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Activation of Prodeath Bcl-2 Family Proteins and Mitochondrial Apoptosis Pathway by Sanguinarine in Immortalized Human HaCaT Keratinocytes¹

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ABSTRACT

Sanguinarine, derived from the root of *Sanguinaria canadensis* and other poppy *fumaria* species, possesses strong antimicrobial, anti-inflammatory, and antioxidant properties. We earlier showed that sanguinarine kills human epidermoid carcinoma A431 cells via an induction of apoptosis [N. Ahmad *et al.*, *Clin. Cancer Res.*, 6: 1524–1528, 2000]. In this study, using immortalized human keratinocytes (HaCaT cells), we provide information about mechanism of the antiproliferative effect of sanguinarine. Sanguinarine [0.1 (M-2 (M))] treatment to HaCaT cells was found to inhibit in a dose-dependent manner the cell proliferation and induce apoptosis, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and ELISA, respectively. Sanguinarine treatment also resulted in a significant cleavage of poly(ADP-ribose) polymerase in HaCaT cells. Because mitochondrial pathway is critical for the regulation of apoptosis, we studied the involvement and regulation of mitochondrial events in sanguinarine-mediated apoptosis of HaCaT cells. As shown by the immunoblot analysis, our data clearly demonstrated that sanguinarine treatment to HaCaT cells resulted in a dose-dependent (a) increase in the level of Bax with a concomitant decrease in Bcl-2 levels and (b) increase in Bax/Bcl-2 ratio. Sanguinarine also resulted in significant increases in the proapoptotic members of Bcl-2 family proteins, *i.e.*, Bak and Bid. This was accompanied by increase in (a) protein expression of cytochrome *c* and apoptotic protease-activating factor-1 and (b) activity and protein expression of caspase-3, caspase-7, caspase-8, and caspase-9. Taken together, our

data showed the involvement of mitochondrial pathway and Bcl-2 family proteins during sanguinarine-mediated apoptosis of immortalized keratinocytes. We suggest that sanguinarine could be developed as a drug for the management of hyperproliferative skin disorders, including skin cancer.

INTRODUCTION

More than 1 million new cases of nonmelanoma skin cancers are diagnosed annually in the United States (1). Therefore, there is an urgent need to develop mechanism-based approaches for the management of skin cancer. In the recent past, agents obtained from herbs and plants have gained considerable attention for the prevention and/or treatment of certain cancer types, including skin cancer. Studies of naturally occurring plant-based agents often provide opportunities to unravel important biology, which may provide leads for specific cellular targets and strategies for the management of cancer and other diseases (2). Sanguinarine (13-methyl (1,3)benzodioxolo [5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium), derived from the root of *Sanguinaria canadensis* and other poppy-*fumaria* species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine and has been shown to possess antimicrobial, antioxidant, and anti-inflammatory properties (3). It is widely used in toothpaste and mouth washes (4–6). There is a suggestion for the antitumor properties of this alkaloid (3, 7). In a recent study, we have shown that sanguinarine, at micromolar concentrations, imparts cell growth inhibitory response in human squamous carcinoma (A431) cells via an induction of apoptosis (7). The important observation of this study was that sanguinarine treatment did not result in apoptosis of the normal human epidermal keratinocytes at similar dose (7).

Indeed, in recent years, the regulation of apoptosis has become an area of extensive study in cancer research as the life span of both normal and cancer cells within a living system is regarded to be significantly affected by the rate of apoptosis (8–12). Many apoptosis-causing agents target the mitochondria, thereby triggering the execution phase of apoptosis, often the activation of caspases, which are the proteolytic enzymes responsible for the execution of apoptosis (13–16). It has been shown that in the process of apoptosis-induction, the Bcl-2 family of cytoplasmic proteins play a critical role (16–18). Its interacting pro- and antiapoptotic members integrate the diverse survival and distress signals to decide the fate of the cell (16–18). In the process of apoptosis, mitochondria plays a central but complex role (19). This complex role of mitochondria was unraveled when studies identified several mitochondrial proteins that are able to activate the cellular apoptotic process directly (19). Normally, these proteins reside in the intermembrane space of mitochondria, and in response to a variety of apoptotic stimuli, they are released to the cytosol and/or to the nucleus where they initiate apoptosis (19). Thus,

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mitochondria is increasingly appreciated as a target for the management of cancer, and the agents that can modulate mitochondrial events and the process of apoptosis (thereby being able to affect the steady-state cell population) may be useful in the management of cancer (2, 20).

In this study, because of our interest in skin biology and skin cancer, we used immortalized human keratinocytic (HaCaT) cells to evaluate the involvement of apoptosis in antiproliferative effects of sanguinarine. We assessed the involvement of mitochondria-associated events and Bcl-2 family of proteins during the antiproliferative effects of this alkaloid. Our findings may have implications for the management of skin cancer and other hyperproliferative skin conditions.

MATERIALS AND METHODS

Cell Culture. We used immortalized human keratinocytes (HaCaT cells) for this work. Our choice of these cells is based on the fact that HaCaT cells are immortalized keratinocytes with very high proliferative potential. HaCaT cells possess many features of premalignant or cancerous cells (Ref. 21 and references therein). For example, like many cancer cells, HaCaT cells (*a*) exhibit mutations in both the alleles of the p53 gene (as do a number of skin carcinomas), (*b*) have significantly high telomerase activity, (*c*) show colony formation efficiency, and (*d*) shown very high sensitivity for a complete tumorigenic conversion by genetic alterations or many external stimuli such as elevated temperature and altered growth factors (Ref. 21 and references therein). The cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment.

Treatment of Cells. Sanguinarine (dissolved in ethanol) was used for the treatment of cells. For our studies, the cells (70–80% confluent) were treated with sanguinarine at 0.1, 0.25, 0.5, 1, and 2 μM concentrations for 24 h in complete cell medium. The control cells were incubated with vehicle (ethanol) only for 24 h.

Cell Growth/Cell Viability. The effect of sanguinarine on the viability of cells was determined by MTT³ assay. The cells were plated at 2×10^5 cells/well in 200 μl of DMEM complete medium containing 0.1, 0.25, 0.5, 1 and 2 μM concentrations of sanguinarine in a 96-well microtiter plate. Each concentration of sanguinarine was repeated in 10 wells. After incubation for 24 h at 37°C in a humidified incubator, cell viability was determined using the MTT assay. Briefly, 4 μl of MTT reagent (5 mg/ml in PBS) was added to each well and incubated for 2 h, and the plate was centrifuged at 1800 rpm for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. Formazan crystals were dissolved in 150 μl of DMSO, and the absorbance was recorded on a microplate reader at 540-nm wavelength. The effect of sanguinarine on growth

inhibition was assessed as percentage cell viability where vehicle-treated cells were taken as 100% viable.

Measurement of Apoptosis by ELISA. The induction of apoptosis was evaluated with a Cell Death Detection ELISAPlus kit purchased from Roche Molecular Biochemicals USA (Indianapolis, IN). This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after the apoptotic death of the cells. For determination of apoptosis by ELISA, the HaCaT cells (1×10^4 cells/well) were treated with sanguinarine at 0.1, 0.25, 0.5, 1, and 2 μM concentrations for 24 h in a 96-well plate. Each concentration of sanguinarine was repeated in 5 wells. The extent of apoptosis was evaluated by assessing the enrichment of nucleosomes in the cytoplasm exactly as per the manufacturer's protocol.

Preparation of Cell Lysates, SDS-PAGE, and Immunoblot Analysis. For the immunoblot analysis, after the treatments of cells with sanguinarine, the medium was aspirated, and the cells were washed twice with cold PBS (10 mM, pH 7.4). The cells were incubated with ice-cold lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor mixture (Protease Inhibitor Cocktail Set III, Sigma Chemical Co., St. Louis, MO) for 30 min over ice. The cells were then scraped, and the lysate was collected in a microfuge tube and passed through a 21-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at $14000 \times g$ for 15 min at 4°C, and the supernatant (total cell lysate) was either used immediately or stored at -70 °C. The protein concentration was determined using DC Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) as per the manufacturer's protocol.

For immunoblot analysis, 25–50 μg of protein were resolved over 12% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked with blocking buffer [5% nonfat dry milk in 1% Tween 20 in 20 mM Tris-buffered saline (pH 7.5)] by incubating for 1 h at room temperature followed by incubation with appropriate primary antibody (at the dilutions recommended by the manufacturer) in blocking buffer for overnight at 40°C. The blot was then incubated with appropriate secondary antibody horseradish peroxidase conjugate (Amersham Life Science, Inc., Arlington Height, IL), and the protein expression was detected by chemiluminescence using enhanced chemiluminescence kit (Amersham Life Science, Inc.) and autoradiography with XAR-5 film (Eastman Kodak Co., Rochester, NY).

Determination of Cytosolic Cytochrome *c*. After treatment with sanguinarine as described above, the cells were washed with PBS and collected by centrifugation at $700 \times g$ for 10 min at 4°C. The pellet was resuspended in extraction buffer containing [220 mM mannitol, 68 mM sucrose, 50 mM PIPES-NaOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, with freshly added protease inhibitors]. The cells were incubated over ice for 30 min followed by sonication. The resulting mixture was centrifuged at $14,000 \times g$ for 15 min at 4°C, and the supernatants were removed and stored at -80 °C until analysis by gel electrophoresis. For gel electrophoresis for cytochrome *c*, 20 μg of cytosolic protein extracts were subjected to 12% SDS-PAGE followed by immunoblot analysis as dis-

³ The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Apaf-1, apoptotic protease-activating factor-1; PARP, poly(ADP-ribose) polymerase.

cussed above using anticytochrome *c* antibody (PharMingen, San Diego, CA; at a dilution of 1:5000) and antimouse secondary horseradish peroxidase conjugate (Amersham Life Science, Inc.), and the protein expression was detected by chemiluminescence using enhanced chemiluminescence kit (Amersham Life Science, Inc.) and autoradiography with XAR-5 film (Eastman Kodak Co.).

Caspase 3 (DEVDase) Activity Assay. After the treatment of cells with sanguinarine as described above, the activity of caspase-3-like proteases was measured using a kit obtained from Biomol Research Laboratories (Plymouth Meeting, PA) according to the manufacturer's protocol. This assay is based on spectrofluorometric detection (excitation, 400 nm; emission, 505 nm) of the chromophore *p*-nitroaniline that is linked to the end of the caspase-specific substrate. Active caspase causes cleavage of the substrate and release of free *p*-nitroaniline that is then measured. For the measurement of caspase-3 like activity, Ac-DEVD-AFC is used as the substrate.

RESULTS

Sanguinarine Treatment Results in an Inhibition of Cell Proliferation and Induction of Apoptosis in HaCaT Keratinocytes. Our first goal was to assess whether sanguinarine treatment imparts antiproliferative effects in immortalized human keratinocytes HaCaT cells. Thus, we first evaluated the effect of sanguinarine on the growth of these cells by MTT assay. As shown in Fig. 1A, sanguinarine treatment (0.1–2 μM for 24 h) of HaCaT cells resulted in a dose-dependent inhibition of cell growth.

Because our earlier studies (7) have shown that sanguinarine induces apoptosis in A431 cells, using multiple methods, we assessed the induction of apoptosis by this alkaloid in HaCaT cells. As shown by the formation of DNA ladders (Fig. 1B), sanguinarine treatment resulted in significant DNA fragmentation, a hallmark of apoptotic cell death. The induction of apoptosis was additionally confirmed by ELISA and the cleavage of PARP. Using ELISA, we characterized the enrichment of nucleosomes in the cytoplasm of the cells where our data (Fig. 2A) clearly demonstrated that compared with vehicle treated control, sanguinarine treatment of HaCaT cells resulted in significant dose-dependent apoptosis reaching to a maximum at 2 μM concentration of sanguinarine. Furthermore, as shown by the immunoblot analysis of PARP (Fig. 2B), sanguinarine treatment of cells resulted in a dose-dependent increase in the cleavage of PARP to its M_r 85,000 fragment, which is indicative of apoptotic cell death.

Sanguinarine Treatment Results in Dose-dependent (a) Increase in Bax, Decrease in Bcl-2, and (c) Increase in Bax/Bcl-2 Ratio in HaCaT Keratinocytes. The Bcl-2 family of proteins plays a central regulatory role via its interacting pro- and antiapoptotic members, which integrate a wide array of diverse upstream survival and distress signals to decide the fate of the cells (16–18). The Bax and Bcl-2 are the key players of this family (16–18). We evaluated the effect of sanguinarine treatment on the levels of Bax and Bcl-2 protein expression. As shown by the data (Fig. 3A), sanguinarine treatment was found to result in a dose-dependent increase in protein level of Bax and decrease in protein level of Bcl-2. A densitometric analysis of

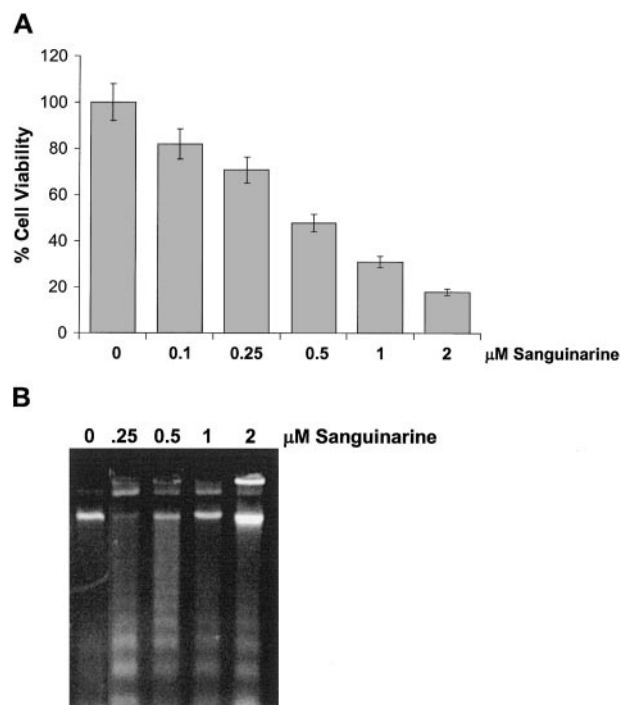


Fig. 1 A, effect of sanguinarine on the growth of HaCaT keratinocytes. As detailed under "Materials and Methods," the cells were treated with sanguinarine, and the viability of cells was determined by MTT assay. The data are expressed as percentage cell viability and represent the means (SE of three experiments where each treatment was performed in 10 wells). B, induction of apoptosis by sanguinarine in HaCaT keratinocytes: DNA ladder formation. The cells were treated with sanguinarine at the specified concentrations (for 24 h), harvested, cellular DNA isolated, and subjected to agarose gel electrophoresis followed by visualization of bands as described in "Materials and Methods." The data shown here is from a representative experiment repeated three times with similar results.

the bands revealed that sanguinarine-treatment to the HaCaT cells resulted in a dose-dependent increase in Bax/Bcl-2 ratio that favors apoptosis (Fig. 3B).

Sanguinarine Treatment Results in Dose-dependent Increases in the Protein Levels of Bak and Bid in HaCaT Keratinocytes. The regulation of apoptosis by Bcl-2 family proteins is a complex process, often with the involvement of only some of the members of this family (16–18). To assess the role of other members of Bcl-2 family in sanguinarine-mediated apoptosis, we evaluated its effect on the protein levels of (a) antiapoptotic members, *i.e.*, Bcl-xl, Bag, and Bid, and (b) proapoptotic members, *i.e.*, Bcl-xs, Bad, and Bak. As shown in Fig. 4, our immunoblot analysis data demonstrated that sanguinarine treatment to the HaCaT keratinocytes results in a significant time-dependent increase in the protein levels of the proapoptotic members Bak and Bid. The other members of this family of proteins were found to be unaffected by sanguinarine treatment (data not shown).

Sanguinarine Treatment Results in Dose-dependent Increases in the Protein Levels of Cytosolic Cytochrome *c* and Apaf-1 in HaCaT Keratinocytes. The Bcl-2 family proteins are the known regulators of cytochrome *c* from the mitochondria into the cytosol, an event known as mitochondrial-initiated

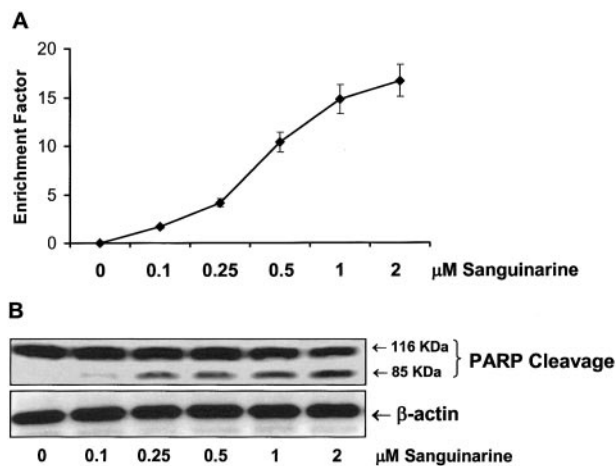


Fig. 2 *A*, induction of apoptosis by sanguinarine in HaCaT keratinocytes: enrichment of nucleosomes in the cytoplasm. As described in "Materials and Methods," cells were treated with increasing concentrations of sanguinarine in a 96-well microtiter plate, and the enrichment of nucleosomes in the cytoplasm was determined using Cell Death Detection ELISAPlus kit as per the manufacturer's protocol. The data are expressed as mean (SE of three separate experiments in which each treatment was performed in multiple wells). *B*, effect of sanguinarine on the cleavage of PARP in HaCaT keratinocytes. The cells were treated with specified concentrations of sanguinarine for 24 h and then harvested. Total cell lysates were prepared, and 25 μ g of protein were subjected to SDS-PAGE followed by immunoblot analysis using anti-PARP antibody from Upstate Biotechnology, Inc. (Waltham, MA) as described in "Materials and Methods." Equal loading of protein was confirmed by stripping the blot and reprobing with β -actin antibody. Data shown are from a representative experiment repeated three times with similar results.

caspase activation (22). The involvement of Apaf-1 in the execution phase of apoptotic process is also very important (23). Therefore, in our next series of experiments, we evaluated the effect of sanguinarine-treatment on the release of cytochrome *c* from mitochondria into the cytosol and the activation of Apaf-1 in the cytosol. As shown in Fig. 5, sanguinarine-treatment of HaCaT keratinocytes was found to result in a significant increase in the protein levels of both cytochrome *c* and Apaf-1. These results clearly suggested the role of mitochondrial events in sanguinarine-mediated apoptosis of HaCaT keratinocytes.

Sanguinarine Treatment Results in Dose-dependent Activation of Caspase-9, Caspase-3, Caspase-7, and Caspase-8 in HaCaT Keratinocytes. The mitochondrial damage causes the release of cytochrome *c* and activation of Apaf-1, thereby initiating the activation of caspase machinery that, in fact, is believed to be the execution phase of apoptosis (19, 22, 23). Therefore, we evaluated the involvement of various caspases during sanguinarine-mediated apoptotic death of HaCaT keratinocytes. As shown by the immunoblot analysis, sanguinarine treatment was found to result in a significant increase in the active form of caspase-9, caspase-3, and caspase-8 in a dose-dependent fashion. The proform of these caspases was not found to be affected by sanguinarine treatment of the keratinocytes. In case of caspase-7, both the proform as well as the active form showed a dose-dependent increase in the protein levels because of sanguinarine treatment (Fig. 6).

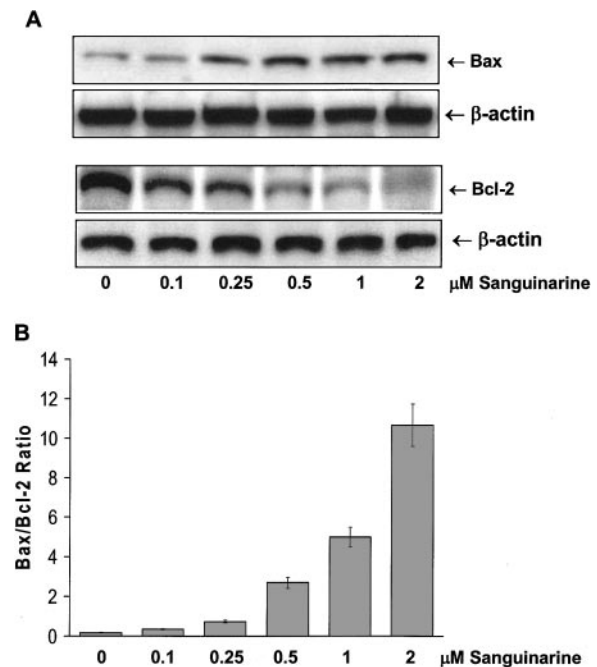


Fig. 3 *A*, effect of sanguinarine on the protein expression of Bax and Bcl-2 in HaCaT keratinocytes. Cells were treated with specified concentrations of sanguinarine for 24 h and then harvested. Total cell lysates were prepared, and 25 μ g of protein were subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection as described in "Materials and Methods." Equal loading of protein was confirmed by stripping the blot and reprobing with β -actin antibody. Data shown are from a representative experiment repeated three times with similar results. *B*, effect of sanguinarine on the Bax/Bcl-2 ratio in HaCaT keratinocytes. The data obtained from the immunoblot analyses of Bax and Bcl-2 were used to evaluate the effect of sanguinarine on the Bax/Bcl-2 ratio. The densitometric analysis of Bax and Bcl-2 bands was performed using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT), and the data (relative density normalized to β -actin) were plotted as Bax/Bcl-2 ratio. Data are expressed as mean (SE of three separate experiments with similar results).

We also evaluated the effect of sanguinarine treatment on the activity of caspase-3-like proteases using commercially available caspase assay kit (Biomol Research Laboratories). As shown by data in Fig. 7, sanguinarine treatment to the HaCaT cells was found to result in a concentration-dependent increase in caspase-3-like protease activity.

DISCUSSION

Many studies have established the role of mitochondria and mitochondria-associated events in the process of apoptosis (16, 18, 19, 22). In the recent past, mitochondria have been appreciated as targets for cancer chemoprevention as well as chemotherapy (2, 20). Dependent or independent from mitochondria, the Bcl-2 family proteins have been shown to play critical role in apoptosis (16–18). In this study, we have assessed the role of mitochondria-associated events and Bcl-2 family of proteins in the apoptotic death of immortalized human HaCaT keratinocytes induced by naturally occurring alkaloid sanguinarine.

Because nonmelanoma skin cancer represents the largest group of cancer patients in the United States and because the

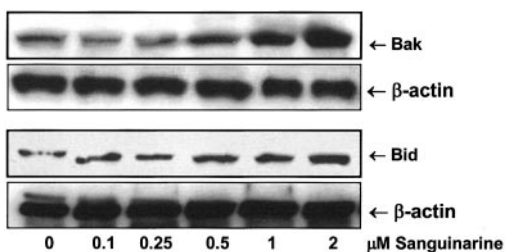


Fig. 4 Effect of sanguinarine on the protein expression of Bak and Bid in HaCaT keratinocytes. Cells were treated with specified concentrations of sanguinarine for 24 h and then harvested. Total cell lysates were prepared, and 25 μg of protein were subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection as described in "Materials and Methods." Equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody. Data shown are from a representative experiment repeated three times with similar results.

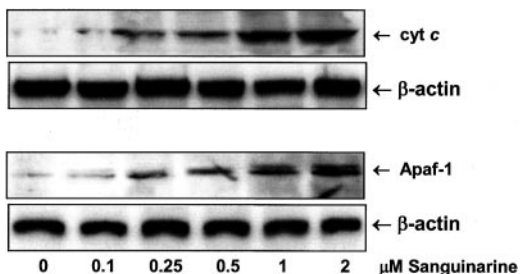


Fig. 5 Effect of sanguinarine on the protein expression of cytochrome c and Apaf-1 in HaCaT keratinocytes. Cells were treated with specified concentrations of sanguinarine for 24 h and then harvested. Total cell lysates were prepared, and 25 μg of protein were subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection as described in "Materials and Methods." Equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody. Data shown are from a representative experiment repeated three times with similar results.

treatment options and surgery have not been able to deal with the growing incidence of this cancer type, there is a need to develop mechanism-based novel agents for its management (1). Sanguinarine is a benzophenanthridine alkaloid predominantly found in Papaveraceae such as the roots of the blood root plant *Sanguinaria canadensis* L, *Chelidonium majus* L, and the seeds of the *Argemone mexicana* L (24). Extracts of *S. canadensis* have been shown to possess antioxidative, antitumor, antibacterial activities, and anti-inflammatory properties in animals and to reduce gingival inflammation and supragingival plaque when used clinically (3, 7, 24–26). The *Sanguinaria* extract, sanguinarine, has been used in many over-the-counter products, including toothpaste, mouthwash, cough and cold remedies, and homeopathic preparations (24). Sanguinarine has a broad *in vitro* activity against Gram-positive and Gram-negative bacteria, fungi, and some protozoa (24).

Some studies suggested that sanguinarine may be an effective anticancer/cancer chemopreventive agent (3, 7, 24). In ancient times, sanguinarine containing herbs such as bloodroot were believed to possess anticancer activity. These herbs have

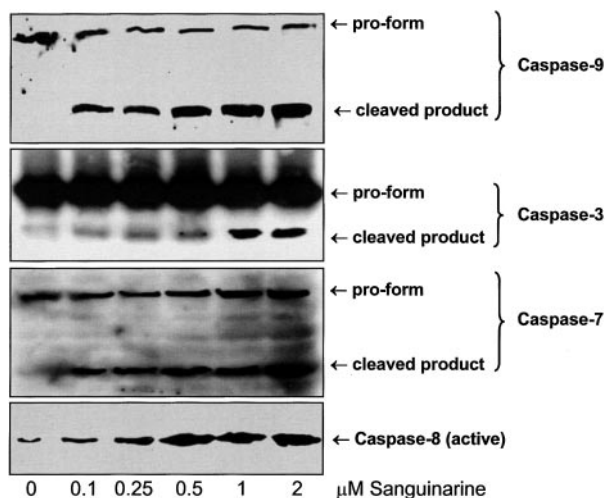


Fig. 6 Effect of sanguinarine on the protein expression of caspase-9, caspase-3, caspase-7, and caspase-8 in HaCaT keratinocytes. Cells were treated with specified concentrations of sanguinarine for 24 h and then harvested. Total cell lysates were prepared, and 25 μg of protein were subjected to SDS-PAGE followed by immunoblot analysis and chemiluminescence detection as described in "Materials and Methods." Equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody (data not shown). Data shown are from a representative experiment repeated three times with similar results.

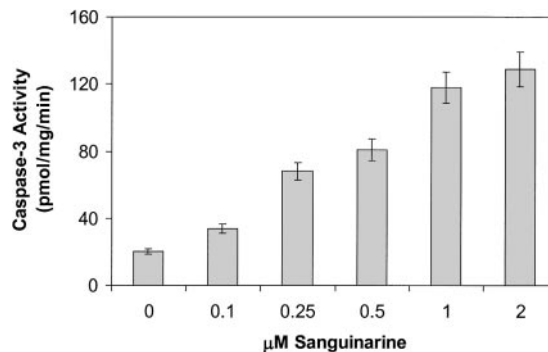


Fig. 7 Effect of sanguinarine on the activity of caspase-3-like proteases in HaCaT keratinocytes. Cells were treated with specified concentrations of sanguinarine for 24 h, and the activity of caspase-3 like proteases was determined using DEVDase assay kit obtained from Biomol Research Laboratories following the manufacturer's protocol. The data are expressed as mean (SE of three separate experiments).

long been used by Native American healers to treat cancer. Sanguinarine has been shown to inhibit several enzymatic activities (such as lipoxygenase, cholinesterase, and Na⁺/K⁺-ATPase) and signaling pathways such as nuclear factor-κB pathway (3, 24). We have earlier shown that sanguinarine, at micromolar concentrations, imparts cell growth inhibitory response in human squamous carcinoma A431 cells via an induction of apoptosis (7). The important observation of this study was that sanguinarine treatment did not result in apoptosis of the normal human epidermal keratinocytes at similar concentration.

This study demonstrated that sanguinarine treatment to human immortalized HaCaT keratinocytes results in significant

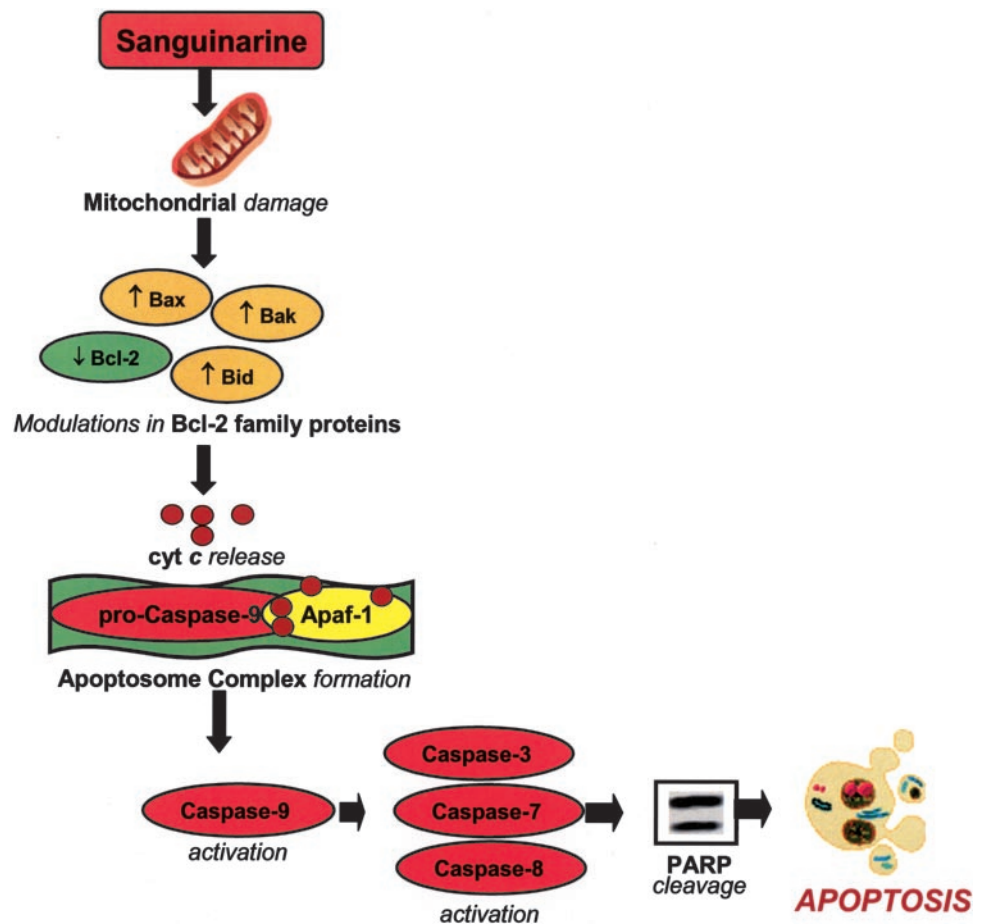


Fig. 8 Schematic illustration of the proposed model for sanguinarine-mediated apoptosis in HaCaT keratinocytes.

cell growth inhibition and induction of apoptosis (Figs. 1 and 2). Our next aim was to identify the mechanism of sanguinarine-mediated apoptosis in these cells. Bcl-2 is an antiapoptotic gene, and in fact, the link between apoptosis and cancer emerged when Bcl-2 (B-cell lymphoma 2), which is the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death (27). This unexpected discovery gave birth to the concept, now widely embraced that impaired apoptosis is a crucial step in the process of cancer development (9, 17, 28). In this study, we have shown that sanguinarine treatment to the HaCaT keratinocytes results in significant decrease in the levels of antiapoptotic Bcl-2 protein and increase in the proapoptotic Bax protein, thus shifting the Bax/Bcl-2 ratio in favor of apoptosis (Fig. 3). Studies have shown that Bcl-2 forms a heterodimer with Bax and might thereby neutralize its proapoptotic effects (29–31). In addition, Bcl-2 is also known to prevent the release of caspases (29). Our studies have also shown the increase of protein levels of other proapoptotic members of Bcl-2 family, *i.e.*, Bak and Bid, by sanguinarine treatment (Fig. 4).

Furthermore, sanguinarine treatment of HaCaT keratinocytes resulted in increase in the levels of cytochrome *c* and Apaf-1 (Fig. 5) and caspase-3, caspase-7, caspase-8, and caspase-9. These are important observations as it is known that the Bcl-2 family proteins regulate the release of cytochrome *c*

from the mitochondria into cytosol (19, 22). Cytochrome *c* resides in the intermembrane space of mitochondria, whereas its cofactors, Apaf-1 and procaspase-9, are both cytosolic proteins (22). The overexpression of Bcl-2 has been shown to block cytochrome *c* release in response to a variety of apoptotic stimuli (22). On the contrary, the proapoptotic members of Bcl-2 family proteins such as Bax, Bak, and Bid promote cytochrome *c* release from the mitochondria. (19, 22). The execution mechanism of apoptosis is mediated by caspases (cysteinyl aspartate-specific proteinases), which carry out the apoptotic program through a sequential activation cascade of initiator and executioner caspases (22). Apaf-1 induces activation of initiator caspase-9 (17, 18, 32). Apaf-1 binds caspase-9 via the caspase recruitment domains at their NH₂ termini, triggering the formation of a supramolecular complex called the apoptosome (23, 33). When activated, initiator caspase-9 triggers subsequent proteolytic activation of executioner caspase-3, caspase-7, and caspase-8 (16–18, 23, 32, 33). This whole process results in the cleavage of PARP and subsequent DNA degradation and apoptotic death (16–18, 23, 32, 33).

Thus, based on our data and the available literature, we suggest that as shown in Fig. 8, sanguinarine-caused apoptosis of immortalized HaCaT keratinocytes is mediated via caspase activation triggered by modulations in Bcl-2 family proteins-mediated cytochrome *c* release and the associated events. Al-

though, the modulation of Bcl-2 by sanguinarine has been shown earlier in some cell types (34–36), to our knowledge this is the first study showing the complete cascade of events that lead to apoptotic cell death by sanguinarine.

Because mitochondria is increasingly appreciated as a target for the management of cancer, our study showing the modulation of mitochondrial events and the process of apoptosis by sanguinarine may be useful in the management (chemoprevention as well as chemotherapy) of skin cancer and possibly other hyperproliferative skin disorders by promoting endogenous apoptosis-inducing mechanisms. On the basis of our work, it is conceivable to design sanguinarine-containing emollient or a patch for the treatment of skin cancer or other hyperproliferative cutaneous disorders. Such approaches have recently been advocated for the chemoprevention/chemotherapy of skin lesions (2).

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