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Preclinical report

Induction of apoptosis in human leukemia K-562 and gastric carcinoma SGC-7901 cells by salvicine, a novel anticancer compound

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Salvicine (a novel diterpenoid quinone compound) exhibited a marked antitumor activity on human solid tumor cell lines and BALB/c-*nu* human carcinoma xenografts in our earlier studies, and it has been chosen as a candidate anti-carcinogenic compound in the preclinical research stage. The present study was undertaken in order to observe whether or not the antitumor effect of salvicine is associated with its ability to induce apoptosis. Our results show that salvicine is capable of inhibiting cell proliferation and inducing characteristic changes of apoptosis in both human leukemia K-562 and gastric carcinoma SGC-7901 cells. These effects are dose and time dependent. The results of this study strongly suggest that the antitumor effect of salvicine is associated with its ability to induce apoptosis. Meanwhile, this study also shows that the activity of salvicine against K-562 and SGC-7901 cells is similar with regards to both growth inhibition and apoptosis induction, further indicating that salvicine causes these particular effects on solid tumor cells. [© 2001 Lippincott Williams & Wilkins.]

Key words: Apoptosis, K-562 cell, salvicine, SGC-7901 cell.

Introduction

Carcinogenesis can be involved in the malfunctioning of cell apoptosis. It is well known that most of the anticancer drugs in current use induce apoptosis in susceptible cells.[1] The fact that disparate agents interacting with different targets seem to induce cell death through some common mechanism suggests that antitumor activity is determined by the ability of the cell to engage in apoptosis.[2–7] Therefore, finding new compounds that influence the ability of inherent apoptosis susceptibility may open new strategies for improving chemotherapy.

Salvicine [4,5-seco-5,10-friedo-abieta-3,4-dihydroxy-5(10),6,8,13-tetraene-11,12-dione] is a novel diterpenoid quinone compound obtained by structural modification of a natural product lead isolated from a chinese medicinal plant *Salvia prionitis* used traditionally as an antibacterial, antitubercular and anti-phlogistic drug in folk medicine (Figure 1). We have isolated more than 40 diterpenoid compounds from this plant and systematic chemical modification of some compounds led to the preparation of pharmacologically active derivatives of which salvicine is one. In our previous studies, salvicine exhibited high *in vitro* and *in vivo* antitumor activities.[8,9] In the present study, we chose human leukemia K-562 and human gastric carcinoma SGC-7901 cells as models to determine the effects of salvicine in inhibiting cell growth and inducing apoptosis, and whether or not its antitumor effects are related to its ability to induce apoptosis.

Materials and methods

Preparation of salvicine

Tangerine-colored crystalloid salvicine was provided by the Phytochemistry Department of our Institute. A stock solution of 10^4 μM of salvicine was made in 50% dimethylsulfoxide (DMSO) and 50% normal saline. The

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final concentration of DMSO for all treatments was below 0.25%. Stock solution was aliquoted and stored at −20°C, thawed just before the test, and diluted with the medium.

Cell culture and treatment

The human chronic myelogenous leukemia cell line K-562 was obtained from ATCC (Rockville, MD), and human moderate-differentiated gastric adenocarcinoma cell line SGC-7901 was derived from a Chinese cancer patient and was established *in vitro* in 1983.[10] Both cell lines were grown in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated bovine serum, 2 nM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES, pH 7.4. Cells were kept at 37°C in a humidified 5% CO_2 incubator. For this study, cells were incubated in the absence or presence of various concentrations of salvicine for 24 h or exposed to salvicine at 25 μM for various time periods.

Measurement of cell growth inhibition

Growth inhibition by salvicine on tumor cells was measured by microculture tetrazolium (MTT) assay[11,12] with minor modifications.[13] Briefly, tumor cells were seeded into 96-well microculture plates at appropriate densities to maintain the cells in an exponential phase of growth during the period of the experiment. Cells were either exposed to salvicine at 6.25, 12.5, 25, 50 and 100 μM for 24 h or exposed to salvicine at 25 μM for 1, 6, 10, 24, 34 and 48 h; each concentration was tested in triplicate wells. At the end of the exposure, 20 μl of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; Sigma, St Louis, MO] was added to each well and the plates were incubated for 4 h at 37°C, then 'triplex solution (10% SDS–5% isobutanol–0.012 M HCl)' was added and the plates were incubated for 12–20 h at 37°C. The optical density (OD) was read on a plate reader at a wavelength of 570 nm. Media and DMSO control wells, in which salvicine was absent, were included in all the experiments. The inhibitory rate of cell proliferation was calculated by the following formula: Growth inhibition (%)=$OD_{control} - OD_{treated}/OD_{control} \times 100$. Results were obtained from two replicate tests.

Observation of nuclear morphology

Salvicine-treated cells were harvested by centrifugation, resuspended in the medium and dripped onto a glass slide. The cells on a glass slide were stained with DNA binding fluorochrome 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Boehringer, Mannheim, Germany) after fixation with 95% ethanol. The nuclear morphology was observed under an Olympus IX70 UV light fluorescence microscope.

DNA extraction and gel electrophoresis

The total cellular DNA was extracted from K-562 and SGC-7901 cells untreated and treated with salvicine by the method described by Slin and Stafford with minor modifications.[14] Briefly, cells were washed in phosphate-buffered saline (PBS) and lysed overnight at 37°C in lysis buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.4% sodium dodecylsulfate and 1 mg/ml proteinase K. After complete digestion, saturated phenol was added to the cell lysates and mixed fully. Samples were then centrifuged at 6000 r.p.m. for 5 min. An equal volume of chloroform was added to the supernatant isolated from the previous step, mixed fully and centrifuged as above. Supernatant was mixed with 2.5-fold volume of absolute ethanol and NaCl at 0.2 M final concentration for DNA precipitation. The DNA pellets were obtained by centrifugation at 12 000 r.p.m. for 10 min and were then air dried, dissolved in TE buffer (10 mM Tris–HCl, pH 7.8 and 1 mM EDTA) containing 0.5 mg/ml RNase (Sigma) for 30 min at 37°C. Electrophoresis was performed on 1.5% agarose gel in TBE buffer (90 mM Tris, pH 8.0, 90 mM boric acid and 2 mM EDTA). At the end of electrophoresis, gel was stained in 0.5 μg/ml ethidium bromide (EB) for 30 min. DNA fragments were visualized by UV fluorescence.

Flow cytometry

Cellular DNA content was quantified by flow cytometry via determination of propidium iodide (PI).[15] After treatment, the cells were collected, washed in PBS and fixed in 70% ethanol (4°C for 12 h or longer, usually overnight). After fixation, the cells were washed in PBS, and cell pellets obtained by centrifugation were stained with PI staining solution containing 50 μg/ml PI, 10 μg/ml RNase, 0.5% (v/v) Triton X-100 and 0.1% (w/v) trisodium citrate for 30 min at room temperature in the dark. DNA content was determined on a Becton Dickinson (Mountain View, CA) FACSCalibur flow cytometer. The cell populations in the sub-G_1 area (the position where apoptotic cells are located) were quantified from a standard count of 10 000 cells using CellQuest, ModFIT LT for Mac version 1.01 (Becton Dickinson).

Results

Cytotoxic effect

Salvicine inhibited the proliferation of K-562 and SGC-7901 cells in a dose- and time-dependent manner. After exposure of K-562 cells to 6.25, 12.5, 25 and 50 μM of salvicine for 24 h, cell proliferation was inhibited by 5.83, 54.17, 65.83 and 88.33%, respectively (Figure 2A). After exposure of SGC-7901 cells to 6.25, 12.5, 25, 50 and 100 μM of salvicine for 24 h, cell proliferation was inhibited by 0.9, 39.82, 60.18, 87.61 and 100%, respectively (Figure 2A). On exposing K-562 cells to 25 μM of salvicine for 1, 6, 10, 24, 34 and 48 h, cell proliferation was inhibited by 7.21, 33.22, 51.73, 74.15, 78.78 and 88.79%, respectively (Figure 2).

Morphological changes

Untreated SGC-7901 cells displayed extended and flat cell bodies with uniform chromatin across the nuclei (Figure 3A). The nuclei of SGC-7901 cells treated with salvicine for 24 h showed characteristic nuclear morphological changes of apoptosis, including DNA fragmentation and nuclear condensation. DNA fragmentation was observed at 12.5 μM (Figure 3B), and with an increase in dose the apoptotic changes appeared more apparent and extensive (Figure 3C). The changes in the nuclei of K-562 cells are similar to those in SGC-7901 cells (not shown).

DNA fragmentation

The typical 'DNA-ladder' pattern, indicating the presence of DNA equivalent to the size of single and oligo nucleosomes, could be seen in both K-562 and SGC-7901 cells treated with salvicine for 24 h. In K-562 cells, DNA ladder became obvious at the concentration of 50 μM (Figure 4A), while in SGC-7901 cells salvicine appeared to be more effective as the agarose gel electrophoresis revealed apparent DNA ladder at this concentration (Figure 4B). No chromosomal DNA cleavage was observed in untreated control cells.

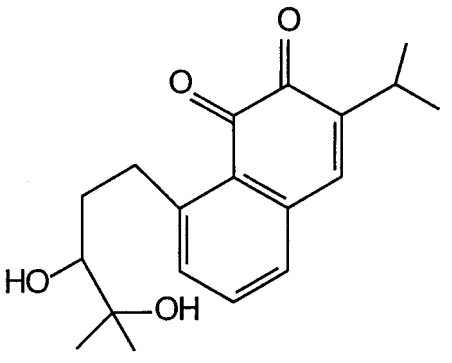

Figure 1. Chemical structure of salvicine.

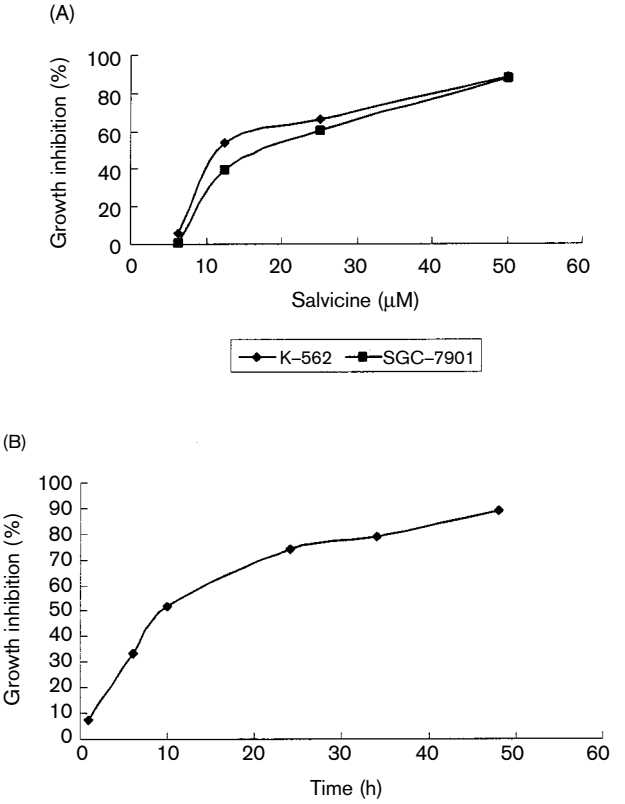

Figure 2. Effects of salvicine on the growth of human tumor cells. (A) Dose-dependent effects of salvicine on the inhibition of K-562 and SGC-7901 cell growth (exposure: 24 h). Time-dependent effects of salvicine on the inhibition of K-562 cell growth (salvicine: 25 μM).

Quantitative detection of apoptotic cells

Apoptotic cells can be recognized by their diminished stainability with the DNA-specific fluorochrome PI due to DNA degradation and its subsequent leakage from the cells. The hypodiploid population formed by cells having a reduced DNA content in DNA content frequency histograms (sub-G_1 peak) can be quantified by flow cytometry. Salvicine induced the apoptosis of K-562 and SGC-7901 cells in a dose-dependent manner. In untreated K-562 cells, the mean apoptotic population was 2.3% which increased to 15.76, 29.56, 26.84 and 40.28% after treatment with 6.25, 12. 5, 25 and 50 μM salvicine for 24 h, respectively. In untreated SGC-7901 cells, the mean apoptotic population was 0.7%, and it increased to 13.2, 38.51 and 64.6%

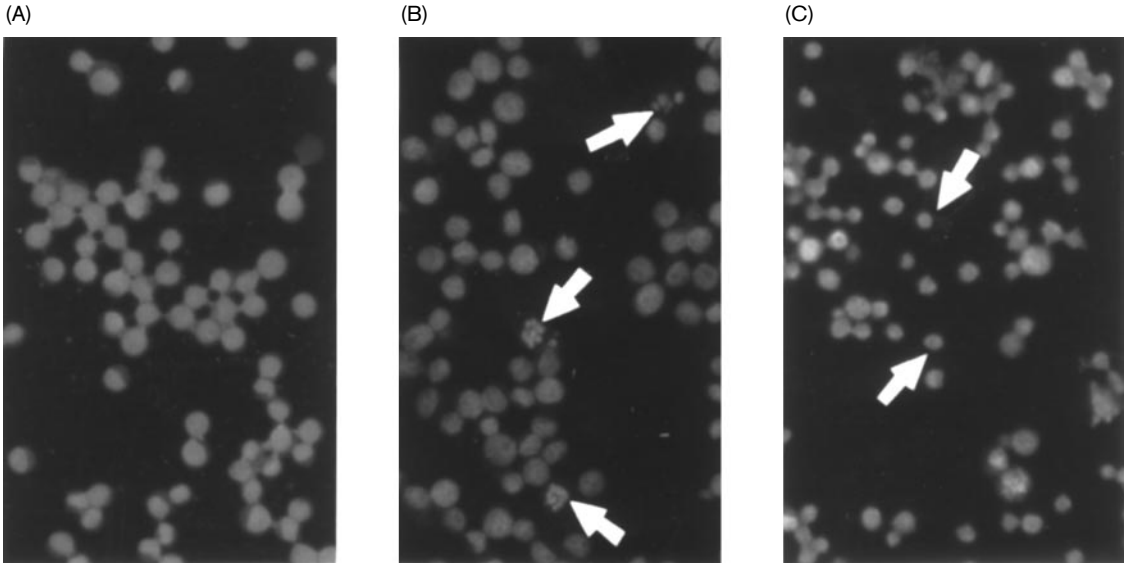

Figure 3. Morphological changes in SGC-7901 cell nuclei treated without (A) or with 12.5 (B) and 100 (C) μM salvicine for 24 h. Arrows denote condensed and fragmented nuclei. Magnification $\times$200.

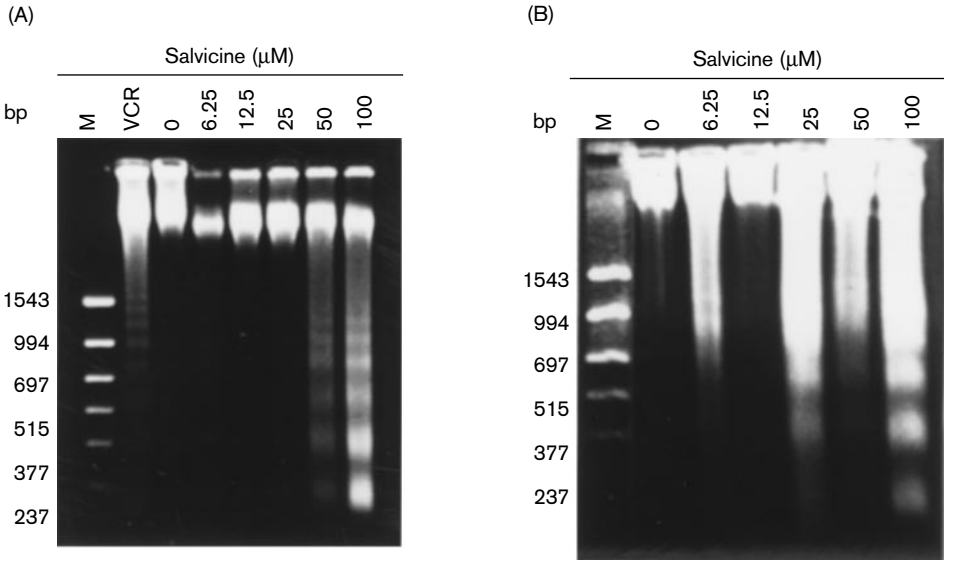

Figure 4. Agarose gel electrophoresis of DNA extracted from K-562 (A) and SGC-7901 (B) cells treated with 6.25, 12.5, 25, 50 and 100 μM salvicine for 24 h. M, DNA size marker; VCR, positive control.

after treatment with 25, 50 and 100 μM of salvicine for 24 h, respectively. The number of apoptotic SGC-7901 cells was not significantly increased at low concentrations (6.25 and 12.5 μM) of salvicine, For the time–effect relationship, the number of apoptitic K-562 cells increased with prolonged incubation duration to salvicine (Figure 5). The apoptotic K-562 cells increased to 25.5, 78.8 and 92.8% after 24, 34 and 48 h of exposure to 25 μM salvicine, respectively. While apoptitic K-562 population was not increased after a short time exposure (less than 10 h) to salvicine.

Discussion

In this study, we have shown that salvicine has a cytotoxic effect on K-562 and SGC-7901 cells in a dose-

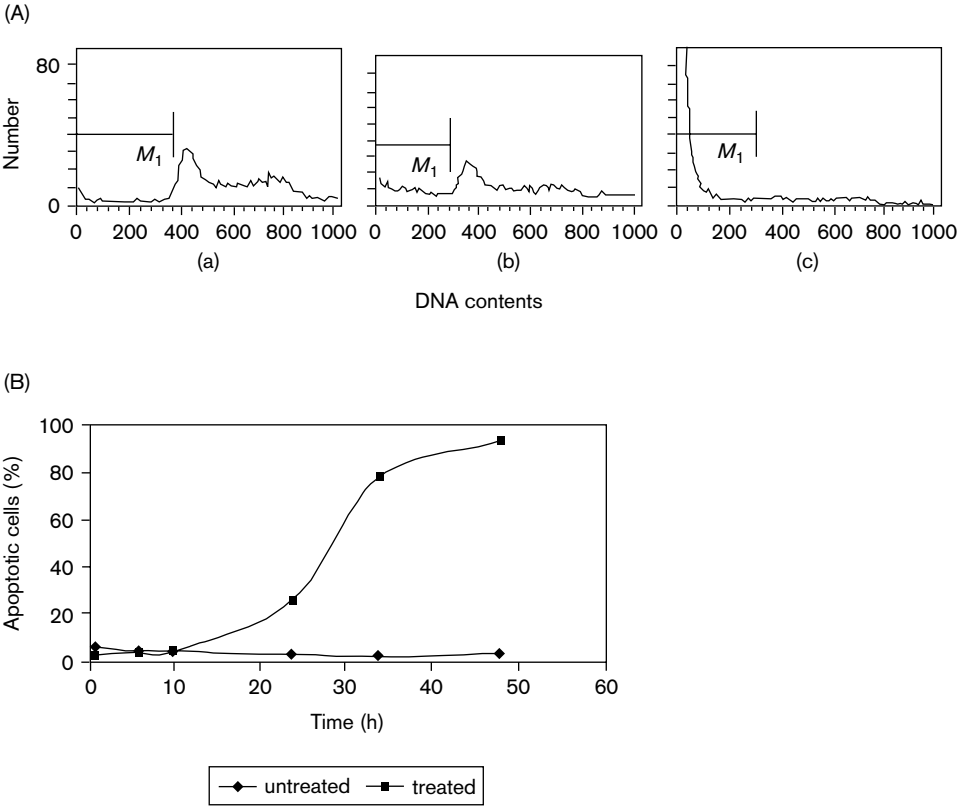

Figure 5. Quantitative detection of apoptotic K-562 cells (cells in the sub-G_1 phase) treated with 25 μM salvicine for different time periods by flow cytometry. (A) DNA histograms of apoptotic cells. M_1 indicates apoptotic cells. (a) Control, (b) 24 h and (c) 48 h. (B) Time-dependent effects of salvicine-induced apoptosis in K-562 cells.

and time-dependent manner. The cytotoxic intensity of salvicine after 24 h of treatment on human leukemia K-562 and human gastric tumor SGC-7901 cells was very similar. These results are in agreement with the results obtained from our previous studies, the IC_{50} of salvicine after 72 h treatment on these two cell lines lie in the same range, 7.79 and 7.82 μM, respectively.[9] Gastric carcinoma is one of the most commonly encountered malignant tumors in China and Asia. Chemotherapy and radiation therapy of gastric carcinoma has met with limited success at present. Therefore, we chose SGC-7901 as an *in vitro* model representing human solid tumors. In general, the antitumor effects of drugs seem to be more potent in human leukemia cells than in solid tumor cells. However, salvicine seems to be different. Previous studies have clearly shown that on average IC_{50} of salvicine in 12 human solid tumor cell lines was over 5.41 and 4.15 times more potent than the common plant-derived antitumor drugs vincristine (VCR) and etoposide (VP-16).[9] For three leukemia cell lines, the mean IC_{50} of salvicine was the same as that of VP-16 and weaker than that of VCR.[9] Thus, salvicine has been chosen as a prototype compound for developing antineoplasmic agents due to its remarkable inhibitory effects on the growth of human solid tumor cells.

Our present results show that salvicine can induce characteristic morphological and biochemical changes associated with apoptosis in both K-562 and SGC-7901 cells, and the ability of salvicine to induce apoptosis in these two cell lines was similar, further indicating salvicine causes this particular effect on solid tumor cells. Moreover, we have noted that salvicine exhibits more potent effects on growth inhibition than apoptosis induction at similar concentrations. Our previous studies have shown that salvicine blocked K-562 cells in the G_1 phase of the cell cycle in a dose- and time-dependent manner,[9] and also arrested SGC-7901 cells in the G_1 phase of the cell cycle (data not shown). Additionally, we found that DNA topoisomerase II is one of the cellular targets of salvicine (unpublished data). Therefore, it can be considered that the antitumor efficiency of salvicine results from multiple mechanisms of action, such as interfering with cell cycle progression, inducing apoptosis and targeting DNA topoisomerase II of tumor cells.

In conclusion, our results demonstrate that salvicine is capable of inhibiting growth and inducing apoptosis in both human leukemia K-562 and gastric tumor SGC-7901 cells, and these effects are dose and time dependent. These results suggest that the antitumor activity of salvicine is related to its ability to induce apoptosis. Considering that both K-562 and SGC-7901 cell lines are resistant to apoptosis and their current therapy is unsatisfactory, salvicine (which is highly cytotoxic in tumor cells) seems to be a promising anti-tumor agent. Salvicine is now ready to enter clinical trials.

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