Cancer Chemother Pharmacol (2005) 55: 286–294
DOI 10.1007/s00280-004-0877-z

ORIGINAL ARTICLE

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DNA damage, c-*myc* suppression and apoptosis induced by the novel topoisomerase II inhibitor, salvicine, in human breast cancer MCF-7 cells

Received: 8 April 2004 / Accepted: 28 May 2004 / Published online: 16 November 2004

Abstract Salvicine, a diterpenoid quinone compound, possesses potent in vitro and in vivo antitumor activity. Salvicine is a novel non-intercalative topoisomerase II poison. In this study salvicine induced evident DNA damage, which was further characterized as double-strand breaks mainly in MCF-7 human breast cancer cells. The degree of damage was highly correlated with growth inhibition of MCF-7. Using a PCR-stop assay we demonstrated that this damage was selective. Preferential damage occurred in the p2 promoter region, but not the 3′-end of the protooncogene c-*myc*. The expression of oncogenes, such as c-*myc* and c-*jun*, was additionally investigated. Salvicine induced a dose-dependent decrease in c-*myc* gene transcription, concomitant with an increase in c-*jun* expression. Furthermore, reverse-transcription PCR and Western blotting data revealed that salvicine failed to stimulate the mRNA and protein levels of p53 and its downstream targets p21 and bax. The phosphorylation degree of serine 15 of p53, which is thought to be an active form of p53 in response to cellular DNA damage, remained in a steady state. In view of these results, we propose that the downregulation of c-*myc* resulting from selective damage plays a role in apoptosis signaling. Moreover, salvicine-induced apoptosis in MCF-7 subsequent to DNA damage seems to be mediated through a p53-independent pathway.

Keywords Salvicine · DNA damage · Apoptosis · c-*myc* · p2 promoter · *p53*

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Abbreviations Topo II: Topoisomerase II · DSBs: DNA double-strand breaks · MDR: Multidrug resistance · RT-PCR: Reverse transcription-polymerase chain reaction · *m*-AMSA: Amsacrine

Introduction

DNA topoisomerase II (Topo II) is a major molecular target for a number of conventional antitumor drugs [1, 2]. One type of Topo II-targeting agent, designated Topo II poison, elicits DNA double-strand breaks (DSBs) due to its ability to convert target enzymes to a DNA-damaging toxin through stabilization of a "cleavable complex" comprising Topo II and DNA. DSBs are the most severe damage on genomic DNA. However, the characteristics of the signal transduction pathways leading to growth arrest and/or cell death subsequent to DNA breakage are yet to be established.

Salvicine, a structurally modified diterpenoid quinone derivative isolated from *Salvia prionitis* [3] (Fig. 1), is a novel Topo II poison. The compound displays potent in vitro and in vivo activity against malignant tumor cells and xenografts [3, 4]. DNA Topo II is the primary molecular target of salvicine [5]. The compound promotes Topo II-DNA binding, and inhibits prestrand and poststrand Topo II-mediated DNA break religation, thus stabilizing the cleavable complexes [6]. Salvicine additionally induces apoptosis in various tumor cell lines [7–9], and exhibits a broad spectrum of antimultidrug resistant (MDR) activity [9]. These findings collectively indicate that salvicine is a promising antitumor drug candidate. Initial clinical trials are currently underway in China.

The signaling pathway leading to growth arrest and/or cell death subsequent to DNA damage induced by salvicine has not been fully elucidated. Targeting Topo II-mediated DNA damage is closely correlated with cytotoxicity. Several lines of evidence show that Topo II

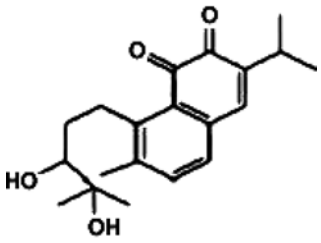


Fig. 1 Chemical structure of salvicine

inhibitors display gene selectivity in damaging DNA. For example, Adriamycin [10], amsacrine [10, 11], and pMC450 [12] induce preferential damage within the c-*myc* locus and suppress c-*myc* expression. The c-*myc* oncogene plays a central role in cell proliferation and apoptosis, and is generally amplified in various human cancers, including breast, lung, and cervical carcinomas [13]. Deregulation of c-*myc* is an element of the DNA damage response pathway in breast cancer cells [14], and leads to apoptosis in various cells. The c-*jun* gene may act as the downstream target of c-*myc* for apoptosis induction [15], and the full myc-mediated apoptotic response requires p53 involvement [16]. In this study, we analyzed the influence of salvicine on DNA damage, and its relationship to the expression of key genes participating in apoptosis of human breast cancer MCF-7 cells.

Materials and methods

Agents

Salvicine (tangerine yellow crystalloid, purity above 99.3%) was kindly provided by Prof. Jin-Sheng Zhang from the Department of Phytochemistry in our Institute. The compound was dissolved at a concentration of 10 mmol/l in dimethyl sulfoxide (DMSO) as a stock solution, stored at −20°C in the dark, and diluted to the desired concentrations with normal saline before use. The final DMSO concentration did not exceed 0.1%.

Cell culture

The human breast cancer cell line, MCF-7, was obtained from American Type Culture Collection (Rockville, Md.). Cells were maintained as a monolayer culture in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum, 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 m*M* sodium pyruvate, and supplemented with 0.01 mg/ml bovine insulin under a humidified atmosphere of air containing 5% CO_2 at 37°C.

Cell survival assessment

Cell survival was assessed using two independent methods. Short-term growth inhibition of MCF-7 by salvicine was evaluated using the sulforhodamine B (SRB) assay [17] with minor modifications. Briefly, cells (5×10^4 ml^{-1}) at 100 μl per well were seeded in 96-well plates. Cells were treated in triplicate with gradient concentrations of salvicine for 4 h at 37°C, washed in phosphate-buffered saline (PBS, pH 7.4), and incubated with fresh medium for an additional 72 h. At the end of culture, cells were fixed in situ by adding 100 μl ice-cold 10% (w/v) trichloroacetic acid (TCA) and incubating for 1 h at 4°C, following removal of the medium. The supernatant was discarded, and plates were washed five times with distilled water and dried at room temperature. SRB solution (4 mg/ml in 1% acetic acid) (100 μl) was added to each well, and the culture was incubated for 15 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid, and plates were air-dried. The bound stain was solubilized with Tris buffer, and optical density was measured by absorbance at 515 nm using a multiwell spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, Calif.). The cytotoxicity of salvicine in MCF-7 cells is expressed as the IC_{50} value, which was determined from the dose-response curve.

The colony-formation assay was used to confirm the result of the SRB assay and further evaluate the effect of salvicine on the ability of MCF-7 cells to continuously divide [18, 19]. Briefly, cells were seeded in a six-well plate at 10^5 per well and incubated overnight to ensure that all cells were in exponential growth. Drug was administered to the cells at the concentrations and for the time intervals specified in the figure legend. Plates were then washed in three complete changes of medium to remove the salvicine, and the cells were trypsinized and replated at 300 cells/well. Following 7 days of incubation, clones were fixed in methanol/acetic acid (3:1), stained with crystal violet (400 mg/ml) for 20–30 min, and washed gently three times with distilled water. Clones were counted under an anatomical microscope and scored if they contained more than 50 cells. The colony-formation rate was determined according to the following expression: colony formation rate (%) = (number of clones containing more than 50 cells)/(number of cells seeded)×100.

DNA damage assay

The alkaline unwinding assay was used to detect DNA damage induced by salvicine, as described previously [20]. Briefly, cells were incubated with salvicine for 4 h at 37°C, and washed twice with ice-cold PBS. The cell pellet was resuspended in PBS, and the cell number was determined prior to analysis. The assay is based on the differential binding to single-strand and double-strand DNA, and fluorescence of bisbenzimide trihydrochloride (Hoechst 33258) after a fixed period of alkaline denaturation. Experiments were performed on three different groups: (1) double-strand DNA control with no alkaline-induced DNA unwinding, (2) cells subjected to

a 30-min alkaline-induced DNA unwinding period, and (3) total single-strand DNA whereby cells were sonicated before alkaline unwinding. The *F*-values, determined according to the expression (alkali-treated DNA−single-strand DNA)/(double-strand DNA control−single-strand DNA), were determined in triplicate.

DNA strand breaks were evaluated using the neutral single-cell microgel electrophoresis assay (comet assay) described previously [21] with minor modifications. Briefly, the cell samples (about 40,000 cells in 30 μl) were carefully resuspended in 70 μl 0.5% low melting point agarose, layered onto microscope slides precoated with 50 μl 1% normal melting point agarose and spread with a cover slip carefully to avoid producing a bubble. After solidification for 10 min at 4°C and removal of the cover slip, the slides were immersed in cold fresh lysing solution (2.5 *M* NaCl, 100 m*M* Na_2EDTA, 10 m*M* Tris, 10% DMSO, 1% Triton X-100 and 1% laurosylsarcosinate) for 1 h at 4°C in a dark chamber. After lysis, the slides were equilibrated for 20 min with TBE buffer (90 m*M* Tris, 90 m*M* boric acid, 2 m*M* EDTA, pH 8.0) and electrophoresis was performed in TBE buffer at 1.67 V/cm for 20 min. After electrophoresis, the slides were dried at room temperature for 5–10 min and then 20 μl 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml in PBS) was pipetted onto the agarose surface under a cover glass. Individual cells were viewed using an Olympus BX51 UV light fluorescence microscope. DNA strand breaks were quantitated by analyzing at least 50 randomly selected comets per slide with Komet 5.5 software (Kinetic Imaging, Nottingham, UK). The parameters [22] taken were tail length (estimated leading edge from the nucleus; μm), L/H (the ratio of tail length to head diameter), and olive tail moment (arbitrary units). The definition of olive tail moment is described in Komet 5.5 user guide.

PCR-stop assay

A PCR-stop assay was performed as previous described [10]. Briefly, MCF-7 cells were incubated with salvicine for 4 h at 37°C, harvested and washed twice with ice-cold PBS. Pellets were resuspended in 100 μl lysis buffer (50 m*M* KCl, 10 m*M* Tris-HCl, pH 9.0, 0.5% Triton X-100, 0.45% Tween-20, 0.06 mg/ml proteinase K). Lysates were incubated at 50°C for 2 h and centrifuged at 10,000 *g* for 3 min. The supernatant was subjected to PCR. Amplification was performed using the following primers derived from the c-*myc* gene. A 283-bp fragment of c-*myc* corresponding to the P2 promoter was amplified with primers spanning the region from nucleotide 2392 (5′-TCGAGAAGGGCAGG-3′) to nucleotide 2674 (5′-CCCTATTCGCTCCGGATC-3′), and a 213-bp fragment of the 3′ region was amplified with the primers 5′-TTCGTTTCTTCCCCCTCCCA-3′ and 5′-CCCTGCTTCTGCCATTCC-3′. A typical 50-μl reaction system contained 50 m*M* KCl, 10 m*M* Tris-HCl (pH 9.0), 1 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 0.2 μ*M* of each primer, 5 μl cell lysate (1 μg DNA), and 1 μl (2 U) HotStart polymerase. The initial heating step was at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 55°C for 30 s, and 72°C for 30 s. The final cycle was followed by an extra 7 min of polymerization at 72°C before cooling to 4°C. Reaction products (10 μl) were separated on a 1.5% agarose gel, stained with ethidium bromide (0.5 μg/ml), and visualized using a GDS8000 Gel Documentation System (UVP, Upland, Calif.). Bands were semiquantitated and analyzed using Gelworks 1D Intermediate version 2.01 software.

RT-PCR assay

Following treatment of cells with various concentrations of salvicine for the required periods, total RNA was extracted using TRIzol reagent (Sangon, Shanghai, China), according to the manufacturer's instructions. RNA yield and purity were assessed by spectrophotometric analysis. Total RNA (1 μg) from each sample was subjected to reverse transcription with random hexamer, dNTPs, and M-MLV reverse transcriptase in a 20-μl reaction mixture. PCR of cDNA was performed using HotStart polymerase, dNTPs, and the following related primers: 5′-CCA TGG AGA AGG CTG GGG-3′ (sense) and 5′-CAA AGT TGT CAT GGA TGA CC-3′ (antisense) for glyceraldehydes-3-phosphate dehydrogenase (GAPDH); 5′-CTG GTG CTC CAT GAG GAG-3′ (sense) and 5′-AGG TGA TCC AGA CTC TGA C-3′ (antisense) for c-*myc*; 5′-AAC GAC CTT CTA TGA CGA TGC CCT C-3′ (sense) and 5′-GCG AAC CCC TCC TGC TCA TCT GTC-3′ (antisense) for c-*jun*; 5′-CCT TCC CAG AAA ACC TAC CA-3′ (sense) and 5′-TCA TAG GGC ACC ACC ACA CT-3′ (antisense) for p53; 5′-GGG GGC ATC ATC AAA AAC TT-3′ (sense) and 5′-ACT GAA GGG AAA GGA CAA GG-3′ (antisense) for p21; and 5′-ACC AAG AAG CTG AGC GAG TGT C-3′ (sense) and 5′-ACA AAG ATG GTC ACG GTC TGC C-3′ (antisense) for bax. Amplified products were separated on a 1.5% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualized using a GDS8000 Gel Documentation System (UVP, Upland, Calif.).

Western blotting

Cells (5×10^5 ml^{-1}) were seeded into six-well plates and treated with salvicine for the desired periods. After washing twice with ice-cold PBS, protein extracts were prepared by lysing cells in RIPA buffer (2 m*M* sodium orthovanadate, 50 m*M* NaF, 20 m*M* Hepes, pH 7.5, 150 m*M* NaCl, 1.5 m*M* $MgCl_2$, 5 m*M* sodium pyrophosphate, 10% glycerol, 0.2% Triton X-100, 5 m*M* EDTA, 1 m*M* PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) on ice for 30 min. Insoluble material was pelleted at 13,000 *g* for 20 min at 4°C. Equal amounts of protein (20 μg) were electrophoresed on 10% or 15%

sodium dodecyl sulfate (SDS) polyacrylamide gels. Next, gels were electroblotted onto nitrocellulose membranes. The following antibodies and concentrations were used: mouse monoclonal anti-human c-*myc* (1:500, Santa Cruz Biotechnology, Santa Cruz, Calif.), rabbit polyclonal anti-human c-*jun* (1:1000, Santa Cruz), mouse monoclonal anti-human p53 (1:2000, Santa Cruz), mouse monoclonal anti-phospho-p53 (ser-15) (1:1000, Cell Signaling Technology, Beverly, Mass.), mouse monoclonal anti-human p21 (1:500, Oncogene, San Diego, Calif.), mouse monoclonal anti-human Bax (1:500, Santa Cruz), goat polyclonal anti-human β-actin (1:1000, Santa Cruz). The secondary antibodies used for c-*myc*, total p53, phospho-p53, p21, bax, c-*jun*, and β-actin were anti-mouse (1:1000), anti-rabbit (1:1000) or anti-goat (1:1000) horseradish peroxidase-labeled antibodies. Results were visualized by enhanced chemiluminescence (ECL, Pierce Biotech, Rockford, Ill.).

Apoptosis assessment

Morphological changes characteristic of apoptosis were evaluated by staining cell nuclei with DAPI followed by viewing with an Olympus UV light fluorescence microscope. Briefly, after treatment with salvicine for 24 h or 48 h, cells were washed twice with ice-cold PBS and fixed with 4% paraformaldehyde for 30 min at room temperature in the dark, stained with 0.5 μg/ml DAPI for 2 min, rewashed twice with ice-cold PBS, and visualized with a UV light fluorescence microscope. Apoptotic MCF-7 cells were quantitated using an Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, Calif.), according to the manufacturer's instructions. At least 10,000 cells from each sample were examined using a FACSCalibur analyzer (Becton Dickinson, San Jose, Calif.). Experiments were repeated twice.

Statistical analysis

Data from independent experiments of growth inhibition and DNA damage were analyzed by Pearson correlation analysis for least significant difference using the statistical software package SAS 8.0. Differences at $P < 0.05$ were considered significant.

Results

DNA damage by salvicine is positively related to growth inhibition

The alkaline unwinding assay was used to detect DNA damage induced by salvicine in MCF-7 cells. Salvicine induced bulk DNA damage at 2.5 μ*M* (Fig. 2), and the *F* value decreased with increasing salvicine concentration. The decreasing *F* value trend indicates that DNA damage induced by salvicine accumulates in a concentration-dependent manner. Bulk DNA damage elicited by salvicine is positively related to its antiproliferative or cytotoxic effects in the same cell line in the range 2.5–40 μ*M*. The growth inhibition rates increased from 7.69% at 2.5 μ*M* to 93.72% at 40 μ*M* as the *F* value decreased from 0.90 to 0.43 in MCF-7 cells (Fig. 2a). In view of the finding that DNA integrity is crucial to maintain cell survival, the observed correlation indicates that salvicine-elicited bulk DNA damage contributes to growth inhibition. Results from the clonogenic survival assay were consistent with those from the SRB assay and further validated the correlation of DNA damage with cell survival (Fig. 2b).

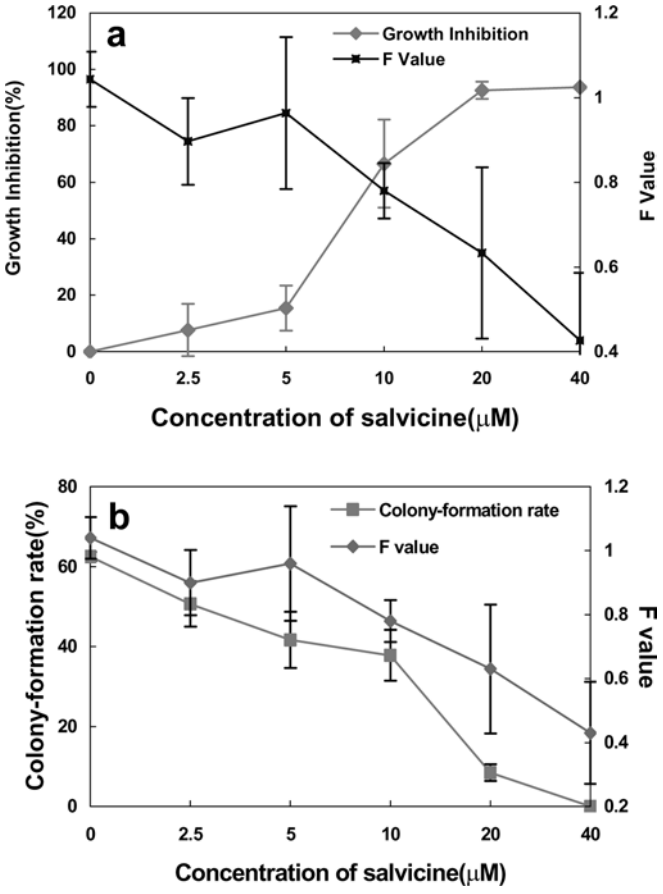


Fig. 2 Induction of DNA damage and growth inhibition by salvicine in MCF-7 cells. MCF-7 cells were treated with various concentrations of salvicine for 4 h. DNA damage was determined by the alkaline unwinding assay, and cell survival was evaluated using the SRB assay and the clonogenic survival assay. Each value is the mean ± SD from three independent experiments. **a** Correlation between DNA damage and growth inhibition detected by the SRB assay (Pearson correlation coefficient $r = -0.939$, $P = 0.0055$). **b** Correlation between DNA damage and colony-formation rate (Pearson correlation coefficient $r = 0.955$, $P = 0.003$)

DNA damage by salvicine is mainly DSBs

The alkaline unwinding assay provides only an average value for DNA damage, but does not identify the type of damage. Thus, we further evaluated the type of damage induced by salvicine using the neutral comet assay,

which permits the detection of DNA DSBs but not single-strand breaks. As shown in Fig. 3a, treatment with 2.5 μ*M* salvicine led to the appearance of an obscure "halo" around the nucleus of the MCF-7 cell. Comet tails became evident when the salvicine concentration exceeded 5 μ*M*, and extended with increasing dose. At salvicine concentrations of up to 20 μ*M*, the nucleus almost disappeared, and was accompanied by the appearance of a "broom-like" tail indicating severe DNA damage. The degree of salvicine-induced DSBs was reflected more clearly using the semiquantitative comet parameters (Fig. 3b). The parameters including tail length, L/H, and Olive tail moment all increased significantly in a dose-dependent manner. This finding is consistent with data from the alkaline unwinding assay (Fig. 2), suggesting that DSBs are the principal form of DNA damage induced by salvicine in MCF-7 cells.

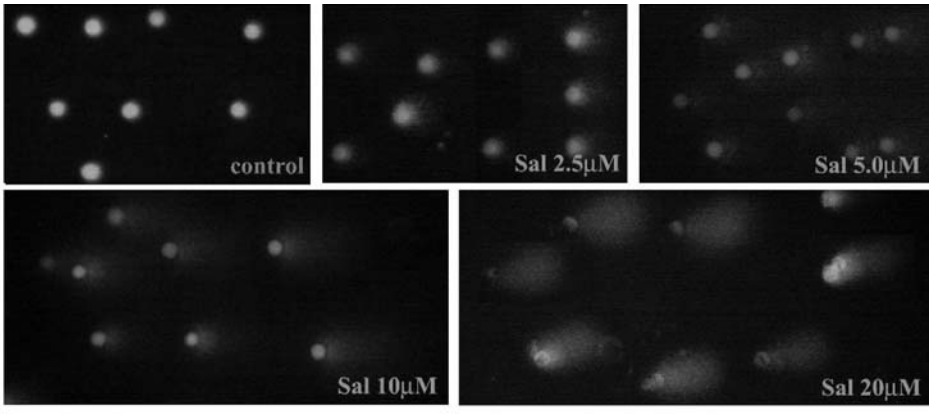
Salvicine elicits selective damage in the p2 promoter region vs the 3′-end of the c-*myc* gene

Several Topo II poisons induce distinct DNA breaks within genes (such as c-*myc*) which are closely associated with cell survival and death. Salvicine-induced non-random DNA damage in MCF-7 cells was evaluated by the PCR-stop assay after 4 h of exposure. We analyzed two fragments of c-*myc*, one at the 3′-end region and the other within the P2 promoter. As shown in Fig. 4a, amplification of the 3′-end region revealed no evident change, whereas the PCR products of the P2 promoter fragment decreased with increasing salvicine concentration. The ratio of the P2 promoter/3′-end region in the presence of 10 μ*M* salvicine was reduced to 19.3% of that measured in untreated cells (Fig. 4b). The results indicate selective cleavable site(s) for salvicine in the P2 promoter of c-*myc*, thus confirming that salvicine-induced DNA damage is not random.

Salvicine inhibits c-*myc* but stimulates c-*jun* mRNA and protein levels

We further investigated the effect of salvicine on the mRNA and protein levels of c-*myc* and c-*jun*. Salvicine induced a decrease in mRNA expression, with a simultaneous enhancement in c-*jun* mRNA levels in a concentration-dependent manner (Fig. 5a). Changes in the protein levels also showed the same trend (Fig. 5b,c). Decreased levels of c-*myc* mRNA may be a result of

a



b

Group	Tail length (∝m)	L/H	Olive tail moment (Arbitrary unit)
control	9.80±0.97	0.67±0.34	0.55±0.06
SAL 2.5∝M	21.43±0.74	1.64±0.08	3.04±0.14
SAL 5.0∝M	31.70±1.09	2.04±0.11	6.60±0.28
SAL 10∝M	53.03±1.69	3.62±0.32	14.00±0.65
SAL 20∝M	56.70±2.40	4.14±0.55	21.56±1.27

Fig. 3 Determination of DNA strand breakage using the neutral comet assay viewed under a fluorescence microscope (×200). MCF-7 cells were incubated at the specified concentrations of salvicine (*SAL*) for 4 h. **a** Morphological appearance of MCF-7 cells after neutral electrophoresis. **b** Semiquantitative analysis of the images in **a** expressed as tail length, L/H, and Olive tail moment. The data are the mean values of two experiments

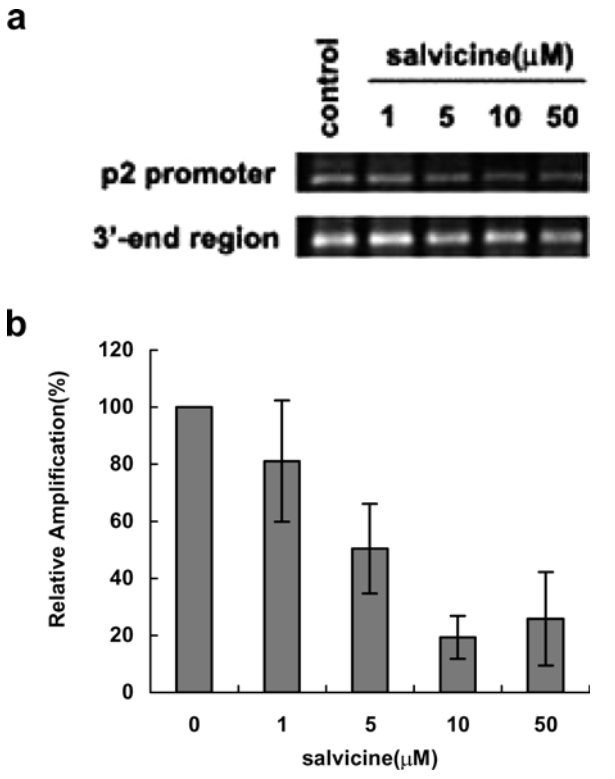


Fig. 4 Effects of salvicine on the amplification signal generated after PCR performed within the P2 promoter and 3′-end region of the c-*myc* gene of MCF-7 cells. **a** Representative image of amplification products. **b** Semiquantitation of the bands. Experiments were repeated at least three times

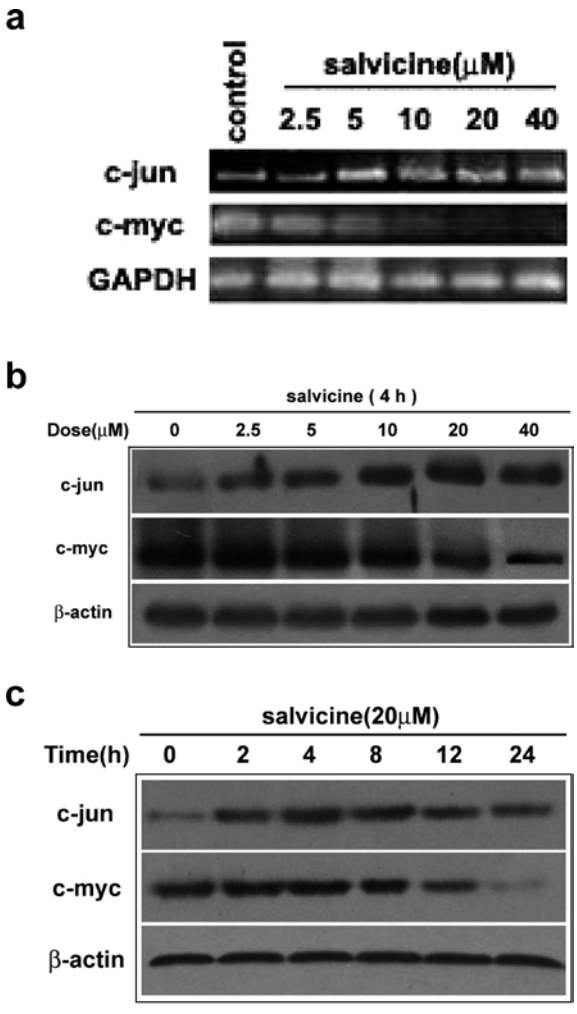


Fig. 5 Effects of salvicine on the c-*myc* and c-*jun* in MCF-7 cells. **a** mRNA levels following salvicine treatment for 4 h. **b** Effect of dose on protein levels following salvicine treatment for 4 h. **c** Effect of time on protein levels following treatment with salvicine at 20 μ*M*. Experiments were repeated at least three times

reduced transcription subsequent to P2 promoter damage induced by salvicine. It is worth noting that the alteration in c-*jun* expression occurred before the alteration in c-*myc* expression (Fig. 5c).

Salvicine does not alter the activation and transcription activity of p53

The p53 tumor suppressor gene plays an important role in cellular responses to various stress signals, particularly in cellular DNA damage response pathways. The regulation of p53, which may act as a transcription factor, is complex. Multiple mechanisms are implicated including protein stability and localization as well as post-translational modification. In particular, phosphorylation of p53 within the amino terminus (at serine 15) is a critical regulator for cellular response to endogenous DNA damage [23, 24]. Therefore, we examined the expression of p53 and its downstream target genes (p21 and bax) in salvicine-treated MCF-7 cells. Salvicine had no effect on the expression of p53, p21 or bax mRNA at concentrations in the range 2.5–40 μ*M* (Fig. 6). Salvicine did not activate the expression of p53 or transcriptional activity of p53 protein. We further examined the influence of salvicine on the levels of p53, p21 and bax protein in MCF-7 cells using Western blotting. Salvicine had no effect on the expression of p53, p21 and bax at concentrations in the range 2.5–40 μ*M* (Fig. 7a) or following incubation for times up to 24 h (Fig. 7b). Moreover, the phosphorylated p53 protein level in MCF-7 cells remained unaffected in the presence of salvicine. The

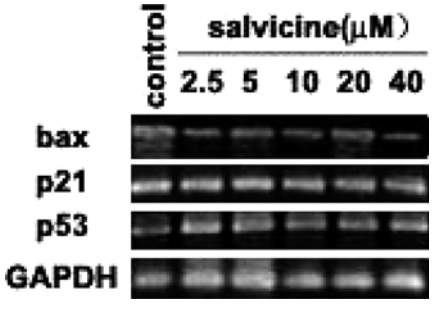


Fig. 6 Effects of salvicine on expression of p53, p21 and bax genes. RT-PCR products of p53, p21, bax and GAPDH transcripts after exposure of MCF-7 cells to salvicine for 4 h. Experiments were repeated three times

results imply that DNA damage induced by salvicine does not involve activation of the p53 response pathway.

Salvicine elicits MCF-7 apoptosis

In our system, salvicine damaged DNA, but did not activate the p53 response pathway in MCF-7 cells. This finding was unexpected, since this pathway generally mediates one of the final effects of DNA damage, specifically, apoptosis. Accordingly, we investigated the ability of salvicine to induce apoptosis in MCF-7 cells. Flow cytometric analysis of cells stained with Annexin V and propidium iodide (PI) showed that the apoptotic population accounted for 8.80% in the control sample, which increased to 13.19%, 29.47%, 81.97%, 92.19%, and 86.91% following incubation with 20, 25, 30, 35, and 40 μM salvicine, respectively, for 48 h (Fig. 8). The result was confirmed by fluorescence microscopy. After treatment with salvicine (20 μM) for 24 h and 48 h, morphological features of apoptosis (such as nuclear condensation and the presence of apoptotic bodies) were observed, and became more abundant with longer exposure times (data not shown). These findings suggest that salvicine induces apoptosis in MCF-7 cells in a dose-dependent manner.

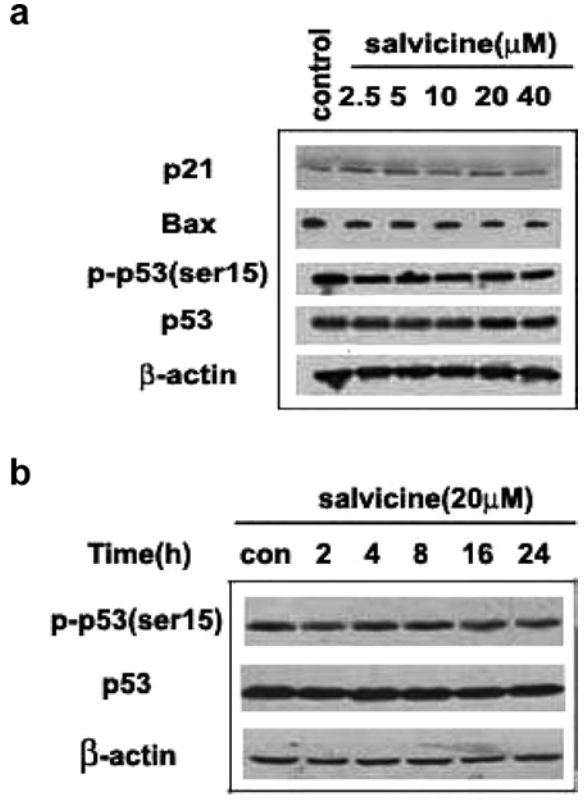


Fig. 7 Effects of salvicine on p53, p-p53 (ser15), p21 and bax protein levels in MCF-7 cells. Cells (5×10^5 ml^{-1}) were treated as indicated and lysates electrophoresed on 10% (for p53, p-p53 and β-actin) or 15% (for p21 and bax) SDS-polyacrylamide gels, respectively. Proteins were electrotransferred to nitrocellulose membranes, which were probed with antibodies appropriate for detecting the indicated proteins (see text). **a** Effect of dose; the duration of exposure to the drug was 4 h. **b** Effect of time. Experiments were repeated three times. (*con* control group)

Discussion

In this study, we established the relationship between DNA damage and growth inhibition induced by salvicine in MCF-7 cells. DSBs are the principal form of DNA breakage in this system. Our finding that salvicine induces preferential damage in the P2 promoter of the c-*myc* gene indicates that such DSBs may not be random. Damage in the P2 promoter results in reduced expression of c-*myc* mRNA and protein. We also observed that salvicine led to enhancement of c-*jun* mRNA and protein. Unexpectedly, salvicine-induced DNA damage did not activate the p53 response pathway to elicit a typical apoptotic response.

Salvicine is a newly discovered Topo II poison. The compound inhibits Topo II via acting on multiple sites of the enzyme catalytic cycle [6]. In this study, we demonstrated that DSBs elicited by salvicine subsequent to Topo II poisoning result in growth inhibition and apoptosis induction in the solid tumor cell line, MCF-7. DNA DSBs are potent inducers of genotoxic effects and cell death. In higher eukaryotes, a single non-repaired DSB inactivating an essential gene is sufficient to induce cell death via apoptosis [25]. Different Topo II poisons generate distinct drug-dependent DNA breaks within given genes. VP-16- and AMSA-induced DNA breaks occur in the p2 promoter region of c-*myc*. Furthermore, DSBs induced by salvicine preferentially occur in the P2 promoter of c-*myc*, and inhibit expression of this gene in our system. The proto-oncogenic *myc* gene product, Myc, exerts biological effects as a transcription factor [13] that promotes cell proliferation, and is required for the efficient induction of apoptosis [26]. Since c-*myc* is generally overexpressed in a variety of lymphomas and solid tumors, and tumor cells rely on the expression of this gene to maintain their transformed phenotype and/or sustain high levels of proliferation, drug-induced DNA breaks within the locus of the active gene may augment activity. c-Myc degradation induced by DNA damage results in apoptosis of CHO cells [27]. In the breast tumor cells, c-*myc* is a critical component of signal transduction pathways responding to DNA damage. Topo II inhibitors, such as teniposide, doxorubicin and amsacrine, as well as ionizing radiation, induce suppression of c-*myc* mRNA, which correlates with growth inhibition of MCF-7 breast tumor cells [14]. It is also worth noting that our results exclude the possibility of the c-*myc* gene regulating the expression of the c-*jun* gene as has been reported [15] because the alteration of c-*jun* expression occurred before that of c-*myc* expression (Fig. 5c) in our situation. The possible relationship between c-*myc* and c-*jun* needs further elucidation.

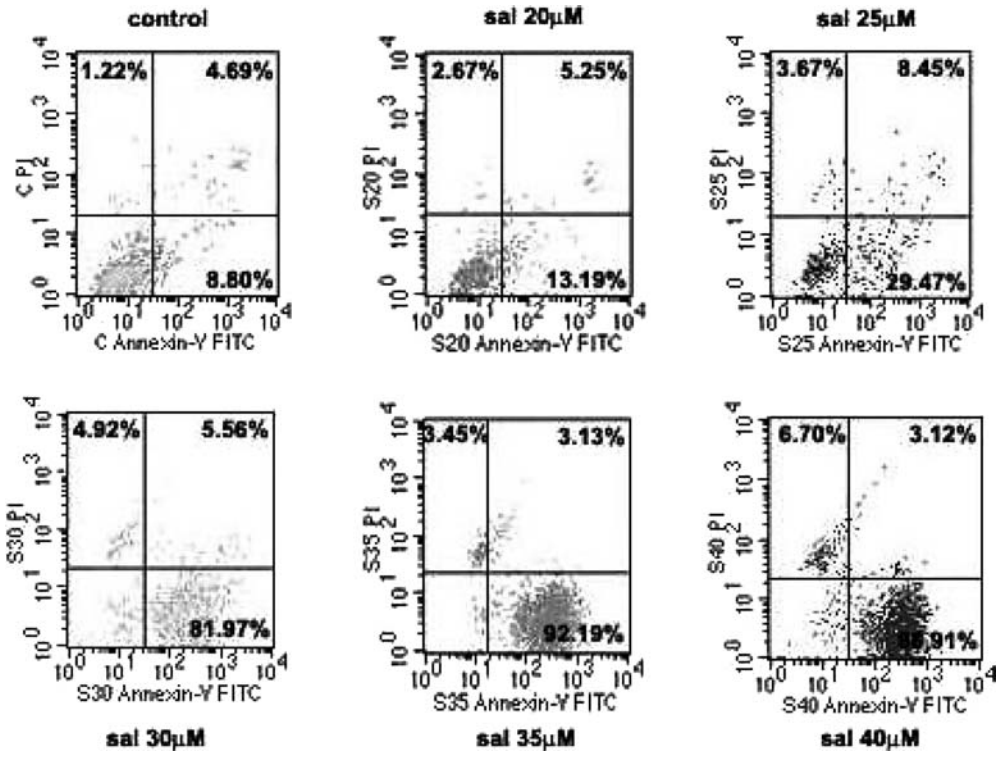


Fig. 8 Detection of apoptosis induced by salvicine using flow cytometry. Cells were treated with salvicine at the desired concentrations for 48 h and analyzed with the Annexin V and PI double-staining method using a FACSCalibur flow cytometer. Annexin V-positive/PI-negative staining represents early apoptotic events

Additionally, p53 and its signaling pathway are involved in the Myc-mediated apoptotic response [16, 28] as well as DNA damage and repair signaling transduction [29, 30]. The unaltered levels of p53 mRNA and protein, the phosphorylated active form, and target gene expression (p21 and bax) indicate that salvicine-induced DSBs do not activate the p53 response pathway. The results suggest that the *myc*-mediated death response pathway uncouples p53 signaling transduction in salvicine-treated MCF-7 cells, thus confirming that salvicine induces p53-independent apoptosis. In contrast, in view of the finding that the transcription factor c-Jun is required for apoptosis induced by various stimuli [31], including salvicine in K562 and multidrug-resistant K562/A02 cells [32], we speculate that increased c-*jun* expression may also contribute to apoptotic induction following DNA damage in our system.

In summary, we demonstrated that salvicine induces DNA DSBs, and consequently apoptosis in the solid tumor cell line MCF-7. The suppression of c-*myc* expression following the selective damage within the p2 promoter as well as the enhancement of c-*jun* expression probably mediates a subsequent apoptotic response. There are several issues that still require elucidation, including the molecular links between c-*jun* stimulation and apoptotic induction, and the signaling cascades in salvicine-stimulated DNA repair when the p53-related pathway is non-responsive.

Acknowledgements This work was supported by grants (No. 30330670, No. 30228032, No. KSCX2-SW-202, and No. 2002AA2Z346A) from the National Natural Science Foundation of China (NSFC), the Chinese Academy of Sciences, and the Ministry of Science and Technology of China, respectively.

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