Evaluation of Anticancer Activities of Benzo[c]phenanthridine Alkaloid Sanguinarine in Oral Squamous Cell Carcinoma Cell Line

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Abstract. While the effects of benzo[c]phenanthridine alkaloids (QBA), known mainly as sanguinarine and chelerythrine, on the inhibition of some kinds of cancer cell proliferation have been established, the effect on oral squamous cell is not known. Here, the antitumor activity of sanguinarine was demonstrated using in vitro assay systems in SAS, a human oral squamous cell carcinoma (OSCC) cell line. The anti-proliferative and -invasive effects were confirmed with IC50 values in the concentration range of 0.75-1.0 μM by MTT assay and invasive assay, respectively. Sanguinarine was also able to suppress cell anchorage-independent growth, whereas it did not affect the cells’ adhering capabilities. Finally, sanguinarine induced apoptotic cell death by activating caspase and altering the Bcl-2/Bax ratio. Taken together, these results indicate that sanguinarine is a potential inhibitor of tumorigenesis and suggest that it may be valuable in the development of new anticancer drugs for the treatment of OSCC.

Oral squamous cell carcinoma (OSCC) is one of the leading causes of cancer-related deaths (1). The five-year survival rate in advanced patients, including those operable to remove OSCC, is about 30-35% (2). Therefore, chemotherapy is used together with surgery because this combination reduces the rates of recurrence and distant metastases (2). However, despite the decrease in distant metastases, no improvement in survival has been reported (3). The severe prognosis for OSCC patients makes it imperative to develop new drugs to improve the efficiency of conventional chemotherapies against OSCC. Many clinically approved cytotoxic and antiproliferative anticancer drugs are available, including those of both synthetic and natural (microbial and plant) origins (4, 5). Although anticancer drug discovery and development focus on mechanism-based agents that act on specific molecular targets associated with the biology of cancer, there is growing interest in the use of natural product compounds to search for useful candidates (4, 5).

Benzo[c]phenanthridine alkaloids (QBA), isolated from members of the Papaveraceae (Chelidonium majus, Macleaya cordata and Sanguinaria canadensis L.), have been reported to have a broad range of biological effects in cell-cycle regulation and cell death in various cell lines, and are known as modulators of the energy-transducing ion pump, Na+/K+-ATPase, which drives excess Na+ out of cells in exchange for K+, thereby maintaining an essential ionic and osmotic balance (6, 7).

Sanguinarine [13-methyl-(1,3)benzodioxolo[5,6-c]-1,3-dioxolo[4,5-i]phenanthridinium] is a phytoalexin with antimicrobial, anti-oxidant, anti-inflammatory and pro-apoptotic effects, and is involved in interactions with cellular biomacromolecules (4, 8-12). Specifically, several studies have indicated that sanguinarine inhibits the proliferation of cancer cells from different origins, including A549 lung cells (9, 13), breast (9, 14, 15), AsPC-1 and BxPC-3 pancreatic cells (9, 14, 16) and HCT116 and SW480 colon cells (9, 17, 18). It is noteworthy that sanguinarine is a novel potential agent for cancer therapy, since it was reported that the apoptosis induction in different malignant cell lines takes place through the extrinsic activation of cell surface receptors and intrinsic cytochrome c release from mitochondria pathways. However, the precise mechanisms by which the anticancer effects of this compound in OSCC occur are still not fully understood. Therefore, SAS, an OSCC cell line, was used to screen sanguinarine for its effects on cell proliferation, cell-cycle progression and apoptosis.
Materials and Methods

Materials. For these in vitro experiments, sanguinarine was purchased commercially from Sigma (St. Louis, MO, USA) and dissolved in MilliQ (Millipore, Bedford, MA, USA) at a concentration of 10 μg/ml for the stock solution and kept at −20°C prior to use.

Cell culture. SAS cells derived from a human oral squamous cell carcinoma (HSRBB, Osaka, Japan) were cultured at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Proliferation assay. Cell proliferation was evaluated using the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Dojindo, Kumamoto, Japan) assay, as described previously (19). The cells (5×10³/well) were seeded with DMEM in 96-well plates (Falcon Laboratories, McLean, VA, USA). After 24 h, the cells were treated with 0.25, 0.5, 0.75, 1.0 μM. sanguinarine and then further cultured for 3 days. Controls were medium alone or 2 mg/ml cis-diamminedichloroplatinum (CDDP; Nippon Kayaku, Tokyo, Japan) as positive control.

Anchorage-independent growth. The cells (5.0×10⁵/ml) were suspended in 0.35% agarose in DMEM containing 5% FBS. This suspension was overlaid onto a solidified layer of 0.4% agarose in a 96-well plate. Fresh DMEM was maintained on top of the plate during 5 days’ incubation, at which time the cells were examined under a microscope (Eclipse TS100/TS100-F; Nicon) equipped with a digital CDD camera (DS-Fil: Nicon) at 20x or 40x magnification. The cells were photographed and counted. Cells incubated for 5 days with or without sanguinarine (0.25-1.0 μM) or 2 μg/ml CDDP (positive control) were examined and photographed.

Migration assay. Cell migration was assayed using cell culture Boyden chambers (Cell BioLabs, San Diego, CA, USA) and vascular endothelial growth factor (VEGF) at a dose of 10 ng/ml as a chemoattractant. Following 24 hours’ incubation, cells adhering to the bottom of the membrane were fixed with 70% ethanol, stained with 0.1% crystal violet and photographed. The inserts were then treated with 10% acetic acid, and absorbance was measured at 560 nm.

Adhesion assay. Assays were carried out using the CytoSelect (Cell BioLabs) 48-well cell adhesion assay extracellular matrix (ECM) array. The cells (1×10⁵) were seeded onto the wells coated with each ECM protein (Fibronectin, Collagen I, Collagen IV, Laminin, Fibrinogen) and bovine serum albumin (BSA). Cells were incubated at 37°C for 60 min in a tissue culture incubator before washing and staining. Absorbance was measured at 560 nm.

Western blot analysis. Western blot analysis was carried out according to the method described previously (8). Anti-phospho-p42/p44 mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinase (ERK) 1/2) and anti-phospho-p38 were obtained from Promega (Madison, WI, USA), and anti-p42/p44 MAPK (ERK1/2), anti-phospho-c-Jun NH₂-terminal kinase and anti-c-Jun NH₂-terminal kinase were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-p38 was obtained from Calbiochem (Bad Soden, Germany). Bcl-2, Bax antibodies and peroxide-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

DNA fragmentation electrophoresis analysis. Genomic DNA was extracted from the SAS cells using a Wizard genomic DNA purification kit (Promega) according to the manufacturer’s protocol. The cells were treated with sanguinarine (0.75, 1.0 μM) and CDDP (2 μg/ml) for 24 h. Approximately 50 mg of DNA was loaded into each well and 3% agarose gel electrophoresis was carried out at 100 V in a TAE buffer (Trisacetate-EDTA) for 30 min. After electrophoresis, DNA was visualized by soaking the gel in TAE buffer containing 0.1 mg/ml ethidium bromide. The gel was observed using UV light and photographed.

Terminal deoxyuridine nick-end labeling (TUNEL) assay. A TUNEL assay was carried out as described previously (20). The cells (5×10⁵) were seeded onto Lab-Tek chamber slides (Thermo Fisher Scientific, Rochester, NY, USA) and incubated for 24 h. The cells were treated with sanguinarine (0.75, 1 μM) for 24 h and then the slides were observed using phase-contrast fluorescence microscopy (ECLIPSE TS100/TS100-F; Nikon, Tokyo, Japan).

Caspase-3 and caspase-7 activities. The SAS cells were seeded in a 96-well plate at a density of 3.0×10⁵/ml. The cells were incubated for 24 h with or without various concentrations of sanguinarine. They were then lysed and the caspase-3 and caspase-7 activities in the lysates were detected by a Caspase-Glo 3/7 assay kit (Promega), according to the manufacturer’s protocol. A dual luciferase system (Promega) was used for the sequential measurement of firefly and Renilla luciferase activities with beetle luciferin and coelenterazine, respectively, as the specific substrates. The quantification of both luciferase activities and the calculation of the relative ratios were carried out manually with a luminometer (GloMax; Promega).

Statistical analysis. Unless otherwise specified, all the experiments were repeated at least twice, and similar results were obtained in the repeated experiments. Statistical analysis was carried out using Student’s t-test. Data are expressed as mean±standard deviation.

Results

Effect of sanguinarine on monolayer- and anchorage-independent growth. As shown in Figure 1A, sanguinarine inhibited the monolayer proliferation of the SAS cells in a dose-dependent manner, with half-maximal inhibition at 0.75-1.0 μM. Sanguinarine was also able to markedly inhibit anchorage-independent growth in a dose-dependent manner (Figure 1B, left and right panel).

Effect of sanguinarine on invasion properties and adhesion to ECM. Sanguinarine inhibited the stimulation of cell migration by VEGF (Figure 2A) in Boyden chamber assay, but had no effect on adhesion to the ECM array (Figure 2B, upper and bottom panel).

Effect of sanguinarine on mitogen-activated protein kinase expression. We investigated the effects of sanguinarine on the activation of three subgroups of MAPK pathways. As shown in Figure 3, the sanguinarine activation of these pathways (p38 kinase, ERK and c-Jun NH₂-terminal kinase) was
observed in the SAS cells. Indeed, the phosphorylation of MAPKs became detectable as early as 15 min after stimulation by sanguinarine.

**Effect of sanguinarine on apoptotic cell death.** To further investigate the molecular mechanism underlying the observed growth-suppressing effects of sanguinarine, assays to detect the induction of apoptotic cell death were conducted. DNA fragmentation was observed in the SAS cells treated with sanguinarine at a dose of 0.75 μM (Figure 4A). Apoptosis was also confirmed by a significant increase in TUNEL-positive cells (Figure 4B) and by increased levels of activated caspase-3 resulting from 24h sanguinarine treatment (Figure 4C). Apoptosis was associated with a noticeable increase of Bax expression, while Bcl-2 expression markedly decreased in the sanguinarine-treated cells (Figure 4D).

**Discussion**

Sanguinarine inhibited monolayer-and anchorage-independent growth of the SAS cells in a concentration-dependent manner. One of the molecular mechanisms elucidated was that sanguinarine activated three subgroups of the MAPK pathways, and another was that sanguinarine treatment concentration-dependently had a pro-apoptotic effect by activating both caspase-3 and caspase-7. Furthermore, sanguinarine reduced the Bcl-2 protein level, but increased that of the proapoptotic Bax protein. Although this study did not directly investigate the role of the MAPK pathways in the induction of both the intrinsic and extrinsic apoptotic pathways by sanguinarine, it is plausible that these sanguinarine-activated MAPK pathways mutually affected other signaling pathways, *i.e.*, between ERK and Akt (21), and some downstream apoptosis-associated molecules, such as Bax, Bcl-2, caspase-3 and caspase-7.

Another possible mechanism underlying sanguinarine action could be the inhibition of Na⁺/K⁺-ATPase activity. Na⁺/K⁺-ATPase inhibitors, such as cardiac glycosides,
Figure 2. Effect of sanguinarine on the invasion of SAS cells. A: Quantified cell migration after VEGF stimulation in Boyden chambers. Means±SD of two independent experiments performed in triplicate. B: SAS cell adhesion to ECM. FN: Fibronectin; Col I: collagen I; Col IV: collagen IV; LN: laminin; FG: fibrinogen; BSA: bovine serum albumin.

Figure 3. Signal transduction profile in sanguinarine-treated SAS cells. Western blot analysis of phosphorylated or total ERK1/2, phosphorylated or total p38, and phosphorylated or total c-Jun NH$_2$-terminal kinase 1/2 (JNK1/JNK2) 5-60 min after stimulation by sanguinarine (1 μM). ERK: Extracellular signal-regulated kinase, JNK: c-Jun NH$_2$-terminal kinase.
disrupt the functioning of this enzyme, leading to an enhanced entry of calcium into cells, which, in the event of failing cardiac myofibrils, helps produce a more efficient myocardial contraction and improves cardiac pump activity(22). Ca^{2+} homeostasis makes this phenomenon interesting in the context of apoptosis studies, since an increased intracellular Ca^{2+} concentration may start apoptosis by itself, and this concentration is a step in several cascades leading to apoptosis after receptor interaction (22). Some epidemiological studies originally reported that a low incidence of cancer was observed among patients taking digitalis, one of the Na^{+}/K^{+}-ATPase inhibitors (22). Interestingly, it has been reported that apoptosis induction in different malignant cell lines occurs as a result of non-toxic
doses of cardiac glycosides. Since chelerythrine, a benzophenanthridine alkaloid, has been reported to inhibit Na⁺/K⁺-ATPase activity, it is plausible that sanguinarine also acts as an apoptosis inducer, like cardiac glycosides, by controlling intracellular Ca²⁺ concentration. Further research is required to clarify the mechanisms underlying the apoptotic action of sanguinarine.

In conclusion, sanguinarine prevents the growth of OSCC cell lines by inducing apoptosis and might be useful as a novel chemotherapy agent for human oral squamous cell carcinoma.

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References


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