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The alkaloid sanguinarine is effective against multidrug resistance in human cervical cells *via* bimodal cell death

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Abstract

Sanguinarine, a benzophenanthrine alkaloid, is potentially antineoplastic through induction of cell death pathways. The development of multidrug resistance (MDR) is a major obstacle to the success of chemotherapeutic agents. The aim of this study was to investigate whether sanguinarine is effective against uterine cervical MDR and, if so, by which mechanism. The effects of treatment with sanguinarine on human papillomavirus (HPV) type 16-immortalized endocervical cells and their MDR counterpart cells were compared. Trypan blue exclusion assays and clonogenic survival assays demonstrated that MDR human cervical cells are as sensitive as their drug-sensitive parental cells to death induced by sanguinarine. Upon treatment of both types of cells with sanguinarine, two distinct concentration-dependent modes of cell death were observed. Treatment with 2.12 or 4.24 μM sanguinarine induced death in most cells that was characterized as apoptosis using the criteria of cell surface blebbing, as determined by light and scanning electron microscopy, and proteolytic activation of caspase-3 and cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase (PARP), as detected by Western blot analysis. However, 8.48 and 16.96 μM sanguinarine caused a second mode of cell death, oncosis, distinguished by cell surface blistering, and neither caspase-3 activation nor PARP cleavage. This study provides the first evidence that sanguinarine is effective against MDR in cervical cells *via* bimodal cell death, which displays alternative mechanisms involving different morphologies and caspase-3 activation status.

Keywords: Alkaloid sanguinarine; Multidrug-resistant cervical cells; Apoptosis; Oncosis; Caspase-3

1. Introduction

Sanguinarine (Scheme 1) is derived from the plant *Sanguinaria canadensis* [1]. Its principal pharmacologic use to date is in dental products based on its antibacterial, antifungal, and anti-inflammatory activities, which reduce gingival inflammation and supragingival plaque formation [2–4]. Sanguinarine also has been reported to have antiviral and tumor-targeting activity [5–7].

Molecular biological studies indicate that sanguinarine has multiple cellular targets [8]. For example, it can interact with and intercalate DNA [9,10], inhibit microtubule assembly [11], affect membrane permeability [12,13], and inhibit a wide variety of enzymes, including Na^+/K^+ ATPase [14]. Most interestingly, it also is a potent inhibitor of protein kinases [15] and NF-κB [16], which are involved in signal transduction pathways leading to cell proliferation and/or cell death [7].

Cell death is important for normal homeostasis, cell proliferation, and differentiation. The importance of cell death is demonstrated by the observation that dysregulation of cell death can lead to cancer, developmental abnormalities, and autoimmune disorders [17–19]. Cells undergoing PCD (or apoptosis) are characterized by morphologic changes, including cellular shrinkage, blebbing, and nuclear DNA condensation with or without fragmentation [20–24]. However, it is stated that apoptosis is rarely observed *in vivo* and may not be the sole mechanism of cell death [25]. The discovery of intact novel forms of cell death pathways

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Abbreviations: PCD, programmed cell death; CDDP, *cis*-diamminedichloroplatinum (II), cisplatin; MDR, multidrug resistance (or resistant); HPV, human papillomavirus; CSC, cigarette smoke condensate; PARP, poly(ADP-ribose) polymerase; BCD, blister cell death/oncosis; mAb, monoclonal antibody; FBS, fetal bovine serum; P-gp, P-glycoprotein.

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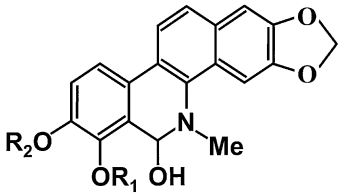

Scheme 1. Structure of sanguinarine.

induced by potential anticancer agents may have an important bearing in overcoming chemoresistance.

Of all neoplasms found in females worldwide, cervical cancer has the third highest incidence and is fourth on the list of the leading causes of death by cancer [26,27]. The available drugs most commonly used for treating cervical malignancies are impeded by frequent progression to chemotherapy resistance. Sanguinarine may be effective against MDR, since the related *Sanguinaria canadensis*-derived alkaloid, chelerythrine, has been shown to be cytotoxic to cancer cells and MDR cells [28]. In this study, we used our recently established *in vitro* cervical cancer model system for MDR [29] to investigate whether sanguinarine is effective against MDR in human cervical cells, and to understand the cellular and molecular mechanisms by which it may induce cell death.

2. Materials and methods

2.1. Cell culture, cell viability assays, and clonogenic survival assays

Most cell culture protocols, the HPV type 16-immortalized human endocervical cell line (HEN-16-2), the CSC-transformed HEN-16-2 cell line (HEN-16-2T), and the MDR HEN-16-2 cell line (HEN-16-2/CDDP) have been described previously [29–31]. Cells were cultured in keratinocyte growth medium (KGM). HeLa cervical carcinoma, CEM-VLB leukemia, CEM-T4 leukemia, K562 erythroleukemia, and JM1 pre-B cell lymphoblastic cells were obtained from the American Type Culture Collection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). CEM-VLB leukemia, CEM-T4 leukemia, K562 erythroleukemia, and JM1 pre-B cell lymphoblastic cells were cultured in RPMI-1640 medium supplemented with 10% FBS. All experiments were performed in triplicate.

Sanguinarine chloride (Sigma) was dissolved in H_2O as a 2.72 M stock solution, aliquots of which were serially diluted with KGM and used when needed to prepare fresh working solutions.

To examine the effect of sanguinarine on cell viability, 5×10^4 cells/well were seeded in 12-well plates, incubated for 4 or 48 hr, treated with various concentrations of sanguinarine, and then assayed for trypan blue exclusion and propidium iodide exclusion under light microscopy, as described previously [32,33]. A hemocytometer was used to count the cells.

Clonogenic survival assays were performed to examine the combined survival and proliferative potential of sanguinarine-treated cervical cells, as previously described [29,34]. Briefly, 10^3 cells were seeded into 60-mm plates, incubated with 0–16.96 μM sanguinarine for 24 hr, washed twice with phosphate-buffered saline, and incubated without sanguinarine for 10–14 days. The cells were stained with 2% (w/v) crystal violet in methanol, and colonies of 50 or more cells were counted using a hemocytometer.

2.2. Cell morphology analysis

To examine the effect of sanguinarine on cell morphology under light microscopy, 5×10^3 cells/chamber were seeded in 8-chamber slides (Nalge Nunc International), incubated for 24 hr, and treated with 0–16.96 μM sanguinarine for 4 hr.

To examine the cell ultrastructural effect of sanguinarine under scanning electron microscopy (SEM), 5×10^4 cells/well were seeded in 12-well plates containing acid-cleaned coverslips (Lux Scientific Corp.), incubated for 24 hr for attachment to coverslips, treated with 0–16.96 μM sanguinarine for 4 hr, fixed in Karnovsky fixative containing 2.5% (v/v) glutaraldehyde (J.B. EM Services) in 0.1 M sodium cacodylate buffer, and then dehydrated in a 25, 50, 75, and 100% (v/v) ethanol series followed by Freon 113 substitution. All samples were dried simultaneously, sputter-coated with gold, and examined under a Hitachi S-570 scanning electron microscope, as previously described [35].

2.3. Western blot analysis

Western blot analysis of the effect of sanguinarine on caspase-3 activation and PARP cleavage was performed as described previously [36]. Briefly, 10 μg of protein was resolved by 10% (w/v) SDS–PAGE and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane under semidry conditions. Immunodetection was performed using the ECL system (Amersham Pharmacia Biotech). Procaspase-3 and caspase-3 were probed using anti-caspase-3 monoclonal antibody (mAb) (Santa Cruz Biotechnology). Full-length and cleaved fragments of PARP were probed using anti-PARP mAb (PharMingen).

3. Results

3.1. Evasion of MDR of human cervical HEN-16-2/CDDP cells by sanguinarine

We examined the chemotherapeutic potential of sanguinarine for MDR cervical cancer cells in a human cervical *in*

Table 1
Bimodal cell death characteristics induced by sanguinarine and Ukrain in cervical cells and leukemia cells

Cell line	Sanguinarine cell death/caspase-3 activation/PARP cleavage		Ukrain cell death/caspase-3 activation/PARP cleavage	
	(4.24 μM)	(16.96 μM)	(4.24 μM)	(16.96 μM)
HEN-16-2[a]	PCD/+/+	BCD/−/−	PCD/+/+	BCD/−/−
HEN-16-2/CDDP[b]	PCD/+/+	BCD/−/−	PCD/+/+	BCD/−/−
HEN-16-2T[c]	PCD/+/+	BCD/−/−	PCD/+/+	BCD/−/−
HeLa[d]	PCD/+/+	BCD/−/−	PCD/+/+	BCD/−/−
CEM-T4[e]	PCD/NA/NA	BCD/NA/NA	NA/NA/NA	NA/NA/NA
CEM-VLB[f]	PCD/NA/NA	BCD/NA/NA	NA/NA/NA	NA/NA/NA
JM1[g]	PCD/NA/NA	BCD/NA/NA	PCD/NA/NA	BCD/NA/NA
K562[h]	PCD/NA/NA	BCD/NA/NA	PCD/NA/NA	BCD/NA/NA

Cells were treated with a dilution series of sanguinarine or Ukrain for 4 hr and morphologic changes were observed by microscopy. For examining caspase-3 activation and PARP cleavage, cell lysates were subjected to Western blotting using anti-caspase-3 mAb and anti-PARP mAb. PCD programmed cell death or apoptosis; BCD, blister cell death or oncosis; NA, not available.

[a] Human endocervical (HEN) immortalized with HPV16.
[b] HEN-16-2 transformed by cisplatin and MDR.
[c] HEN-16-2 transformed by cigarette smoke condensate.
[d] Endocervical carcinoma.
[e] Leukemia P-gp-negative.
[f] Leukemia P-gp-positive.
[g] Pre-B cell lymphoblastic, Bc1-2 high level.
[h] Erythroleukemia, Bc1-2 low level.

vitro system, which is composed of MDR HEN-16-2/CDDP cells and their drug-sensitive parental HEN-16-2 cells [29]. Cell viability, measured by the trypan blue exclusion assay, was similar in both types of cells treated with 0, 0.13, 0.26, 0.53, 1.06, 2.12, and 4.24 μM sanguinarine for 4 or 48 hr (Table 1; Fig. 1). The propidium iodide exclusion assay also showed no significant difference in cell viability between MDR HEN-16-2/CDDP cells and their parental HEN-16-2 cells after treatment with these concentrations of sanguinarine for 4 or 48 hr (data not shown). Treating the MDR HEN-16-2/CDDP and HEN-16-2 cells with 0–1.06 μM sanguinarine produced no significant increase in cell viability; however, 2.12 μM (Fig. 2) and 4.24 μM sanguinarine treatment caused the death of most of the cells (data not

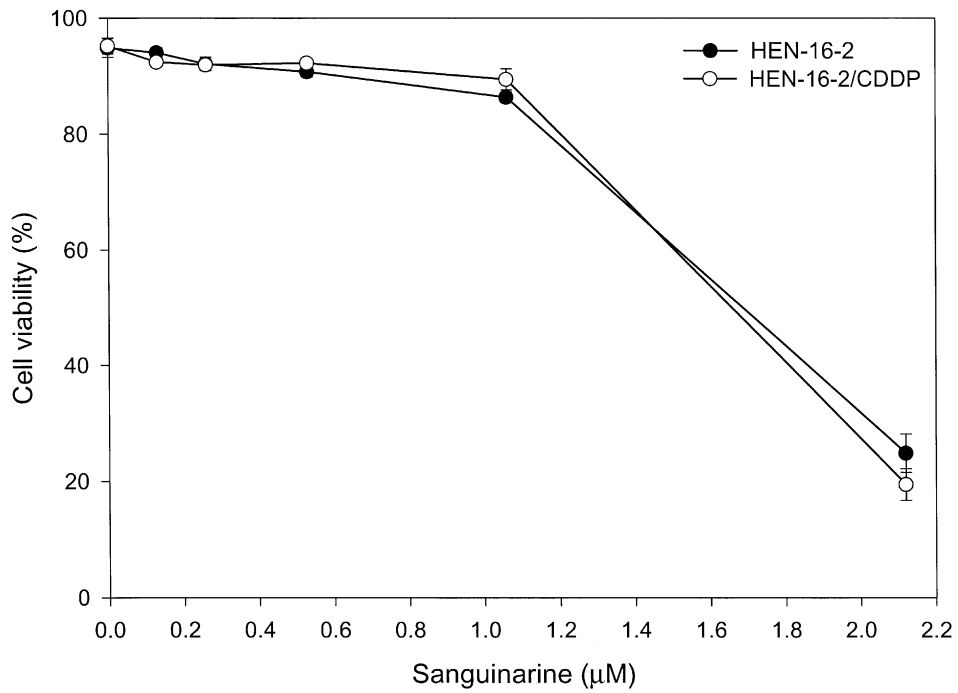

Fig. 1. Concentration-dependent effect of sanguinarine on HEN-16-2 and HEN-16-2/CDDP cell viability. Cells were incubated with 0, 0.13, 0.26, 0.53, 1.06, and 2.12 μM sanguinarine for 48 hr. Cell viability represents the percentage of treated compared with untreated cells that excluded trypan blue dye. The results represent the means ± SD of three independent experiments.

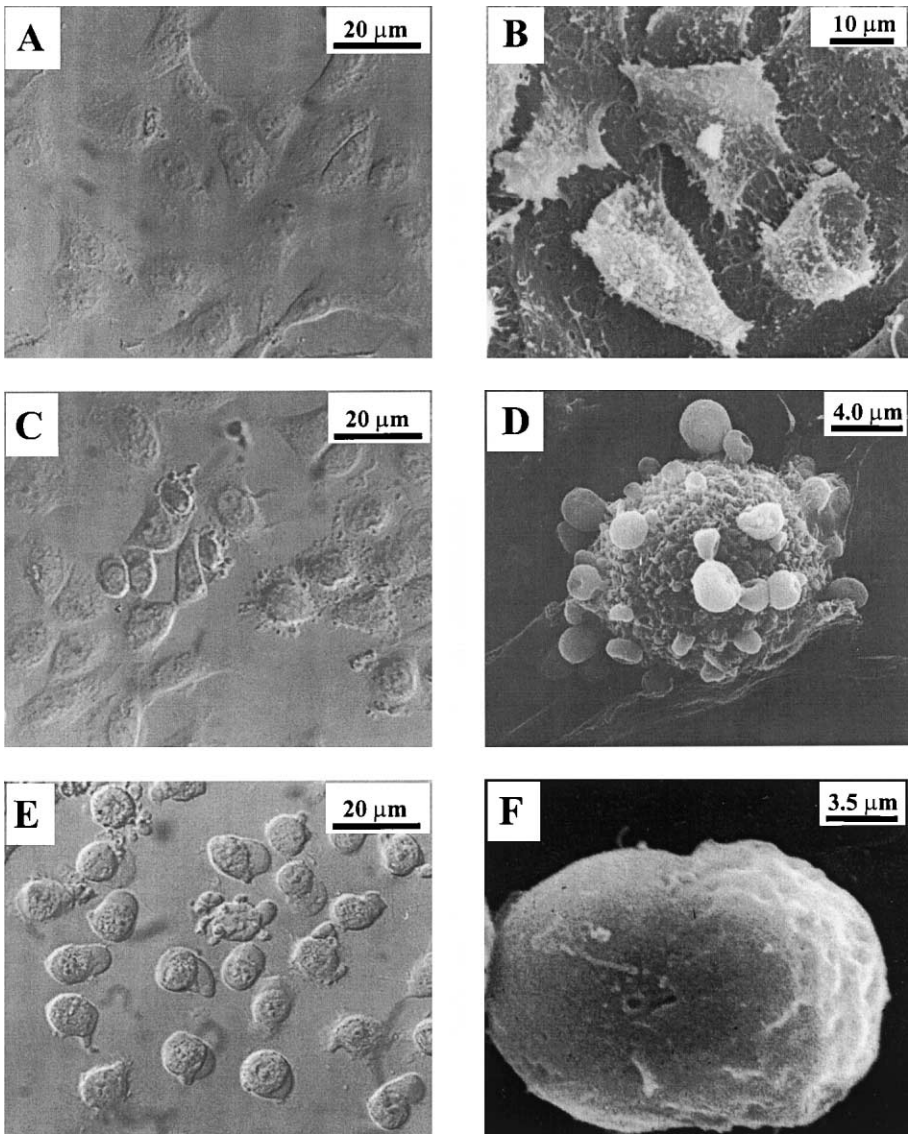

Fig. 2. Concentration-dependent bimodal effect of sanguinarine on the morphology of MDR HEN-16-2/CDDP cells. The panels represent untreated control cells under light microscopy (A) and scanning electron microscopy (SEM) (B); 4 hr, 2.12 μM sanguinarine-treated cells under light microscopy (C) and SEM (D); and 4 hr, 8.48 μM sanguinarine-treated cells under light microscopy (E) and SEM (F).

shown). Treating HEN-16-2/CDDP and HEN-16-2 cells with 8.48 and 16.96 μM sanguinarine resulted in 100% cell death within 48 hr (data not shown). Clonogenic survival assays (also called colony-forming assays) revealed no significant difference in clonogenic survival between HEN-16-2/CDDP and HEN-16-2 cells (data not shown), showing an equally effective potential of sanguinarine in this assay to kill cells and inhibit their growth.

3.2. Induction of concentration-dependent apoptosis and oncosis in MDR HEN-16-2/CDDP and drug-sensitive HEN-16-2 cervical cells by sanguinarine

To evaluate the concentration-dependent effect of sanguinarine on cell death morphology, cells were treated with different concentrations of sanguinarine for 4 hr and observed microscopically. Both cell lines treated with 0–1.06 μM sanguinarine were observed to have normal cell morphology, similar to the morphology of untreated MDR cells (Fig. 2A and B; data not shown). Cell plasma membrane blebbing, a characteristic of PCD or apoptosis, was observed in cells treated for 4 hr with 2.12 μM sanguinarine (Fig. 2C and D) and 4.24 μM sanguinarine (Table 1). However, most cells exhibited single and rare double cell surface blisters after sanguinarine treatment for 4 hr with 8.48 μM (Fig. 2E and F) and 16.96 μM (Table 1). Similar bimodal apoptosis and BCD (or oncosis) were observed at the same respective sanguinarine concentrations and time in CSC-transformed HEN-16-2T, HeLa cells, MDR CEM-VLB leukemia, drug-sensitive CEM-T4 leukemia, K562 erythroleukemia, and JM1 pre-B cell lymphoblastic cells (Table 1).

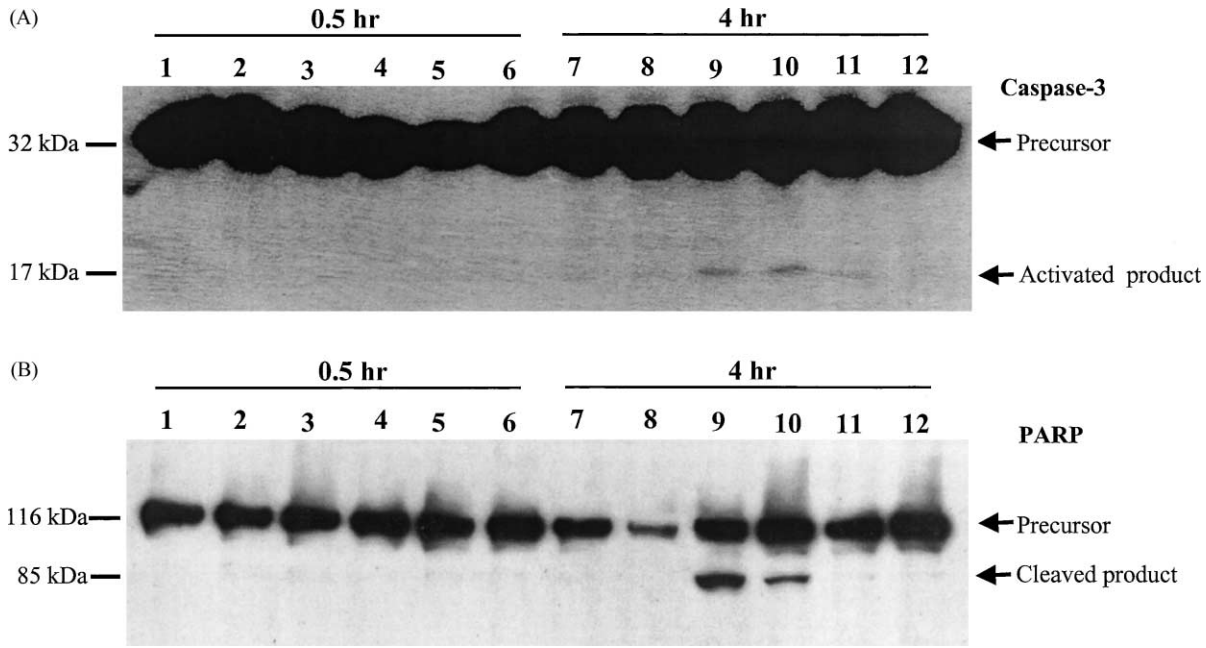

Fig. 3. Concentration- and time-dependent caspase-3 activation and PARP cleavage in sanguinarine-treated MDR HEN-16-2/CDDP cells. Western blot analysis is shown for caspase-3 (A) and PARP (B) using 10 μg protein/lane from cells treated for 0.5 or 4 hr with sanguinarine at 0 μM (lanes 1 and 7), 1.06 μM (lanes 2 and 8), 2.12 μM (lanes 3 and 9), 4.24 μM (lanes 4 and 10), 8.48 μM (lanes 5 and 11), and 16.96 μM (lanes 6 and 12).

3.3. Induction of caspase-3 activation in apoptosis but not oncotic cell death in both MDR HEN-16-2/CDDP and drug-sensitive HEN-16-2 cells by sanguinarine

To study the molecular mechanism by which sanguinarine induces cell morphologic changes, sanguinarine-treated cells were examined for the proteolytic activation of caspase-3, a downstream effector in apoptosis pathways. Sanguinarine induced time- and concentration-dependent activation of caspase-3 in MDR HEN-16-2/CDDP cells, as observed by Western blotting (Fig. 3A). Treatment for 0.5 hr with 0–16.96 μM sanguinarine did not cause detectable proteolytic activation of caspase-3; a 4-hr treatment with 2.12 and 4.24 μM sanguinarine, but no other concentration from 0 to 16.96 μM, induced cleavage of procaspase-3 to the activated 17 kDa caspase-3 fragment. These results are consistent with a previous demonstration that apoptosis requires caspase-3 activation [37].

PARP is a critical cellular substrate for proteolysis by activated caspase-3 [38]. Therefore, we also studied whether the activation of caspase-3 by sanguinarine may lead to increased cleavage of PARP. In a time- and concentration-dependent analysis of PARP cleavage that parallels the one for caspase-3 activation, cleaved PARP fragments were found at only 4 hr in 2.12 and 4.24 μM sanguinarine-treated MDR cells (Fig. 3B) and drug-sensitive cells (Table 1). For several other cervical cell lines, including HEN-16-2T, similar sanguinarine concentration- and time-dependent caspase-3 and PARP results were observed indicating apoptosis; both results were absent in BCD/oncosis (Table 1). Overall, these results suggest that sanguinarine may be equally effective against MDR and drug-sensitive human cervical cells, and act despite MDR through bimodal apoptosis and BCD/oncosis pathways having mechanisms that involve differential morphologies and caspase-3 activation status.

4. Discussion

We established the *in vitro* MDR cervical cell system used in this report by treating HPV16-immortalized human endocervical HEN-16-2 cells with cisplatin [29]. Cell viability was significantly higher in the MDR HEN-16-2/CDDP cells than in the parental cells after treatment with cisplatin, actinomycin D, doxorubicin, etoposide, paclitaxel, 5-fluorouracil, staurosporine, heat shock, or UV radiation [29,39]. However, this study found no significant difference in the effect of sanguinarine on cell viability or clonogenic survival between the MDR HEN-16-2/CDDP cells and their parental drug-sensitive HEN-16-2 cells. Similarly, there was no significant difference in cell death induced by sanguinarine between CEM-VLB leukemia cells in which P-glycoprotein (P-gp) mediates MDR and their wild-type drug-sensitive counterpart CEM-T4 cells, which are P-gp-negative (Table 1). Importantly, sanguinarine has been found to be selectively less toxic to normal cells [7]. Thus, sanguinarine may be regarded as a potential therapeutic agent even for MDR of certain types of transformed cells, which are represented by HEN-16-2/CDDP cells [29].

Proteolytic activation of effector caspases, especially caspase-3, is one of the key events in apoptosis [38,40]. The results presented here show that sanguinarine induced

both apoptosis and BCD/oncosis in cervical MDR HEN-16-2/CDDP cells and drug-sensitive HEN-16-2 cells. Lower concentrations of sanguinarine induced apoptosis, displayed by cell surface blebbing (Fig. 2C and D) and caspase-3 activation, the latter confirmed by induction of proteolytic cleavage of the caspase-3 substrate PARP (Fig. 3). Higher concentrations of sanguinarine induced cell death characterized by blistering, an oncotic late cell death observed previously [41], and the absence of caspase-3 activation (Figs. 2E and F and 3).

Bimodal cell death was also found to be induced by sanguinarine in K562 erythroleukemia cells [42], JM1 pre-B lymphoblastic cells [42], MDR CEM-VLB leukemia, and their wild-type counterpart CEM-T4 cells (Table 1). Ukrain, an alkaloid derived from the same plant family as sanguinarine, has been reported to also induce apoptotic and blister forms during K562 leukemia cell death [21], and in MDR HEN-16-2/CDDP and drug-sensitive HEN-16-2 cells (Table 1). Electronic transmission microscopy of K562 cells showed that sanguinarine-induced apoptosis produced classic morphologic changes, including the formation of apoptotic bodies containing organelles and chromatin condensation [42], whereas sanguinarine-induced oncosis produced blisters that were devoid of organelles and displayed patchy chromatin condensation [42].

BCD/oncosis is a form of cell death that is distinct from apoptosis [43], whereas necrosis refers to the intracellular degradative reactions occurring after cell death by any mechanism, including apoptosis [44]. Oncosis has been documented in many studies [45–50]. The molecular and biochemical mechanisms underlying oncosis are still unclear. Oncosis was believed to result from a failure of plasma membrane ionic pumps and decreased levels of cellular ATP [51]. However, cell surface proteins, including phospholipase A_2 and Porimin, have been documented to be involved in the process of cell membrane injury and membrane structural changes [49,50,52]. Sanguinarine-induced cell death pathways may be initiated that, if not blocked, lead to caspase-3 activation, cleavage of PARP and other caspase-3 substrates, and consequent apoptotic cell death. If these pathways are blocked, then other downstream or parallel steps of a pathway may lead to caspase-independent oncosis [53–58]. Future studies on the sanguinarine-activated cellular factors involved in cell death pathways may provide a greater understanding of the bimodal cell death pathways.

In summary, the data in this report indicate that sanguinarine induces concentration-dependent apoptosis with caspase-3 activation and BCD/oncosis without caspase-3 activation. The ability of this drug to induce bimodal cell death modes at comparable efficiencies in MDR and drug-sensitive human cervical and leukemia cells indicates that sanguinarine was effective against MDR in this *in vitro* system, and that there may be two sanguinarine-induced cell death mechanisms.

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