FACS detection revealed that intracellular ROS levels increased in a time-dependent manner, starting as early as 1 h after treatment, peaking at 4 h, and diminishing after 8 h of treatment with sanguinarine (Fig. 2A). NAC is a widely used thiol-containing antioxidant that scavenges ROS in cells by interacting with OH and H_2O_2 , thus affecting ROS-mediated signaling pathways. The sanguinarine-induced increases in ROS levels were blocked by pretreatment with 10 mmol/L NAC (Fig. 2B).

Recent studies have shown that ROS generated by sulforaphane and hydrogen peroxide up-regulate the expression of DR5, whereas pretreatment with NAC causes a significant inhibition of ROS-induced up-regulation of DR5 gene and protein expression in hepatoma cells (29). In view of these findings, we sought to determine whether sanguinarine-generated free radicals modulate the expression of DR4 and DR5. First, we carried out Western blotting to investigate the induction DR4 and DR5 proteins by sanguinarine. BC1 and BC3 cells were treated with 2.0 $\mu\text{mol/L}$ sanguinarine for various time periods and cell lysates were separated on SDS-PAGE and immunoblotted with antibodies against DR4 and DR5. As shown in Fig. 2C, sanguinarine treatment

of PEL cells selectively up-regulated DR5 but not DR4 protein in a time-dependent manner. We then examined whether sanguinarine treatment of PEL cells regulated DR5 expression at the transcription level. RT-PCR analysis showed that sanguinarine enhanced DR5 up-regulation at the mRNA level in a time-dependent manner (Fig. 2C). We further quantified the level of DR5 expression after sanguinarine treatment by quantitative real-time PCR. As shown in Supplementary Fig. S2, there was a 2-fold increase in the expression level of DR5 in BC1 cells after 2 h of sanguinarine treatment, which further increased to >14-fold after 16 h. A similar pattern of DR5 expression in response to sanguinarine was also seen in the BC3 cell line, as measured by Pfaffl et al.'s (22) method. Altogether, these results indicate that sanguinarine-induced apoptosis involves up-regulation of DR5 expression at the mRNA and protein levels in PEL cells.

To investigate whether ROS generation is directly associated with sanguinarine-induced DR5 up-regulation, we assessed DR5 expression in PEL cells pretreated with NAC for 1 h followed by treatment with 2.0 $\mu\text{mol/L}$ sanguinarine. As shown in Fig. 2D, treatment with sanguinarine significantly increased DR5 protein levels, whereas pretreatment with 10 mmol/L NAC markedly inhibited the sanguinarine-induced DR5 up-regulation. In addition,

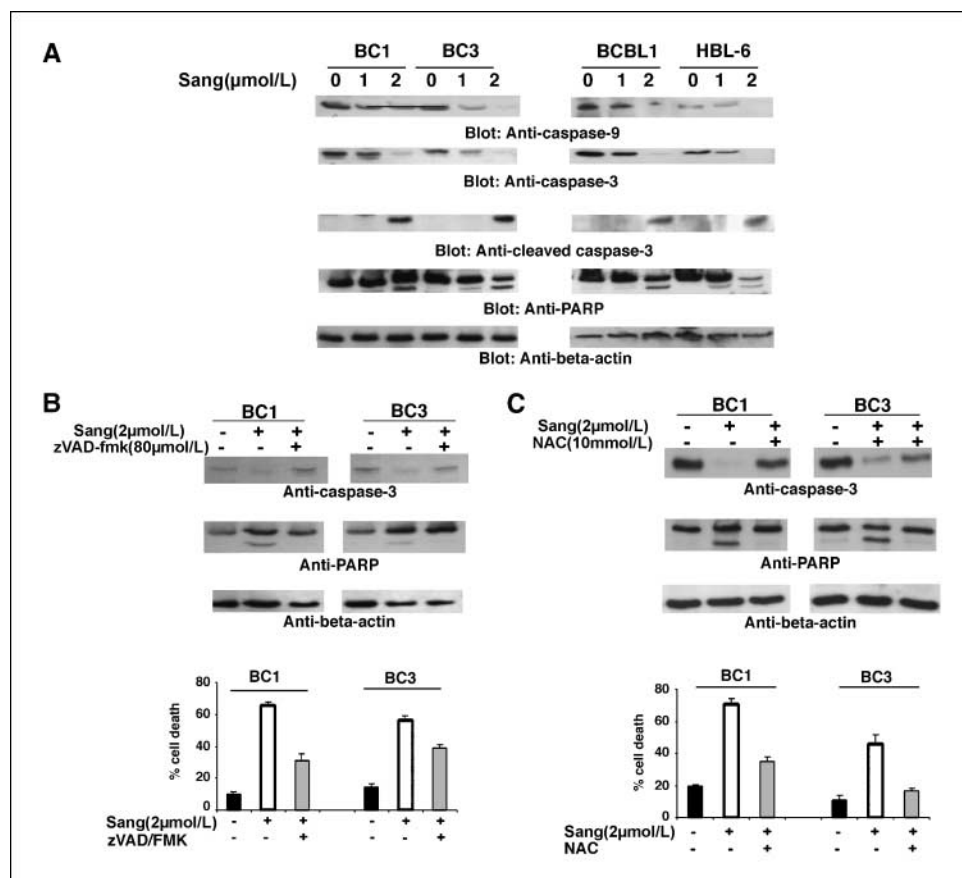
pretreatment with 80 $\mu\text{mol/L}$ of pan-caspase inhibitor z-VAD-fmk followed by sanguinarine treatment did not alter the expression level of DR5, suggesting no active role for caspases in the regulation of DR5 expression. Taken together, these data clearly indicate that ROS generation is critical for sanguinarine-induced DR5 up-regulation.

Sanguinarine-induced activation of caspase-8 and Bid cleavage in PEL cells. The apoptotic signaling cascade starts with activation of caspase-8 and translocation of Bid that translocates to the mitochondrial membrane, allowing activation of proapoptotic proteins and release of cytochrome *c*. Therefore, we sought to determine whether sanguinarine-induced apoptosis involves the mitochondria. PEL cells were treated with various doses of sanguinarine for 24 h and cell lysates were separated on SDS-PAGE and immunoblotted with an antibody against caspase-8. Figure 3A shows that sanguinarine treatment resulted in a reduction in the intensity of the full-length band of procaspase-8, indicating activation of caspase-8. When the blot was exposed for a longer time period, cleaved product of caspase-8 was detected, suggesting that sanguinarine causes apoptosis via activation of the extrinsic mechanism through the cleavage of caspase-8. Bid is a BH3 proapoptotic protein that can be cleaved directly by caspase-8 during apoptosis (30–32). The cleaved Bid or tBid plays a role in the induction of Bax conformational change and subsequent translocation to mitochondria (31–35). Therefore, we examined the role of Bid cleavage after sanguinarine treatment of PEL cells. As shown in Fig. 3A, treatment with 1 and 2.0 $\mu\text{mol/L}$ sanguinarine resulted in a decreased level of full-length of Bid and the appearance of a tBid band. These results suggest that sanguinarine-induced apoptosis in PEL cells may occur via activation of caspase-8 and Bid.

ine-induced apoptosis in PEL cells may occur via activation of caspase-8 and Bid.

Sanguinarine induces Bax conformational changes and oligomerization. As it is known that tBid plays a role in the activation of Bax and apoptosis, we examined the activation of Bax in response to sanguinarine treatment. BC3 cells were treated with 2.0 $\mu\text{mol/L}$ sanguinarine for 4 and 8 h and lysed with 1.0% Chaps lysis buffer; lysates were immunoprecipitated with Bax 6A7 antibody that recognizes only the conformationally changed Bax protein. The detergent Chaps has been shown to retain the Bax protein in its native conformation. As shown in Supplementary Fig. S3A, conformationally changed Bax was detected 4 and 8 h after treatment with 2.0 $\mu\text{mol/L}$ sanguinarine. To confirm these results, we also incubated BC3 cells following sanguinarine treatment with anti-immunoglobulin G and could not detect the conformationally changed Bax. The oligomerization of Bax has previously been shown to occur only in apoptotic cells, possibly playing a role in mediating cytochrome *c* release (36). To test this possibility, we sought to determine whether sanguinarine treatment of PEL cells could trigger Bax oligomerization. BC3 cells were treated with sanguinarine for different time periods and, after harvesting, cells were subsequently exposed to the membrane-permeable cross-linking agent disuccinimidyl suberate. The cross-linked proteins were separated on SDS-PAGE and immunoblotted for the analysis of Bax oligomerization. As shown in Fig. 3B, an immunoreactive band of 42 to 46 kDa, previously reported as a Bax homodimer (37), could be detected from the sanguinarine-treated BC3 cell lysate. In addition, 88- to 90-kDa bands, which could be Bax tetramer or oligomer, were also detectable. These bands were

Figure 4. Activation of caspase-9 and caspase-3 and cleavage of PARP induced by sanguinarine treatment in PEL cells. **A**, caspase-9 and caspase-3 activation and PARP cleavage following sanguinarine treatment. BC1, BC3, BCBL1, and HBL6 cells were treated with and without 1 and 2.0 $\mu\text{mol/L}$ sanguinarine for 24 h. Cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against procaspase-9, procaspase-3, cleaved caspase-3, PARP, and β -actin. Effect of z-VAD-fmk (**B**) or NAC (**C**) on sanguinarine-induced activation of caspase-3 and cleavage of PARP. BC1 and BC3 cells were pretreated with either 80 $\mu\text{mol/L}$ z-VAD-fmk or 10 mmol/L NAC for 2 h and subsequently treated with 2.0 $\mu\text{mol/L}$ sanguinarine for 24 h. After cell counts using trypan blue exclusion dye, cells were lysed and equal amounts of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against procaspase-3, PARP, and β -actin. Live and dead cells were scored using trypan blue exclusion dye. *Columns*, mean of three independent experiments; *bars*, SD.



not detected in DMSO-only cross-linked samples, suggesting specificity of the experiment. In addition, a nonspecific band above 32 kDa was detected in all the samples. These results suggest that sanguinarine treatment of PEL cells involves Bax dimerization/oligomerization, which plays an important role in sanguinarine-induced apoptosis.

Next, we attempted to investigate the mechanism of Bax activation initiated by sanguinarine. BC1 and BC3 cells were pretreated with either 10 mmol/L NAC or 80 μ mol/L z-VAD-fmk for 1 h followed by treatment with and without 2 μ mol/L sanguinarine for 8 h, followed by lysis in 1% Chaps lysis buffer and immunoprecipitation with Bax 6A7 monoclonal antibody. As shown in Fig. 3C, expression of conformationally changed Bax was only detected in sanguinarine alone treated samples. Interestingly, NAC and z-VAD-fmk pretreatment was completely able to block the expression of Bax in the presence of sanguinarine. These data strongly suggest that Bax translocation in sanguinarine-induced apoptosis is downstream of caspase-8 activation. These data also confirm that sanguinarine treatment of PEL cell lines activates the extrinsic apoptotic pathway via release of ROS, leading to the up-regulation of DR5 and the recruitment and cleavage of caspase-8 and Bid.

Sanguinarine induces loss of mitochondrial membrane potential and subsequently release of cytochrome *c* to cytosol.

We further tested the effect of sanguinarine on mitochondrial membrane potential using JC1 dye. BC1, BC3, BCBL1, and HBL6 cells were treated with 2 μ mol/L sanguinarine for 24 h. As shown in Fig. 3D, sanguinarine treatment of these cells resulted in loss of mitochondrial membrane potential as measured by JC1 staining, with green fluorescence depicting apoptotic cells. Cytochrome *c* release from mitochondria in BC1 and BC3 cells treated with sanguinarine was examined by Western blot analysis. Cells were treated in the presence and absence of 2.0 μ mol/L sanguinarine for 24 h. Cytosolic-specific, mitochondrion-free as well as mitochondrial lysates was prepared as described in Materials and Methods. Cell fractionation results showed that cytochrome *c* translocated from mitochondria to cytosol following sanguinarine treatment. As shown in Supplementary Fig. S3B, the protein levels of cytochrome *c* increased in the cytosolic fractions and, concomitantly, decreased in mitochondria-enriched heavy membrane fractions of PEL cells after sanguinarine treatment. These results suggest that sanguinarine treatment of PEL cells causes apoptosis via the release of cytochrome *c* from the mitochondria. These results further support our notion that sanguinarine-induced apoptosis in PEL cells involves signaling at the mitochondrial level.

Sanguinarine-induced signaling results in caspase-9 and caspase-3 activation and PARP cleavage in PEL cells. Because caspases are important mediators of apoptosis in response to various apoptotic stimuli (33), we investigated whether sanguinarine treatment also caused their activation. BC1, BC3, BCBL1, and HBL6 cells were treated with 1.0 and 2 μ mol/L sanguinarine for 24 h and immunoblotted with anti-caspase-3, anti-cleaved caspase-3, and anti-PARP antibodies. As shown in Fig. 4A, sanguinarine treatment of PEL cells induced activation of caspase-9 and caspase-3 cleavage in all cell lines. PARP, a downstream target of caspase-3, was also cleaved in all the cell lines, a hallmark of cells undergoing apoptosis. Furthermore, pretreatment of PEL cells with 80 μ mol/L z-VAD-fmk (Fig. 4B), a universal inhibitor of caspases, or 10 mmol/L NAC (Fig. 4C) abrogated caspase-3 and PARP activation and prevented cell death

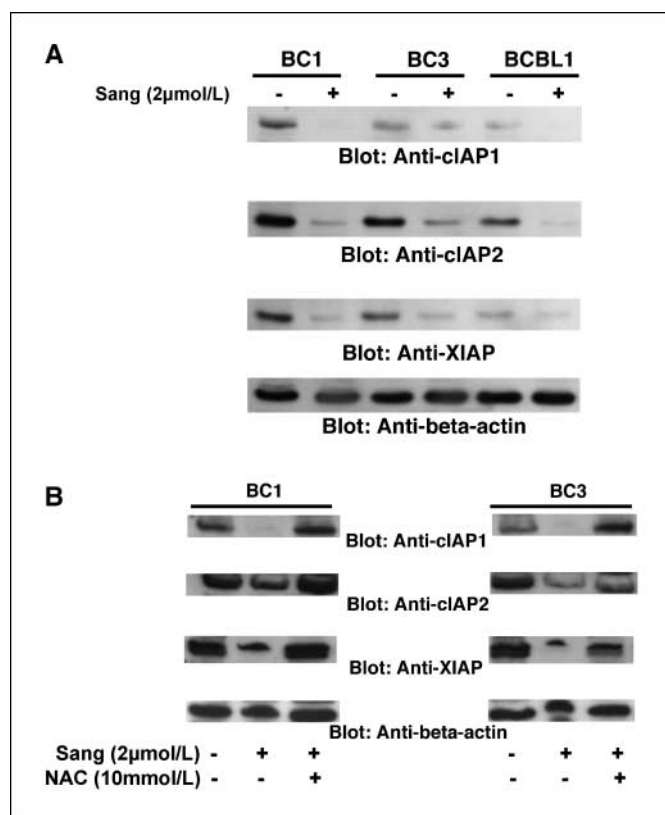


Figure 5. Sanguinarine-induced down-regulation of clAP1, clAP2, and XIAP expression. **A**, BC1, BC3, and BCBL1 cells were treated with and without 2.0 μ mol/L sanguinarine for 24 h. Cells were lysed and equal amounts of proteins were separated on SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against clAP1, clAP2, XIAP, and β -actin. **B**, NAC inhibits down-regulation of IAPs induced by sanguinarine treatment. BC1 and BC3 cells were pretreated with 10 mmol/L NAC for 2 h followed by treatment with 2 μ mol/L sanguinarine for 24 h. Cells were lysed and equal amounts of proteins were separated on SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antibodies against clAP1, clAP2, XIAP, and β -actin.

induced by sanguinarine, clearly indicating that release of ROS as well as caspases play a critical role in sanguinarine-induced apoptosis in PEL cells.

Modulation of inhibitor of apoptosis protein family in sanguinarine-induced apoptosis in PEL cells. We also examined whether sanguinarine induces cell death by modulating the expression of inhibitor of apoptosis protein (IAP) family members, which ultimately determine the cell response to apoptotic stimuli. BC1, BC3, and BCBL1 cells were treated with 2.0 μ mol/L sanguinarine for 24 h and expression of clAP1, clAP2, and XIAP was determined by Western blotting. As shown in Fig. 5A, sanguinarine treatment caused down-regulation of clAP1 and clAP2 as well as XIAP protein, whereas pretreatment with 10 mmol/L NAC restored the expression of the IAPs. These results indicate that IAP proteins may also be involved in sanguinarine-induced apoptosis.

Based on our findings in this article, as well as the studies of other investigators, we propose a model (Fig. 6) in which we show the relationship between the release of ROS and up-regulation of DR5, activation of the caspase cascade, and involvement of the mitochondrion, ultimately leading to DNA fragmentation and apoptosis following sanguinarine treatment in PEL cell lines.

Discussion

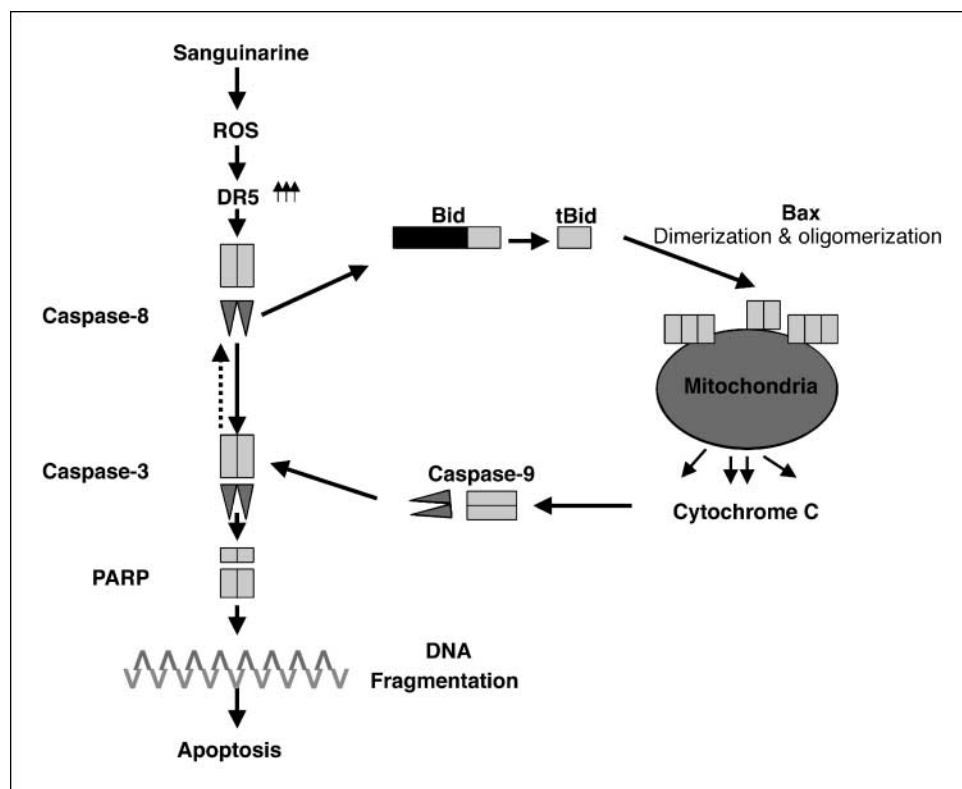
In the present study, we investigated the direct effects of sanguinarine on PEL cells *in vitro*. Our results show that sanguinarine exhibits potent, dose-dependent proapoptotic effects on PEL cells and provide evidence that such apoptosis occurs via generation of ROS, leading to up-regulation of DR5. We found that exposure of PEL cells to sanguinarine induces rapid and transient ROS generation that was evident as early as 1 h after treatment and diminished within 8 h (Fig. 2A). The release of ROS by sanguinarine was blocked by pretreatment of PEL cells with NAC, suggesting a role of superoxide radical in the process. The demonstration of up-regulation of DR5 by sanguinarine is of high interest because DR5 plays a critical role in the recruitment of caspase-8 and the activation of the extrinsic apoptotic pathway (38). This is the first study showing that sanguinarine treatment causes up-regulation of DR5 protein expression by a positive regulatory effect at the transcriptional level, providing evidence for the generation of the proapoptotic effects of this agent in PEL cells. Interestingly, a recent study by Singh et al. (39) showed that sulforaphane-induced apoptosis is also mediated by ROS via up-regulation of DR5 (29), whereas pretreatment with NAC inhibits such sulforaphane-induced apoptosis. Thus, both sulforaphane and sanguinarine seem to use a common effector mechanism for the induction of apoptosis in malignant cells involving ROS-mediated up-regulation of DR5.

It is well documented that the death receptor-mediated apoptotic signaling pathway requires recruitment of Fas-associated death domain and caspase-8, which results in caspase-8 activation and subsequent activation of its downstream caspase cascades and apoptosis (40, 41). In addition, for efficient apoptosis, the activation of intrinsic apoptotic pathway is critical. Our results show that

sanguinarine induces activation and cleavage of caspase-8, resulting in the truncation of Bid, a BH3-only proapoptotic protein (31, 32). The resulting tBid plays a role in the generation of Bax conformational changes and subsequent translocation to mitochondria (33), leading to the formation of mitochondrial pores. Based on our experimental results, Bax conformational changes and its translocation to the mitochondria are dependent on the activation of the extrinsic apoptotic pathway and truncation of Bid. We also clearly establish that pretreatment of PEL cells with universal caspase inhibitor z-VAD-fmk completely blocks the sanguinarine-dependent conformational changes of the Bax protein in PEL cell lines. If the intrinsic apoptotic pathway alone was being activated, z-VAD-fmk treatment would not have been able to block Bax conformational change, suggesting caspase dependency. Pretreatment of NAC also blocked Bax conformational changes following sanguinarine treatment in both cell lines. These results clearly indicate that sanguinarine-induced apoptosis requires not only release of ROS but also up-regulation of DR5 and activation of caspase-8 to function efficiently. It should also be pointed out that previous studies have shown that sanguinarine is a potent inhibitor of NF- κ B activation and blocks the phosphorylation and degradation of I κ B α (13). It is therefore possible that such effects of sanguinarine on NF- κ B activation also participate in the generation of proapoptotic signals and/or cooperate with ROS-mediated up-regulation of DR5 to induce cell death, but this remains to be directly examined in future studies.

There are reports indicating that overexpression of Bax enhances cytochrome *c* release from mitochondria to the cytosol (42), whereas direct addition of recombinant Bax protein to isolated mitochondria has been previously shown to induce cytochrome *c* release (43). Our results establish that sanguinarine induces the

Figure 6. Schematic diagram depicting sanguinarine-induced apoptosis in PELs.



loss of mitochondrial potential in most of the PEL cells. Loss of mitochondrial membrane potential is the root mechanism responsible for cytochrome *c* release in response to different cytotoxic stimuli. More recently, ample evidence suggests (44–46) that some apoptogenic agents induce Bax translocation to the mitochondrial membrane, followed by cytochrome *c* release. In mammalian cells, the release of cytochrome *c* from the mitochondria has been proposed as a critical event for cells to initiate the apoptotic cascade. In cytosol, cytochrome *c* plays a key role in the formation of apoptosome complex by activating the binding of procaspase-9 and Apaf-1 in the presence of ATP (47). The formation of the apoptosome then causes cleavage of caspase-9, which propagates the death signal by activating caspase-3 and causing cleavage of PARP. Activation and cleavage of PARP is the hallmark of apoptosis that in turn causes DNA fragmentation and cell death.

Expression of IAPs correlates with apoptosis resistance in transformed cell types and in a variety of human tumors including lymphoid malignant cells (48, 49). IAPs have been shown to suppress caspase activity and protect cells from apoptosis induced by a variety of agents. Our results show that all the PEL cell lines express IAPs, including cIAP1, cIAP2, and XIAP, and that sanguinarine treatment decreases the expression level of these molecules, indicating that down-regulation of IAPs may be involved

in the activation of caspase-9 and caspase-3 in sanguinarine-induced apoptosis.

PEL is an incurable, aggressive B-cell malignancy, and most of the patients respond poorly to traditional chemotherapy and develop chemoresistance. Several agents have been tested in the search for a more effective treatment of PEL. Our study provides the first evidence that sanguinarine is capable of inducing apoptosis of PEL and raises the possibility that this novel agent may prove to be of future value in the treatment of PEL. These data also raise the possibility that sanguinarine may also have activity against other malignant phenotypes via its ability to activate the extrinsic apoptotic pathway. These findings have important clinical-translational implications because they raise the possibility that sanguinarine may be useful against PEL *in vivo*. Future investigations aimed at determining the efficacy of sanguinarine and possibly other inhibitors of the mitochondrial-induced pathway in PEL are warranted and may lead to the future development of new effective therapies for the treatment of these uniformly fatal lymphomas.

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