Induction of bulk and c-*myc* P2 promoter-specific DNA damage by an anti-topoisomerase II agent salvicine is an early event leading to apoptosis in HL-60 cells

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Abstract Salvicine is a novel diterpenoid quinone derivative possessing strong antitumor activities and was demonstrated to stabilize the DNA topoisomerase II (Topo II) cleavage complex in vitro and in vivo. In the present work we investigated the possible mechanism through which disturbance of Topo II by salvicine led to cell death. We found that salvicine induced DNA strand breaks in human promyelocytic leukemia HL-60 cells and DNA damage correlated with cell growth inhibition. DNA damage induced by brief exposure to salvicine could be partially reversed, but early DNA breaks triggered the process of apoptosis. Preferential damage in the P2 promoter region of the oncogene c-*myc* was detected, whereas no obvious DNA damage was found in the 3′ region of the same gene. Furthermore, the expression of some protooncogenes such as c-*myc*, c-*fos* and c-*jun* was examined, showing that salvicine produced a reduction in the transcription rate of c-*myc* in a dose-dependent manner and a marked induction of c-*fos* and c-*jun* expression was observed. It appears possible that DNA damage within such genomic regions is an early event, which could lead to growth inhibition mediated by alterations of the expression of selected proliferation regulatory genes, such as c-*myc*, c-*fos* and c-*jun*, and ultimately cell death.

Key words: Salvicine; P2 promoter; DNA damage; c-*myc*; DNA topoisomerase II inhibitor

1. Introduction

Leukemia is one of the most threatening diseases today. With the recognition that most adult leukemia patients are not candidates for transplantation, and that a more rational therapy is not adequately defined, leukemia patients are treated with regimens that focus on (or at least contain) chemotherapy. Drugs targeting DNA topoisomerase II (Topo II) are among the most active and widely prescribed anticancer agents for the treatment of human leukemia [1]. However, the nature of the signaling events leading to growth arrest and/or cell death subsequent to DNA breaks have not been fully resolved.

Salvicine (4,5-seco-5,10-friedo-abieta-3,4-dihydroxy-5(10), 6,8,13-tetraene-11,12-dione, Fig. 1), a diterpenoid quinone compound, is a new Topo II inhibitor and induces Topo II-mediated DNA breaks [2]. It is a structurally modified derivative of a natural product from the Chinese traditional herb *Salvia prionitis* Hance (Labiatae) [3]. Its pharmaceutical activity was evaluated against a panel of human tumor cells [2] and xenografts [5]. Salvicine is equipotent to etoposide against three leukemia cell lines [4]. Additionally, salvicine has a prominent cytotoxic effect on multidrug-resistant (MDR) cell lines with an average resistance factor of 1.2 on three MDR cell lines, accompanied by a decrease in the expression of MDR-1 mRNA and P-glycoprotein in MDR tumor cells (unpublished data of studies in our laboratory). Salvicine, like most chemotherapeutic drugs, exerts its anti-tumor effect by inducing cancer cell apoptosis [6], and salvicine is equally effective at inducing apoptosis in K-562 and K-562/A02 cells. Considering that both of these cell lines are resistant to apoptosis, salvicine appears to be a very promising antitumor candidate, and is entering clinical trial in China.

Though the mechanism of salvicine-induced tumor cell apoptosis has not been fully established, it is believed that salvicine acts through trapping the Topo–DNA cleavage complex. Induction of DNA damage by Topo II inhibitors has been shown to correspond closely to drug cytotoxicity and/or antiproliferative activity [7]. However, the signaling pathways leading to cell death subsequent to DNA breaks have not been fully described. Previous work has indicated that Topo II inhibitors such as VM26 [8], AMSA [9] and more recently pMC540 [10] induce preferential damage within the c-*myc* locus. Another study found that Topo II cleaved c-*fos* around the region that possessed enhancer-like properties in a cell-free system [11]. These findings suggest that some protooncogenes may be closely linked with the signal transduction pathway leading to growth arrest or cell death.

Amplification of the c-*myc* gene was observed in the human promyelocytic leukemia HL-60 cells [12] and was believed to be critical to the proliferation of this cell line. Previous research indicated that salvicine displayed high cytotoxicity to HL-60 cells in vitro [4]. It remains unclear whether salvicine induces damage in the c-*myc* locus, or if c-*myc* and other

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Abbreviations: Topo, DNA topoisomerase; MDR, multidrug-resistant; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; Act. D, actinomycin D; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TFO, triple helix-forming oligonucleotide; AP-1, activator protein-1

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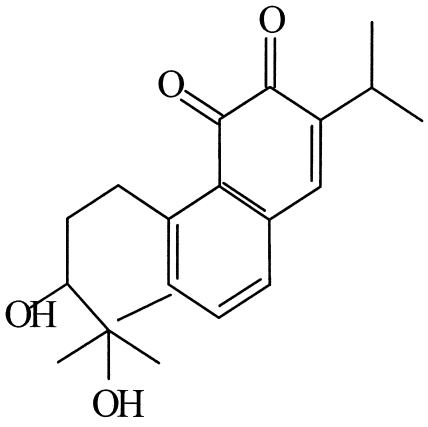

Fig. 1. Chemical structure of salvicine.

protooncogenes are involved in the apoptosis pathway of HL-60 cells. In this report, we study the influence of salvicine on bulk and gene-specific damage, and its effect on the expression of c-*myc*, c-*fos* and c-*jun* in HL-60 cells to explore the possible mechanisms of salvicine-induced apoptosis.

2. Materials and methods

2.1. Chemicals

Salvicine, presenting as a tangerine yellow color crystalloid, was provided by the Phytochemistry Department of Shanghai Institute of Materia Medica. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co; M-MLV reverse transcriptase, RPMI 1640 medium and TRIzol reagent were from Gibco-BRL. Random hexamers were from Promega. Salvicine was solubilized at 0.1 M in DMSO as a stock solution and diluted with normal saline before use. The maximum final concentration of DMSO, employed as vehicle control, was 0.5% (v/v). All other chemicals were reagent grade.

2.2. Cell culture

The HL-60 promyelocytic leukemia cell line was from American Type Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 kIU/l benzylpenicillin, 100 mg/l streptomycin and 10 mM HEPES (pH 7.4) in a humidified atmosphere of 95% air with 5% CO_2 at 37°C.

2.3. Growth inhibition assay

The capacity of salvicine to interfere with the growth of HL-60 cells was determined using the MTT dye assay [13] with minor modifications. Briefly, cells seeded at a density of 5×10^4 cells/ml in 96-well microplates were treated with salvicine for 2 h at 37°C, washed free of drug with phosphate-buffered saline (PBS, pH 7.4), then incubated with fresh medium for an additional 72 h. At the end of culture, 20 μl of MTT (5 mg/ml) was added to each well and the plate was placed at 37°C for 4 h; then 100 μl 'triplex solution' (10% SDS–5% isobutanol–12 mM HCl) was added and the cells were incubated at 37°C for 12–20 h. The plates were measured by the absorbance at 570 nm using a multiwell spectrophotometer (VERSAmax, Molecular Devices, USA). The cytotoxicity of salvicine on tumor cells was expressed as IC_{50}, which was calculated using the logit method.

2.4. Alkaline unwinding assay

Bulk damage to DNA was determined using the alkaline unwinding procedure [14]. Exponentially growing cells were exposed to salvicine for 2 h at 37°C. Following exposure to the drug, the cells were collected and washed with ice-cold PBS. The cell pellet was then resuspended in PBS, and the number of cells was determined prior to analysis for strand breaks. This assay is based on the differential binding and fluorescence of bisbenzimide trihydrochloride (Hoechst 33258) to single- and double-strand DNA after a fixed period of alkaline denaturation. Each condition (4.5×10^6 cells/condition) was subdivided into three different groups: (a) double-strand DNA control, with no alkaline-induced DNA unwinding; (b) cells treated for a 30-min alkaline-induced DNA unwinding period; and (c) total single-strand DNA, where cells were sonicated before alkaline unwinding. F values, defined as F = (alkali-treated DNA minus single-strand DNA)/(double-strand DNA control minus single-strand DNA), were determined in triplicate.

2.5. Secondary DNA fragmentation assay

Exponentially growing HL-60 cells were treated with 10 μM salvicine for 30 min. Salvicine was removed by washing with PBS three times, after which cells were further cultured in drug-free medium for 30 min, 1, 2, 3 or 6 h. Total DNA was extracted according to the procedure of Slin et al. [15]. The DNA samples were subjected to electrophoresis in a 1.5% agarose gel. The DNA bands was stained with ethidium bromide and photographed using the GDS8000 Gel Documentation System (UVP, USA).

2.6. PCR stop assay

HL-60 cells were incubated with salvicine for 30 min, then cells were washed and resuspended in 100 μl of lysis buffer (50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.5% Triton X-100, 0.45% Tween-20, 0.06 mg/ml proteinase K). The lysate was incubated at 50°C for 2 h followed by a cycle at 94°C for 12 min. After centrifugation at $10\,000\times g$ for 3 min, the supernatant was used for PCR. PCR stop assay was performed using the procedure of Clary et al. [16]. The PCR was carried out using the following primers, derived from the c-*myc* human gene. A 283-bp fragment of this gene, corresponding to the P2 promoter, was amplified with primers spanning the region from nucleotide 2392 (5′-TCGAGAAGGGCAGG-3′) to 2674 (5′-CCCTATTCGCTCCGGATC-3′), and a 213-bp fragment of the 3′ region of the same gene was amplified with the following primers: 5′-TTCGTTTCTTCCCCCTCCCA-3′ and 5'-CCCTGCTTCTGCCATTCC-3′. A typical 50-μl reaction mixture contained 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1 mM $MgCl_2$, 0.2 mM of each of the dNTPs, 0.2 μM of each primer, 5 μl (1 μg DNA) of cell lysate, and 1 μl (3 U) of Taq polymerase. The initial heating step was performed at 95°C for 5 min followed by 35 cycles: 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. The reaction products (10 μl) were separated on a 1.5% agarose gel. The gel was stained and visualized, quantitations of the bands were determined and analyzed using Gelworks 1D Intermidiate Version 2.01 software.

2.7. RT-PCR assay

To determine c-*myc*, c-*fos* and c-*jun* expression, HL-60 cells were incubated with various concentrations of salvicine for 2 h before RNA isolation. To estimate the half-life of c-*myc* mRNA, HL-60 cells were treated with 5 μM actinomycin D (Act. D) alone or 5 μM Act. D and 10 μM salvicine simultaneously for the specified times. Total RNA was isolated from HL-60 cells using TRIzol reagent according to the manufacturer's instructions. RNA yields and purity were assessed by spectrophotometric analysis. Total RNA (1 μg) from each sample was subjected to reverse transcription with random hexamer, dNTPs, and 10 U of M-MLV reverse transcriptase in a 20-μl reaction volume. The synthesized cDNA was either used immediately for PCR amplification or stored at −20°C for further analysis. PCR was carried out using the following primers: 5′-CCATGGAGAAGGCTGGGG-3′ (sense), 5′-CAAATGTGTCATGGATGACC-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [17]; 5′-CTGGTGCTCCATGAGGAG-3′ (sense) and 5′-AGGTGATCCAGACTCTGAC-3′ (antisense) for c-*myc* [18]; 5′-GGGTGAATGGAGGTGATGGCAGACA-3′ (sense), 5′-AGGAAGTCATCAAAGGGCTCGGTCT-3′ (antisense) for c-*fos*; 5′-AACGACCTTCTATGACGATGCCCTC-3′ (sense), 5′-GCGAACCCCTCCTGCTCATCTGTC-3′ (antisense) for c-*jun*. Each PCR reaction was performed in a 50-μl volume containing 2 μl of reverse transcription reaction mixture, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1 mM $MgCl_2$, 0.2 mM of each of the dNTPs, 0.2 μM of each primer, and 1 μl (3 U) of Taq polymerase. Samples were denatured at 95°C for 4 min and cycled 30 times through a 30-s denaturation step at 94°C, a 30-s annealing step at 57°C, and a 30-s extension step at 72°C. After a final PCR

extension of 5 min at 72°C, the samples (10 μl) were subjected to electrophoresis on 1.5% agarose gel in TBE. The DNA bands were stained, visualized and analyzed as described earlier.

3. Results

3.1. Induction of DNA damage/inhibition of growth by salvicine

The growth inhibition by salvicine was evaluated using the MTT assay. The results presented in Fig. 2 indicate that the growth of HL-60 cells is inhibited in a concentration-dependent manner after acute exposure to salvicine, with an IC_{50} value of approximately 4 μM. Growth inhibition by Topo II inhibitors is thought to be a consequence of the induction of DNA damage, resulting from disturbance of the activity of Topo II [19,20]. DNA damage was determined by the alkaline unwinding assay, which assessed combined single- and double-strand breaks. As shown in Fig. 2, bulk DNA damage could be detected at a concentration of 0.5 μM, and the *F* value decreased with increasing salvicine concentration, indicating that salvicine-induced DNA damage accumulated in a concentration-dependent manner. Bulk DNA damage was found to correspond to the antiproliferative or cytotoxic effects of salvicine in this cell line in the lower range of drug concentration (<5 μM). For instance, between 1 μM and 5 μM of salvicine, the growth inhibitory rate increased by 32.5% with a corresponding decrease in the *F* value of 0.20. In contrast, drug concentrations in the higher range (5–10 μM) caused an insignificant change in growth inhibition (12.2%), accompanied by a decrease in the *F* value of 0.28.

3.2. Reversal of salvicine-induced DNA damage and secondary DNA fragmentation

Previous work showed that formation of the Topo II inhibitor-induced cleavage complex reversed rapidly after drug removal [2]. It is of interest to explore whether salvicine-induced bulk DNA damage could be reversed in HL-60 cells. The *F* values were determined at specified times of further incubation in drug-free medium. The results of this trial, shown in Fig. 3, indicate that DNA damage is poorly repaired after drug removal. The curve appears biphasic: during the first 30 min, DNA breaks partially resealed, whereas after this initial period the breaks persisted and even increased, suggesting the occurrence of secondary DNA fragmentation.

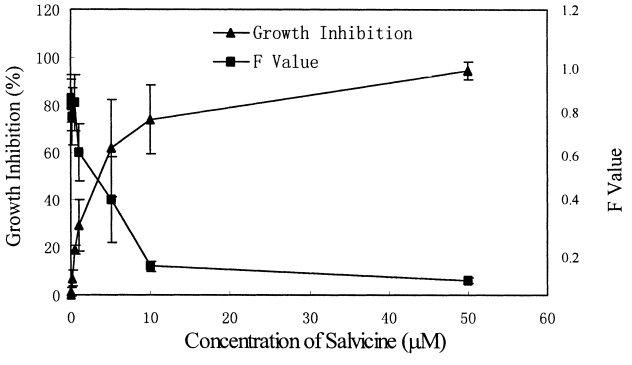

Fig. 2. Induction of DNA damage and growth inhibition by salvicine in HL-60 cells. HL-60 cells were incubated at the specified concentrations of salvicine for 2 h. DNA damage was evaluated by the alkaline unwinding assay, and growth inhibition was monitored using the MTT dye assay. Each value represents the mean ± S.D. of three experiments for strand breaks and growth inhibition.

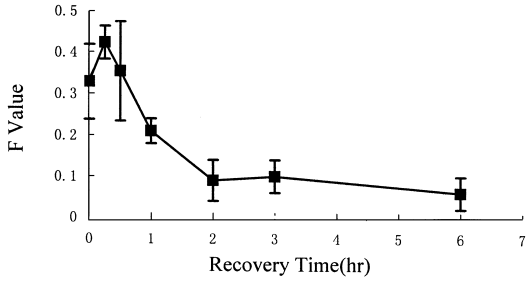

Fig. 3. Repair of salvicine-induced DNA damage in HL-60 cells. HL-60 cells were treated for 30 min with 10 μM salvicine. The drug was washed out with PBS twice, and cells were incubated further in drug-free medium. At the desired times, DNA breaks were assayed by the alkaline unwinding assay. Points represent mean ± S.D. of three independent experiments.

To verify the occurrence of secondary DNA fragmentation, chromatin DNA fragmentation was measured by agarose electrophoresis at various times after drug removal. As shown in Fig. 4, at the time of removal of salvicine (lane 3) there was an increase in high molecular DNA fragments migrating about 1 cm into the gel compared to the vehicle control (lane 2). These fragments might result from the Topo II–DNA cleavage complex induced by salvicine. The high molecular DNA fragments disappeared gradually upon removal of the drug (lanes 4–8), whereas the internucleosomal DNA fragment began to appear 1 h after removal of salvicine (lane 5), indicating the occurrence of secondary DNA fragmentation. Internucleosomal DNA breaks are typical of cells undergoing apoptosis. The observations reported above suggest that apoptosis occurs subsequent to DNA damage induced by acute treatment with salvicine. This result is consistent with reports that Topo II inhibitors could trigger HL-60 cell apoptosis by in-

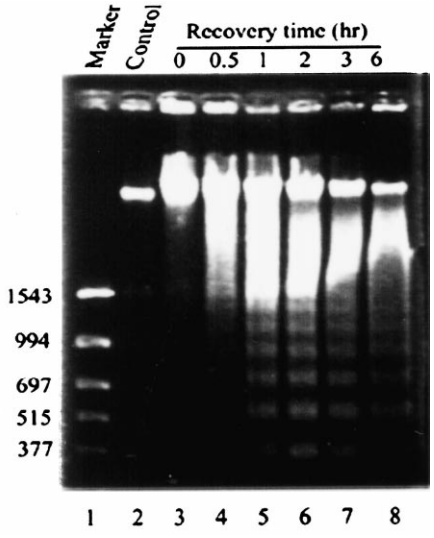

Fig. 4. Secondary DNA fragmentation following salvicine treatment on HL-60 cells. Exponentially growing cells were treated with 10 μM salvicine for 30 min. The drug was removed and cells were incubated further in drug-free medium for the indicated times before DNA extraction. Lane 1: molecular weight markers; lane 2: no drug; lane 3: HL-60 cells that were incubated with 10 μM salvicine for 30 min; lanes 4–8: HL-60 cells that were further incubated in drug-free medium for 30 min, 1, 2, 3 and 4 h, respectively.

ducing DNA fragmentation into oligonucleosome-like fragments [21,22].

3.3. Salvicine-induced gene-specific DNA damage

Gene-specific DNA damage in HL-60 cells was analyzed by PCR stop assay after 30 min exposure to salvicine at various concentrations. Two fragments of the c-*myc* gene were studied, one in the P2 promoter and the other in the 3′ end of the gene. Fig. 5A shows the amplification products of the two fragments. There is no obvious modification in the amplification of the 3′ fragment. In contrast, the amplification of the P2 promoter fragment decreased with increasing salvicine concentration. Quantifications of the DNA bands are shown in Fig. 5B, the P2 promoter/3′ region ratio was down to 15% of that measured in untreated cells in the presence of 10 μM salvicine. These results show that specific drug-induced cleavage site(s) exist(s) in the P2 promoter of the c-*myc* gene, indicating that salvicine treatment resulted in a non-random distribution of DNA damage. Thus, the profile of salvicine-induced DNA damage suggests that there might be heterogeneity in the susceptibility of discrete chromatin regions.

3.4. Effect of salvicine on expression of the c-myc gene

It is possible that the induction of lesions within the locus of the c-*myc* gene could cause alterations in the expression of this gene, and the c-*myc* gene is particularly critical for DNA replication and for the growth of HL-60 cells. To test this possibility, steady-state c-*myc* mRNA was examined by the RT-PCR method, by comparison to a housekeeping gene, *gapdh*. The effect of salvicine on the expression of c-*myc* was determined after 2 h of drug treatment. Fig. 6A represents the result of a typical experiment, where lane 1 is the vehicle control, and lanes 2–5 are samples treated with various concentrations of salvicine. Salvicine produced a specific decrease in expression of the c-*myc* gene compared to the *gapdh* gene. The inhibition of c-*myc* expression, described as $[(\text{c-}myc/gapdh)_{\text{treated}}/(\text{c-}myc/gapdh)_{\text{control}}]\times 100$, is graphed in Fig. 6B. Data from two experiments showed that the relative amplification of c-Myc decreased in a concentration-dependent manner. The maximum inhibition reached ~100% at a concentration of 10 μM.

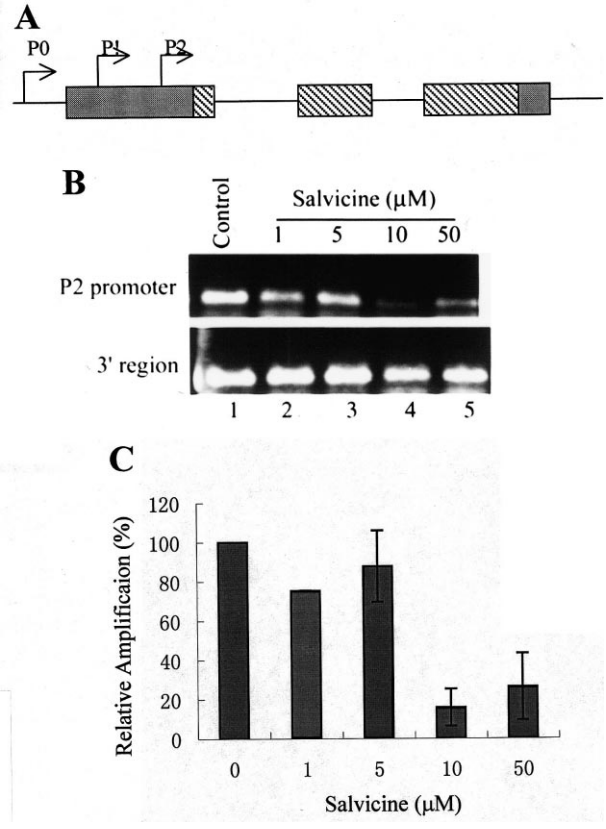

Fig. 5. Effects of salvicine on the amplification signal generated after PCR performed within the P2 promoter and the 3′ regions of the c-*myc* gene of HL-60 cells. A: Map of the c-*myc* gene. Exons 1–3 are represented by boxes, with the coding region hatched. Transcriptional start sites are indicated by arrows. B: Lane 1: vehicle control; lanes 2–5: samples from incubation with 1, 5, 10, 50 μM salvicine. C: The bands in the gels of two independent experiments were quantified and expressed as percentage of the amplification obtained from undamaged DNA. Results are presented as mean ± S.D.

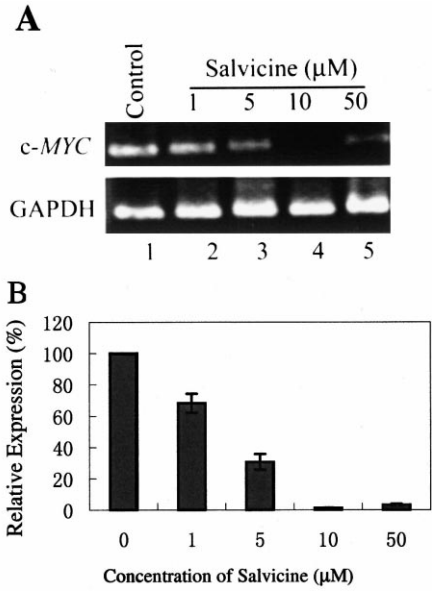

Fig. 6. Effect of salvicine on expression of the c-*myc* gene. A: RT-PCR products of c-Myc and GAPDH transcripts after exposure of HL-60 cells to various concentrations of salvicine for 2 h. Lane 1: no drug; lanes 2–5: 1, 5, 10, 50 μM salvicine respectively. B: Quantification of the DNA bands from two experiments. Results are presented as mean ± S.D.

3.5. Effect of salvicine on the stability of c-myc mRNA

To determine whether the decrease in c-Myc transcript was due to an altered turn-over of the mRNA, we evaluated the influence of salvicine on the stability of the c-*myc* transcript by measuring the rate of disappearance of the c-*myc* mRNA in control and drug-treated HL-60 cells. Because of the long half-life of *gapdh* [23], its transcript was analyzed simultaneously as an inner standard. Data from two independent experiments were pooled and analyzed. The half-life of the c-*myc* transcript was 36.1 ± 9.8 min in the absence of drug, and 32.3 ± 2.8 min with 10 μM salvicine. There was no statistically significant difference in the half-lives of the c-*myc* mRNA upon exposure to salvicine, as calculated using Student's unpaired *t*-test ($P > 0.05$), indicating that salvicine failed to alter the transcript stability.

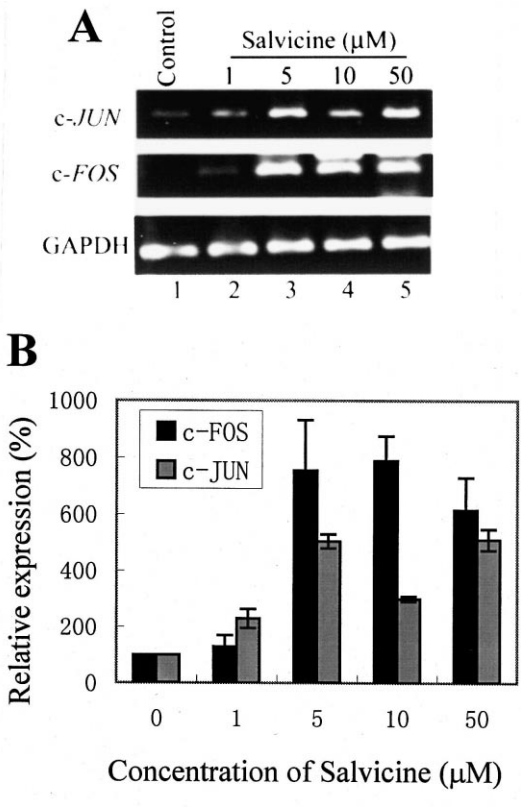

Fig. 7. Effect of salvicine on the expression of c-*fos* and c-*jun*. A: RT-PCR products of c-*fos*, c-*jun* and *gapdh* transcripts after exposure of HL-60 cells for 2 h to various concentrations of salvicine. Lane 1: no drug; lanes 2–5: 1, 5, 10, 50 μM salvicine, respectively. B: Quantification of the DNA bands from two experiments. Results are presented as mean ± S.D.

3.6. Effect of salvicine on the expression of c-fos and c-jun

Besides c-*myc*, c-*fos* and c-*jun* have also been found to be within the immediate early response genes after DNA damage in myeloid leukemia cells [24]. We examined the expression of the two genes in HL-60 cells after treatment with salvicine at mRNA level. As shown in Fig. 7A, treatment with salvicine for 2 h brought out a significant increase in the expression of both genes. Using a semi-quantification method, we observed a concentration–response relationship for c-*fos* and c-*jun* induction in HL-60 cells (Fig. 7B). The mRNA of c-*fos* increases ~1.3-fold in the presence of 1 μM salvicine, with an increase in expression of ~7.9-fold observed for 5 μM salvicine, followed by a plateau between 10 μM and 50 μM. Similar results were obtained for the case of c-*jun* expression.

4. Discussion

Salvicine is a newly discovered diterpenoid quinone compound displaying prominent antitumor activity. Recent research on the antitumor mechanism of salvicine indicates that salvicine is a new anti-Topo II agent that inhibits Topo II-mediated kDNA decatenation and supercoiled pBR322 relaxation [2]. Salvicine was found to stabilize the DNA–Topo II cleavage complex mainly by inhibiting the DNA religation step in the catalytic cycle, resulting in DNA double-strand breaks [2]. Recent research on a yeast genetic system indicates that Topo II is the primary cellular target of salvicine and that cell killing is due to stimulation of Topo II-mediated DNA breaks [25].

Previous studies have demonstrated a correspondence between double-strand breaks of DNA and the cytotoxicity effects of some DNA Topo poisons [26]. Ryan et al. [7] reported that double-strand breaks in nascent DNA are closely related to the activity of camptothecin against SV-40-transformed skin fibroblast. However, it has long been evident that induction of DNA breaks throughout the genome is associated with cytotoxicity or antiproliferative activity of Topo II inhibitors [8,27]. The results in the present study demonstrated a close relationship between DNA bulk damage and cytotoxicity in the lower range of concentrations of salvicine in HL-60 cells. The DNA damage could be partially repaired, but apoptosis was ultimately induced, suggesting that the early DNA break triggers the process of cell apoptosis. It is of interest that DNA damage did not parallel growth inhibition at higher salvicine concentrations (>5 μM). This lack of correspondence was probably due to two reasons. First, bulk DNA damage that was detected after 2 h incubation was a combination of direct topoisomerase II-mediated damage and secondary DNA fragmentation. Second, it is possible that random DNA damage was detected by the alkaline unwinding assay, whereas only breaks which are induced at genomic sites involved in proliferating functions could be responsible for compromised cell growth and/or loss of viability.

It is well known that Topo II poisons induce preferential DNA breaks within the c-*myc* locus, and further research has demonstrated that VP16 and AMSA induce prominent Topo II-mediated cleavage in the P2 promoter in a cell-free system [28]. In this study, a PCR stop assay was utilized as an effective and sensitive method for measuring whether salvicine induced preferential breaks in some regions of c-*myc*. The overall sensitivity of this assay is reflected in its capacity to detect region-specific damage produced by 1 μM salvicine in the locus of the P2 promoter. The PCR stop assay provides an additional advantage in being able to detect a more precise localization of the cleavage sites. We have shown that there was preferential damage in the P2 promoter of the c-*myc* gene, in comparison to the 3′ region of the same gene. The cleavage patterns mediated by Topo II poisons observed in living cells are thought to be determined by two factors: chromatin structure and specific DNA sequence. Although we did not check all regions of the c-*myc* gene, our results provide a clue that chromatin structure might be more essential for the DNA cleavage specificity of salvicine. Since c-*myc* is an active gene in HL-60 cells and the P2 promoter is a strong promoter, its chromatin structure might be more accessible to Topo II. Because DNA synthesis and transcription contribute to the formation of permanent Topo II-associated DNA breaks, DNA breaks that occur within the locus of some active genes might enhance the drug activity. This result proves our hypothesis that some 'critical' DNA fragments (e.g. c-*myc*) have an increased susceptibility to salvicine. On the other hand, damage to the P2 promoter of the c-*myc* gene did not parallel growth inhibition in HL-60 cells, suggesting that DNA breaks at other sites of the c-*myc* locus (e.g. P1 promoter) or another critical gene locus might exist. Improved knowledge on the DNA specificity of salvicine may have a profound influence on the design and development of novel Topo II-based therapeutics or regimens.

Since salvicine-mediated DNA breaks, especially at the crit-

ical gene locus, appear to be an early signal causing cell death, we wished to know what happened in the interval between complex formation and the presence of internucleosomal DNA ladders. To determine if damage in the c-*myc* gene locus leads to an alteration in the expression of this gene, c-*myc* mRNA was examined at transcriptional and post-transcriptional levels. The results showed that the stability of mRNA was not responsible for the down-regulation of c-*myc* expression. We postulated that down-regulated steady levels of c-*myc* should result from DNA breaks in the P2 promoter. Clary et al. [16] reported that Topo II-mediated DNA breaks within the P2 promoter by AMSA are probably responsible for the down-regulation of the c-*myc* gene, which in turn is involved in the apoptosis pathway in K-562 cells. More recently, a triple helix-forming oligonucleotide (TFO) targeted to a homopurine-homopyrimidine sequence in the P2 promoter of the c-*myc* gene was synthesized. Incubation of leukemia or lymphoma cells with TFO reduced c-*myc* RNA and protein levels and induced cell death by apoptosis [29,30]. Combining past results with those presented here, we consider that the P2 promoter should be further studied in antitumor research and that inactivation of the P2 promoter might provide a new strategy for tumor therapy.

On the other hand, reduction in the expression of c-*myc* by salvicine did not correspond closely to growth inhibition. It is possible that Topo II-mediated DNA damage in the c-*myc* and other genes may induce replication-associated damage which could be lethal to the cells independent of the effects on the transcription and c-Myc expression. Our experimental results also showed that other related genes might be involved in the signaling pathway leading to apoptosis. It has been reported that Topo II inhibitor-induced apoptosis is accompanied by the up-regulation of c-*fos* and c-*jun* genes in human myeloid leukemia cells [31]. In the present study, we observed that salvicine activated the transcription of the protooncogenes c-*fos* and c-*jun* in HL-60 cells, and this induction was accompanied by subsequent formation of internucleosomal DNA ladders. However, it is still unknown whether up-regulation of their mRNA is the result of DNA damage. A better understanding of the mechanism will be helpful for further development of salvicine-like analogues. The Jun-family proteins are known as transcription factors that form homodimers or heterodimers with Fos-family proteins, referred to as activator protein-1 (AP-1). AP-1 may control the expression of the gene programs ultimately responsible for the triggering and execution of specific cellular processes, such as proliferation, differentiation and cell death [32]. Our results suggest that AP-1 may be connected to the transcriptional activation of other downstream DNA damage-inducible genes in the signaling pathway leading to apoptosis.

In summary, the new Topo II inhibitor salvicine induced DNA damage and early DNA breaks and triggered the process of apoptosis in HL-60 cells. Salvicine preferentially caused DNA breaks in the P2 promoter of the c-*myc* gene, thereafter inhibiting the expression of c-*myc*. Salvicine also induced a marked increase in the transcription of c-*fos* and c-*jun*. These results suppose that DNA damage, especially in some gene-specific regions such as the P2 promoter of c-*myc*, is an early signal leading to salvicine-mediated HL-60 cell apoptosis. We further suggest that inactivation of the P2 promoter is a valid approach to inhibit c-*myc* expression and tumor cell growth, with potential to be a new target for leukemia and lymphoma.

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