Acta Pharmacologica Sinica 2005 Oct; 26 (10): 1248–1252

Full-length article

Anticancer activity of sodium caffeate and its mechanism[1]

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Key words

caffeic acid; anticancer activity; cell division; cell apoptosis; cell cycle; phytogenic antineoplastic agent; flow cytometry; Western blotting

[1]Project supported by the National Key Basic Research Development Program of China (No 2002CB513108).
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Received 2005-03-16
Accepted 2005-06-24

doi: 10.1111/j.1745-7254.2005.00196.x

Abstract

Aim: To study the anticancer activity of sodium caffeate (SC). **Methods:** A nucleoside transport assay was used to analyze the inhibitory effects of SC on nucleoside rescue. The MTT assay was used to measure cell proliferation. Flow cytometry was used to measure the apoptosis of BEC-7402 induced by SC and the cell cycle distribution change . Western blotting analysis was employed to investigate Bcl-2, caspase and Bax expression. Intracellular Ca^{2+} and mitochondrial membrane potential were determined by flow cytometry. *In vivo* anti-tumor activity was measured using a tumor transplantation model in mice. **Results:** SC inhibited the nucleoside transport of BEL-7402 cells with an IC_{50} of 1.02 mg/mL. SC inhibited tumor cell proliferation with an IC_{50} between 100 μg/mL and 200 μg/mL. SC induced BEL-7402 cell apoptosis in a time- and dose-dependent manner, which was induced by arresting cells in S phase. The *in vivo* study showed that tumor growth was inhibited in a dose-dependent manner. Activated caspase-3 and Bax expression were up-regulated after treatment with SC, while Bcl-2 expression was down-regulated. Intracellular Ca^{2+} was increased while mitochondrial membrane potential was decreased by SC. **Conclusion:** SC is a new anticancer agent with promising potential.

Introduction

There have been an increasing number of anticancer phytochemicals identified in our daily diet. Some of the most promising and extensively investigated are those present in the cruciferous family of vegetables, alliums and tea. Phytochemicals should be considered as an inexpensive and readily applicable, acceptable and accessible approach to cancer control and management for general populations. This is particularly important considering the sluggish progress made in cancer treatment. It is still an urgent task to seek new anticancer drugs from natural resources in oncology pharmacology.

Cinnamic acid is one of the phytochemicals with potential chemopreventive effects in preventing carcinogenesis[1,2]. Cinnamamide, a natural compound containing the cinnamic acid structure, is a new antitumor agent that acts on matrix metalloproteinase, which has been demonstrated by previous work in our laboratory[3]. Caffeic acid (3,4-dihydroxycinnamic acid) is a polyphenol that is found in coffee, fruits, vegetables, grains and many others plants[4–7]. It is also particularly abundant in propolis beehives with 20%–25% content and has various pharmacological activities, such as antioxidant and antiviral effects[8]. The anticancer effect of caffeic acid, however, has not been reported up to now. Because caffeic acid is prone to air oxidation and is only slightly soluble in water, its stable sodium salt (sodium caffeate, SC) was prepared in our laboratory and used in the present study. Here we report the antitumor effect of SC both *in vitro* and *in vivo*.

Materials and methods

Reagents RPMI-1640 medium was purchased from Gibco BRL (Gaithersburg, Maryland, USA). Fetal calf serum (FCS) was purchased from Hyclone (Logan, Utah, USA). MTT, nonidet P-40(NP-40), phenylmethylsulfonyl fluoride (PMSF), aprotinin, ponceau S, Triton X-100, propidium

iodide, Fluo-3, rhodamine 123, RNase A, proteinase K, Hoechst 33342 and other reagents were purchased from Sigma (StLouis , Missouri, USA). [H^3]TdR was purchased from Chinese Atomic Energy Institutes (Beijing, China). Annexin V-FITC/PI apoptosis detection kit was purchased from BioVision company (Hannover, Germany). Mouse anti-Bcl-2 monoclonal antibody, mouse anti-caspase-3 monoclonal antibody and rabbit anti-bax polyclonal antibody were products of Calbiochem (San Diego, California, USA). Horseradish peroxidase-conjugated secondary anti-mouse antibody and anti-rabbit antibody were products of Santa Cruz Biotechnology, Inc(Santa Cruz, California,USA). Enhanced luminol reagent and oxidizing reagent were products of NEN Life Science Products (Boston, Massachusetts, USA). Dr Dan-qing SONG in Department of Chemistry, Institute of Medicinal Biotechnology (Beijing, China) synthesized the SC.

Cells and carcinoma Human oral cavity epidermis squamocellular carcinoma cell line (KB), human hepatocarcinoma cell line (BEL-7402) and human acute promyelocytic cell line (HL-60) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and were grown routinely in RPMI-1640 supplemented with 10% heat-inactivated FCS. The medium was supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine, and the cells were incubated in a humidified atmosphere, with 5% CO_2 in air at 37 °C.

Mouse-transplanted hepatocarcinoma H22 and mouse colorectal cancer C26 cell lines were maintained by serial transplantation into mice in our laboratory. Kunming species mice were supplied by the Experimental Animal Center, Chinese Academy of Medical Sciences (Beijing, China).

Nucleoside transport assay[9] Briefly, cells in the logarithmic growth phase were harvested. The cell suspension was prepared with RPMI-1640 medium at 5×10^6 in 0.9 mL in each test tube. Different concentrations of SC in 0.1 mL RPMI-1640 were added and the tube was kept in a water bath at 37 °C for 5 min. Phosphate-buffered solution (PBS) was used as a control. RPMI-1640 0.1 mL (containing 3.7×10^4 Bq of [^{3}H]TdR in medium free of serum) was added for 30 s and 5 mL ice-cold normal saline was added to terminate the reaction. The reaction was filtrated through a GF/B glass fiber filter (Whatman International, Maidstone, UK) under vacuum. The filters were washed with 0.2 mL of 1 mol/L NaOH and 0.5 mL ethanol, dried under vacuum and placed in scintillation vials containing 2 mL of dimethylbenzene with 0.4% PPO/0.01% POPOP. The cpm (counts per minute) were measured using an LS-9800 scintillometer (Beckman Instruments, Fullerton, California, USA).

MTT assay Briefly, cells in the logarithmic growth phase were harvested and seeded in 96-well plates (Costar, Cambridge, Massachusetts, USA) overnight. The test compound was added and cells were further incubated for 72 h. The viability of cells was determined using the MTT assay according to the method described by Carmichael *et al* [10].

Long-term clonogenicity Cell survival was tested using a clonogenic assay, as described by Valduga *et al*[11]. Briefly, cells in the logarithmic growth phase were harvested and 250 cells/mL of a single-cell suspension was prepared with medium. The cell suspension 200 μL was seeded in 96-well plates (50 cells/well) overnight, and the test compound was added. After 1 week of incubation at 37 °C in air with 5% CO_2, colonies were counted.

Flow cytometry After appropriate treatment, cells were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 75% ethanol for 18 h at 4 °C. The cell apoptosis was measured according to the protocol of Annexin V-FITC/PI apoptosis detection kit. Cell cycles change was measured by treatment of the fixed cell suspensions which were washed with PBS and stained with 80 μL of 50 μg/mL propidium iodide and 50 μg/mL RNase A for 30 min in the dark. Samples were run through an EPICS XL flow cytometer (Coulter, Miami, Florida, USA). Results are presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence[12]. The results on flow cytometry represented the average of 3 individual experiments.

Western blotting assay The cells were lysed in lysis buffer at 4 °C with sonication. The lysates were centrifuged at 15 000×*g* for 15 min and the concentration of protein in each lysate was determined using Coomassie Brilliant Blue G-250. Loading buffer was added to each lysate, which was subsequently boiled for 3 min and then electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and incubated with anti-Bcl-2, anti-caspase-3 or anti-Bax antibodies and then with peroxidase-conjugated secondary antibodies. Detection was carried out using an enhanced chemiluminescence agent[13,14]. The results on Western blot analysis represented the average of 3 individual experiments.

Intracellular Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi$) Following appropriate treatment, cells were collected by centrifugation and incubated at 37 °C with Fluo-3 for 40 min and then with rhodamine 123 for 20 min. Cells were then washed 3 times with cold PBS, and the intracellular Ca^{2+} concentration and $\Delta\psi$ were measured by flow cytometry[15–17].

Tumor transplantation and drug administration H22 as-

cites were diluted to 7.5×10^5/mL suspension and 0.2 mL of the cell suspension was inoculated subcutaneously into the right axilla of each mouse of the Kunming species (weighing 20±2 g). A C26 tumor-cell suspension was prepared by gently suspending tumor tissue in normal saline (1 g of tumor tissue with 3 mL of normal saline) in a cold water bath and inoculated as above. After 24 h of tumor cell inoculation, SC was administered intraperitoneally for 10 d. Normal saline was used as a control. On the d 11 the mice were killed, and body weight and tumor tissue were weighed.

Statistical analysis The data are the mean values of at least 3 experiments and are expressed as mean±SD. The Student's *t*-test was used to compare data. $P<0.05$ was considered to be statistically significant.

Results

Inhibition of nucleoside transport by SC SC inhibited nucleoside transport in the hepatocarcinoma BEL-7402 cell line with an IC_{50} of 1.02 mg/mL. However, the inhibiting effect on nucleoside transport was not very strong.

Inhibition of proliferation and induction of apoptosis by SC SC inhibited tumor-cell proliferation with an IC_{50} of between 100 μg/mL and 200 μg/mL (Table 1). Inhibition of BEL-7402 cells proliferaction by SC was dose-dependent and time-dependent (Figure 1).

Table 1. Inhibition of proliferation by sodium caffeate treatment for 72 h in different cell lines. *n*=3. Mean±SD.

Cell line	IC_{50} /μg·mL^{-1}
HL-60	135±19
KB	157±22
BEL-7402	192±28

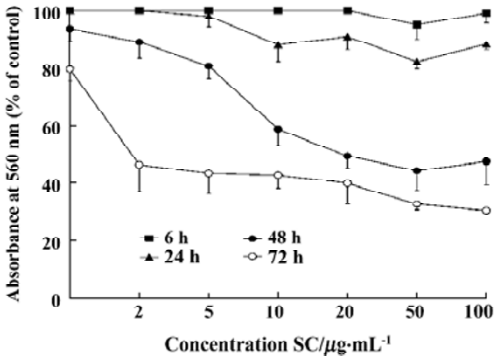

Figure 1. Inhibition of proliferation by SC in BEL-7402 cells. n=3. Mean±SD.

Flow cytometry showed that SC induced BEL-7402 cell apoptosis in a time- and dose-dependent manner (Table 2). After 24 h of treatment with SC, the cell cycle changed. The percentages of cells in S phase increased markedly while percentages of cells in G_2/M phase decreased, which suggested the apoptosis was induced by arresting the cells in S phase (Table 3).

Table 2. SC-induced apoptosis (%) in BEL-7402 cells. *n*=3. Mean±SD.

SC /μg·mL^{-1}	24 h	48 h	72 h
0	2.3±1.2	2.4±1.1	7.8±5.2
25	13.2±4.5	15.0±2.8	42.7±15.5
100	27.4±3.9	44.4±10.1	55.9±16.3
400	41.1±10.1	62.4±12.5	77.7±18.5

Table 3. Effect of sodium caffeate on cell cycle of BEL-7402 cells. *n*=3. Mean±SD.

SC /μg·mL^{-1}	G_1	S	G_2/M
0	55.0±7.2	22.3±2.3	22.7±2.3
10	64.4±10.0	26.7±5.6	8.9±2.2
25	65.1±11.1	28.8±6.9	6.1±1.7
100	65.7±8.9	34.3±7.5	0.0±0.0
400	66.5±7.1	33.5±5.6	0.0±0.0

Inhibition of clonogenicity by SC SC inhibited cell clonogenicity with an IC_{50} between 0.5 μg/mL and 3 μg/mL (Table 4). SC was capable of inhibiting the different cell lines to different extents.

Table 4. Clonogenicity inhibition by sodium caffeate. *n*=3. Mean±SD.

Cell line	IC_{50} /μg·mL^{-1}
KB	0.45±0.04
BEL-7402	2.89±0.07

Effect of SC on transplanted tumor growth H22 and C26 were inoculated subcutaneously into mice. After SC administration for 10 d, tumor growth was inhibited in a dose-dependent manner. No significant difference in body weight was found between the groups, suggesting that SC does not show toxicity *in vivo* (Table 5 and 6).

Table 5. Anti-tumor activity of sodium caffeate treatment for 10 d on Kunming mice with transplanted H22 cells . $^cP<0.01$ *vs* control. n=10. Mean±SD. No mouse died during the experimental period.

Group	Dose /g· kg^{-1}	Body weight change/g	Tumor weight/g	Inhibition/%
Control	0.00	+4.9	4.13±0.19	0
SC	0.15	+4.3	3.00±0.69	27.4^c
SC	0.50	+5.1	2.90±0.58	29.9^c
SC	1.00	+4.0	2.40±0.64	41.9^c
SC	2.00	+5.5	1.19±0.59	71.2^c

Table 6. Anti-tumor activity of sodium caffeate treatment for 10 d on Kunming mice with on transplanted C26 cells. $^cP<0.01$ *vs* control. n=10. Mean±SD. No mouse died during the experimental period.

Group	Dose /g· kg^{-1}	Body weight change/g	Tumor weight/g	Inhibition/%
Control		+2.5	2.66±0.54	
SC	0.5	+3.8	1.85±0.65	30.5^c
SC	1.0	+5.8	1.22±0.36	54.1^c
SC	2.0	+5.6	1.13±0.42	57.5^c

Effect of SC on expression of apoptosis-associated proteins The bands were scanned with light density. Activated caspase-3 and Bax expression were up-regulated after SC treatment, while Bcl-2 expression was down-regulated (Figure 2).

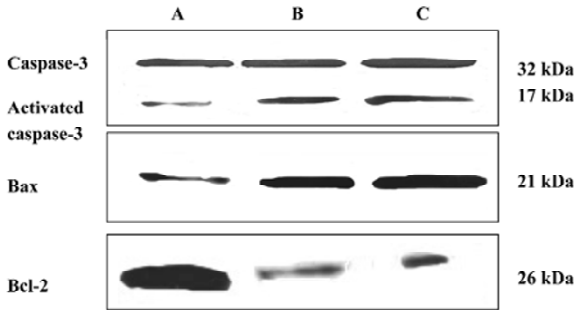

Figure 2. Caspase-3, Bax and Bcl-2 expression in BEL-7402 cells treated with sodium caffeate (SC). (A) Control; (B) 10 μg/mL SC; and (C) 25 μg/mL SC.

Effect of SC on intracellular Ca^{2+} and mitochondrial membrane potential After treatment with 10 μg/mL SC for 24 h, intracellular Ca^{2+} was increased 1.78-fold compared to the control. Δψ was decreased by 22.7% compared to the control. The results showed that SC increased intracellular Ca^{2+} levels and decreased Δψ.

Discussion

Most anti-metabolites in tumor chemotherapy inhibit nucleoside *de novo* synthesis but can not block nucleoside rescue in cancer cells. It is therefore important to control nucleoside rescue by inhibiting nucleoside transport. Previous work found that dipyridamole enhanced the anticancer effect of acivicin by inhibiting nucleoside transport[9].The present study demonstrated that SC was a new member of the nucleoside transport inhibitor family.

Caffeic acid is an active phytophenol that has been found to inhibit rat glutathione-*S*-transferase isoenzymes both *in vitro* and *in vivo*[18]. A large number of population-based studies have found that consumption of wholegrains, vegetables and fruits abundant in caffeic acid reduces the risk of cancer[19–21]. The aqueous extract of *Salvia miltiorrhiza*, a traditional Chinese herb containing caffeic acid was found to strongly inhibit the proliferation of human hepatoma $HepG_2$ cells. It was also observed that its crude extract caused apoptotic cell death[22]. Salvianolic acid A, a caffeic acid trimer, showed synergistic effects in combination with other antitumor agents. Further, salvianolic acid A could increase the antitumor effects of 5-flurouracil without increasing its toxicity in an animal study [2]. However, no report has been published on the anticancer effects of caffeic acid either *in vitro* or *in vivo*. We are the first to report that the sodium salt of caffeic acid inhibits proliferation of cancer cells, with IC_{50} between 100 μg/mL and 200 μg/mL. Further, we showed that it induced the apoptotic cell death and changed cell-cycle distribution by arresting cells in S phase. The *in vivo* study showed that SC inhibited the tumor growth of transplanted H22 and C26 cells in mice with an inhibition rate of 42%–54% when treated with 1 g/kg SC for 10 d.

Preliminary studies on the anticancer mechanism of SC demonstrated that after treatment with SC, Bcl-2 expression was down-regulated and mitochondrial membrane permeability was changed. The mitochondrial permeability transition pore was opened and the mitochondrial membrane potential was broken up. The mitochondrion was swelled and in α state of hyperosmosis before apoptosis was induced. Meanwhile cytochrome c was released, caspase-3 was activated in the presence of Apaf-1 and caspase-9, and apoptosis was induced[23–27].

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