Differential Antiproliferative and Apoptotic Response of Sanguinarine for Cancer Cells *versus* Normal Cells[1]

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ABSTRACT

Sanguinarine, derived from the root of *Sanguinaria canadendid*, has been shown to possess antimicrobial, anti-inflammatory, and antioxidant properties. Here we compared the antiproliferative and apoptotic potential of sanguinarine against human epidermoid carcinoma (A431) cells and normal human epidermal keratinocytes (NHEKs). Sanguinarine treatment was found to result in a dose-dependent decrease in the viability of A431 cells as well as NHEKs albeit at different levels because sanguinarine-mediated loss of viability occurred at lower doses and was much more pronounced in the A431 carcinoma cells than in the normal keratinocytes. DNA ladder assay demonstrated that compared to vehicle-treated control, sanguinarine treatment of A431 cells resulted in an induction of apoptosis at 1-, 2-, and 5-μM doses. Sanguinarine treatment did not result in the formation of a DNA ladder in NHEKs, even at the very high dose of 10 μM. The induction of apoptosis by sanguinarine was also evident by confocal microscopy after labeling the cells with annexin V. This method also identified necrotic cells, and sanguinarine treatment also resulted in the necrosis of A431 cells. The NHEKs showed exclusively necrotic staining at high doses (2 and 5 μM). We also explored the possibility of cell cycle perturbation by sanguinarine in A431 cells. The DNA cell cycle analysis revealed that sanguinarine treatment did not significantly affect the distribution of cells among the different phases of the cell cycle in A431 cells. We suggest that sanguinarine could be developed as an anticancer drug.

INTRODUCTION

The search for novel anticancer drugs continues. Agents that can eliminate the cancerous cells via a programmed cell death but do not affect the normal cells may have a therapeutic advantage for the elimination of cancer cells. In the present study, we provide evidence that sanguinarine is a potential antiproliferative agent that can be developed as a potential agent for skin cancer. Sanguinarine (13-methyl [1,3]benzodioxolo[5,6-*c*]-1,3-dioxolo[4,5-*i*]phenanthridinium; Fig. 1), which is derived from the root of *Sanguinaria canadendid* and other poppy fumaria species, is a benzophenanthridine alkaloid and a structural homologue of chelerythrine (Ref. 1 and the references therein). Sanguinarine has been shown to possess antimicrobial, antioxidant, and anti-inflammatory properties (Ref. 1 and the references therein). A recent study has shown that sanguinarine is a potent inhibitor of the activation of nuclear transcription factor NF-κB[3] (1), which has been implicated to play a key role in the regulation of cell growth, cell cycle regulation, and apoptosis. The antitumor properties of this alkaloid are not well established.

At present, only a few agents are known to possess the potential for selective/preferential elimination of cancer cells without affecting the normal cells (2, 3). This study provides the first evidence that sanguinarine, at micromolar concentrations, imparts a cell growth-inhibitory response in human squamous carcinoma (A431) cells via an induction of apoptosis. In sharp contrast, NHEKs do not show any evidence of apoptosis but undergo necrotic cell death on treatment with higher concentrations of sanguinarine. We suggest that by modulating apoptotic machinery, sanguinarine may be able to affect the steady-state cell population and thus possesses a potential for development as an agent against skin cancer and possibly against other cancer types as well.

MATERIALS AND METHODS

Materials. Sanguinarine was obtained from Sigma Chemical Co. (St. Louis, MO). The human squamous carcinoma cells (A431) were obtained from American Type Culture Collection (Manassas, VA). NHEKs were prepared from human foreskin using standard procedures (2). A431 cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. NHEKs were maintained in keratinocyte-serum-free medium (Life Technologies, Inc.) supplemented with L-glutamine, epidermal growth factor, and bovine pituitary extract. The cells were maintained at 37°C/5% CO_2 in a humid environment.

Cell Viability. The cells were grown to 70% confluence and treated with sanguinarine (0.25, 0.50, 1, 2, 5, and 10 μM) for 24 h, and the cell viability was determined by trypan blue exclusion assay.

Received 10/4/99; revised 1/4/00; accepted 1/4/00.

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[1] Supported in part by NIH Grant CA 78809. N. A. is the recipient of a Research Career Development Award from the Dermatology Foundation.

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[3] The abbreviations used are: NF-κB, nuclear factor κB; NHEK, normal human epidermal keratinocyte; PI, propidium iodide.

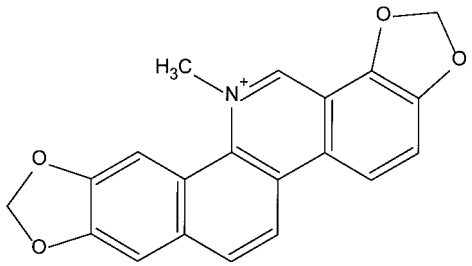

Fig. 1 Structure of sanguinarine.

DNA Fragmentation Assay. The cells were grown to about 70% confluence and treated with sanguinarine (0.25, 0.50, 1, 2, 5, and 10 μM) for 24 h. After the treatments, cells were washed twice with PBS [10 mM PBS (pH 7.2)], suspended in 1 ml of cytoplasm extraction buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100], left on ice for 15 min, and pelleted down by centrifugation (14,000 × *g*) at 4°C. The pellet was incubated with DNA lysis buffer [10 mM Tris (pH 7.5), 400 mM NaCl, 1 mM EDTA, and 1% Triton X-100] for 20 min on ice and then centrifuged at 14,000 × *g* at 4°C. The supernatant obtained was incubated overnight with RNase (0.2 mg/ml) at room temperature and then incubated with proteinase K (0.1 mg/ml) for 2 h at 37°C. DNA was then extracted using phenol:chloroform (1:1) and precipitated with 95% ethanol for 2 h at −80°C. The DNA precipitate was centrifuged at 14,000 × *g* at 4°C for 15 min, and the pellet was air-dried and dissolved in 20 μl of Tris-EDTA buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. The total amount of DNA was resolved over a 1.5% agarose gel containing 0.3 μg/ml ethidium bromide in 1× TBE buffer [(pH 8.3); 89 mM Tris, 89 mM boric acid, and 2 mM EDTA; BioWhittaker, Walkersville, MD). The bands were visualized under an UV trans-illuminator, followed by Polaroid photography.

Detection of Apoptosis and Necrosis by Confocal Microscopy. The ApopNexin apoptosis detection kit (Oncor, Gaithersburg, MD) was used for the detection of apoptotic and necrotic cells. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin V (green fluorescence), and the necrotic cells are stained with PI (red fluorescence). Briefly, the A431 cells and NHEKs were grown to about 70% confluence and then treated with sanguinarine (0.25, 0.50, 1, 2, 5, and 10 μM) for 24 h. Apoptosis and necrosis were detected by the kit according to the vendor's protocol. The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514–540 nm bandpass barrier filter. Images of red PI fluorescence were collected using a 568 nm excitation light from the argon/krypton laser, a 560 nm dichroic mirror, and a 590 nm long pass filter. In a selected field, the cells stained with annexin V and PI as well as unstained cells were counted to ascertain the extent of apoptosis and necrosis.

DNA Cell Cycle Analysis and Quantification of Apoptosis. The cells (70% confluence) were serum-starved for 36 h to synchronize them in the G_0 phase of the cell cycle, and then they were treated with sanguinarine (0.25, 0.50, 1, 2, 5, and 10 μM) for 24 h. The cells were then trypsinized, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 50 μl of cold PBS and 450 μl of cold methanol for 1 h at 4°C. The cells were centrifuged at 1100 rpm for 5 min, pellet-washed twice with cold PBS, suspended in 500 μl of PBS, and incubated with 5 ml of RNase (20 μg/ml, final concentration) for 30 min. The cells were chilled over ice for 10 min, stained with PI (50 μg/ml, final concentration) for 1 h, and analyzed by flow cytometry.

RESULTS AND DISCUSSION

In the present study, we evaluated sanguinarine as a potential agent for possible development as an anticancer drug. The rationale for the selection of sanguinarine for this study is twofold: (*a*) the antioxidant and anti-inflammatory properties of this plant-based alkaloid are well documented (1, 4–6), and certain compounds such as epigallocatechin-3-gallate and curcumin that possess similar properties are believed to prevent the development of cancer (2, 7–11); and (*b*) a recent study has shown that sanguinarine also functions as a potent inhibitor of the oxidant- and/or tumor promoter-mediated activation of NF-κB in a cell culture system (1). NF-κB is a widely distributed pleiotropic nuclear transcription factor that is known to regulate the expression of genes encoding cytokines, cellular adhesion molecules, and growth factors (1). Studies have indicated that NF-κB promotes cell survival by inhibiting apoptosis. Recently, it has been shown that the down-modulation of NF-κB activity in the cytosol and nucleus is associated with an apoptotic response of the eukaryotic cells (12). In recent years, NF-κB has been increasingly appreciated as a target for anticancer drug development (13).

In this study, to evaluate the antiproliferative response of sanguinarine, we used the human squamous carcinoma (A431) cells as model cancer cells and the NHEKs as normal cells. We first determined the effect of sanguinarine on the viability of A431 cells and NHEKs. As shown by data in Fig. 2*A*, sanguinarine treatment resulted in a dose-dependent decrease in the viability of both cell types, albeit at different levels. A striking observation from this data was that in the A431 cells, sanguinarine-mediated loss of viability occurred at lower doses and was much more pronounced than in the NHEKs. This observation suggested a differential response of sanguinarine to cancer cells as compared to normal cells.

We next investigated whether sanguinarine-mediated loss of viability in A431 cells and NHEKs is a result of apoptosis. We first evaluated the induction of apoptosis by sanguinarine via the classical DNA ladder assay. Compared to the vehicle-treated control, sanguinarine treatment of A431 cells resulted in an induction of apoptosis at 1-, 2-, and 5-μM doses (Fig. 2*B*), as evidenced by the formation of internucleosomal DNA fragments. At low doses of 0.1, 0.25, and 0.50 μM, no evidence of apoptosis was observed. It was interesting and important to observe that sanguinarine treatment did not result in the formation of a DNA ladder in NHEKs, even at the very high dose of 10 μM (data not shown).

The induction of apoptosis by sanguinarine was also evident from the analysis of data obtained by confocal microscopy

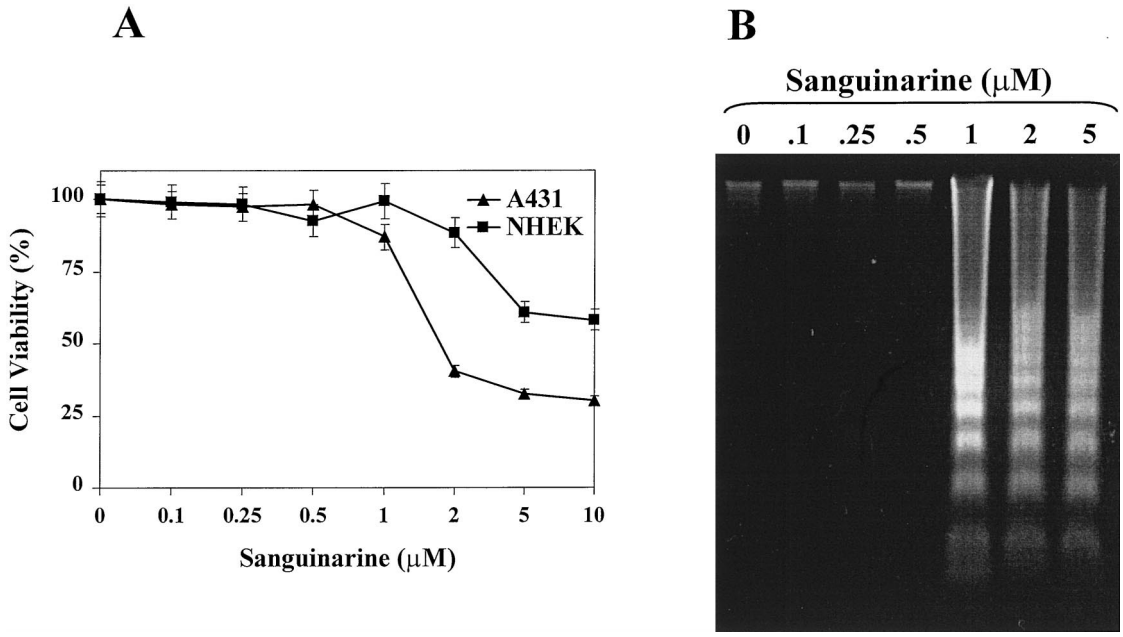

Fig. 2 *A*, effect of sanguinarine on the cell viability of A431 cells and NHEKs. The cells were treated with specified concentrations of sanguinarine for 24 h, and the number of viable cells was counted using the trypan blue exclusion assay. The cell viability is presented as the percentage of viable cells (the cell viability of vehicle-treated cells was regarded as 100%). The data represent the mean ± SE of three experiments done in triplicate. *B*, DNA fragmentation by sanguinarine in A431 cells and NHEKs. The cells were treated with vehicle or with the specified concentrations of sanguinarine, and 24 h later, the cells were collected, and the cellular DNA was isolated and subjected to agarose gel electrophoresis, followed by visualization of bands and Polaroid photography as described previously (2, 18). The data shown here are from a representative experiment repeated three times in which early-passage A431 cells and different batches of NHEKs obtained from different individuals were used.

after labeling the cells with annexin V (Fig. 3, *A* and *B*). We used this method because it identifies the apoptotic (*green fluorescence*) as well as necrotic (*red fluorescence*) cells. As shown by the data in Fig. 3, *A* and *B*, sanguinarine treatment resulted in dose-dependent apoptosis in A431 cells. These data also indicated that sanguinarine treatment also resulted in necrosis of A431 cells. These observations suggest the existence of two intriguing possibilities for the mechanism of sanguinarine action: (*a*) sanguinarine may cause both apoptosis and necrosis of A431 cells; and (*b*) the apoptosis induced by sanguinarine is preceded by a secondary necrosis in A431 cells. Interestingly, on the other hand, no apoptotic morphology was seen in the NHEKs, which showed exclusively necrotic staining, but only at the high doses of 2 and 5 μM. As shown in Fig. 3*B*, the vehicle-treated control as well as the low-dose (0.25, 0.5, and 1 μM) sanguinarine did not cause any alteration in the nuclear morphology of NHEKs. However, at a dose of 2 μM, a small number of cells (mean ± SE, 10.8 ± 0.8% necrotic cells) were necrotic, and significant necrosis (mean ± SE, 38.3 ± 2.7% necrotic cells) was observed only at the highest dose (5 μM). In recent years, apoptosis has become an important issue in biomedical research. The life-span of normal cells as well as cancer cells within a living system is regarded to be significantly affected by the rate of apoptosis. Because apoptosis is a discrete manner of cell death that differs from necrotic cell death and is regarded as an ideal way to eliminate damaged cells, agents that can modulate apoptosis may be used for the management and therapy of cancer by modulating the steady-state cell population. A vast variety of the chemotherapeutic agents currently used in cancer therapy are shown to kill the cells by mechanisms other than apoptosis. This may not always be a preferable form of cancer management. In recent years, many chemotherapeutic and chemopreventive agents have been shown to impart antiproliferative effects via an arrest of cells at certain checkpoints in the cell cycle (2, 14–16). Similarly, the concept of "cell cycle-mediated apoptosis" is also gaining increasing attention, and the anticancer properties of certain agents, are believed to function via this pathway (2, 17, 18). Therefore, we explored the possibility of cell cycle perturbation by sanguinarine in A431 cells. The DNA cell cycle analysis revealed that sanguinarine treatment did not significantly affect the distribution of cells among the different phases of the cell cycle in A431 cells (data not shown). This method is also believed to be a sensitive tool to distinguish between apoptotic and necrotic cell death. A "sub-G_1 " peak in a DNA histogram determined by flow cytometry is considered to be a hallmark of apoptotic cell death. This method provided additional evidence that sanguinarine treatment of A431 cells resulted in dose-dependent apoptosis. Consistent with the confocal microscopic data (Fig. 3*B*), as shown in Fig. 3*C*, DNA cell cycle analysis by flow cytometry also revealed that sanguinarine treatment of A431 cells resulted in dose-dependent apoptosis.

Lastly, to investigate whether the apoptotic response of sanguinarine is specific only to A431 cells, we followed the induction of apoptosis in other human cancer cell types. We found that the apoptotic response of sanguinarine was not lim-

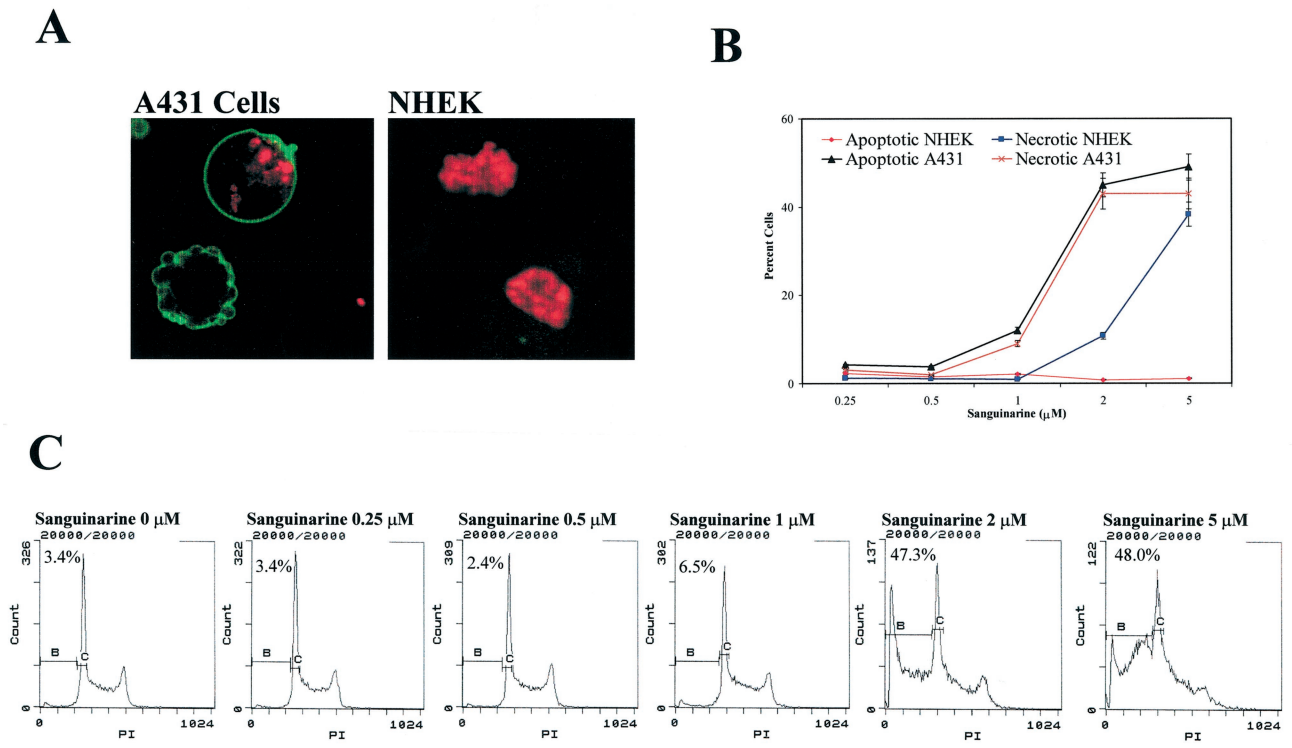

Fig. 3 *A*, morphological changes in A431 cells and in NHEKs after sanguinarine treatment as evidenced by confocal microscopy. The ApopNexin apoptosis detection kit (Oncor) was used for the detection of apoptotic and necrotic cells. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin V (*green fluorescence*), and the necrotic cells are stained with PI (*red fluorescence*). Apoptosis and necrosis were detected by the kit according to the vendor's protocol. The fluorescence was measured by a Zeiss 410 confocal microscope. Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514–540 nm bandpass barrier filter. Images of red PI fluorescence were collected using a 568 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 590 nm long pass filter. The data shown here are from a representative experiment repeated three times (in triplicate) with similar results. *B*, extent of apoptosis and necrosis mediated by sanguinarine in A431 cells and NHEKs. After confocal microscopy, the cells stained with annexin V and PI as well as the unstained cells in a selected microscopic field were counted, and the data are presented as a percentage of the total number of cells. The data represent the mean ± SE of three experiments done in triplicate. *C*, DNA cell cycle analysis. The cells were treated with vehicle or specified doses of sanguinarine for 24 h and analyzed by flow cytometry. The percentage of cells in sub-G_1 (apoptotic cells), G_0-G_1, S phase, and G_2-M phase was calculated using Cellfit computer software. The percentage of apoptotic cells is presented within the histograms. The data shown here are from a representative experiment repeated three times with less than 10% variation.

ited only to the A431 cells because similar treatment also resulted in the apoptotic cell death of other human cancer cell types, *i.e.*, androgen-responsive human prostate (LNCaP) carcinoma cells and androgen-unresponsive human prostate carcinoma (PC-3 and DU145) cells (data not shown).

Taken together, the results of this study suggest that by modulating apoptosis, sanguinarine may be able to affect the steady-state cell population and thus possesses a potential for development as an agent for cancer chemotherapy. To our knowledge, this is the first systematic study showing the cancer therapeutic potential of sanguinarine, and the induction of apoptosis in cancer cells by this alkaloid. However, determining the exact mechanism(s) of apoptosis will require a detailed exploration of genetic and signal transduction pathways. Based on the published study (1), the involvement of the NF-κB pathway could be viewed as a mechanism of sanguinarine-mediated apoptosis in cancer cells. However, additional studies are needed to unravel the differential response of sanguinarine in cancer cells *versus* normal cells. Also, to establish a broader implication, additional studies are needed to verify these data in other normal cells and cancer cell types and to assess the effectiveness of sanguinarine in an *in vivo* model system.

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