

DNA topoisomerase II as primary cellular target for salvicine in *Saccharomyces cerevisiae*¹

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KEY WORDS salvicine; DNA topoisomerase; *Saccharomyces cerevisiae*

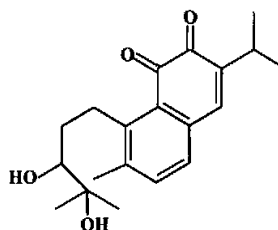
ABSTRACT

AIM: To identify whether DNA topoisomerase II (Topo II) is the primary cellular target of salvicine in *Saccharomyces cerevisiae* (*S cerevisiae*) and the action mode of salvicine. **METHODS:** The catalytic activity of Topo II was determined by Topo II mediated supercoiled pBR322 relaxation. The effects of salvicine on the growth of four strains of *S cerevisiae* were assessed by clone forming assay. **RESULTS:** Salvicine inhibited Topo II mediated supercoiled pBR322 relaxation in cell-free system. Cytotoxicities of salvicine to parent (JN394) and TOP1 deleted (JN394top1⁻) yeast cells were at the same level, suggesting Topo I might not be the cellular target of salvicine. Salvicine displayed high activity against JN394t2-1 cells at 25 °C, while no growth inhibition was observed at 30 °C in the concentration range of interest. Furthermore, JN394t2-5 cells which expressed top2-5 mutant allele were highly resistant to salvicine and etoposide (VP16). **CONCLUSION:** Topo II was the primary cellular target of salvicine *in vivo* and salvicine killed yeast cells mainly by trapping the DNA-Topo II cleavage complex. Salvicine and VP16 might share some similar action locus on Topo II.

INTRODUCTION

Salvicine [4,5-seco-5,10-friedo-abieta-3,4-dihy-

droxy-5(10),6,8,13-tetraene-11,12-dione], a novel diterpenoid quinone compound, was a structurally modified derivative of a natural product lead from Chinese traditional herb *Salvia prionitis* Hance (Labiatae)⁽¹⁾. Salvicine had significant anti-cancer activity both *in vitro*⁽²⁾ and in animal models⁽³⁾. Experiments in our laboratory found that salvicine exerted its anti-tumor effect by inducing cancer cell apoptosis⁽⁴⁾. Furthermore, cytotoxicity of salvicine was not affected by the presence of P-glycoprotein in three multidrug resistant cell lines (unpublished data). All these experimental results make salvicine a very promising antitumor candidate that is entering into clinical trials in China.



Chemical structure of salvicine

Although the cytotoxic mechanism of salvicine has not yet been fully established, recent evidence suggests that it might act through DNA topoisomerase II (Topo II)⁽⁵⁾. Salvicine displayed a potent activity against this essential enzyme, and also stimulated DNA breakage mediated by Topo II. Insight was gained into the mechanisms of the action to demonstrate that the enhancement of cleavage complex induced by salvicine was due to preventing the cleaved DNA from religating. Though the results described above suggest strongly that salvicine might be a new kind of Topo II "poison," it did not exclude the possibility that some effects of the drug were due to actions on other cellular targets. In this study, a

¹Project supported by the National Natural Science Foundation of China (No 39830444 and No 30070877) and Shanghai Research Center of Life Sciences.

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Received 2001-03-13 Accepted 2001-04-26

Saccharomyces cerevisiae (*S cerevisiae*) genetic system was employed to further determine whether Topo II was the primary cytotoxic target of salvicine, and whether this drug killed cells by acting as a Topo II "poison."

MATERIALS AND METHODS

Drugs and reagents Crude Topo II was extracted from rat Ehrlich ascites carcinoma cells harvested from the peritoneal cavity 7 d after tumor innovation by the procedure of de Isabella *et al*^[6]. Supercoiled plasmid pBR322 was isolated from *Escherichia coli* by a method of alkaline lysis. Yeast nitrogen base without amino acids was from Fluka Biochemika. Etoposide (VP16) was obtained from Pudong Pharmaceutical Factory of Shanghai Pharmaceutical Institute (Shanghai, China), and camptothecin (CPT) from Huangshi Pharmaceutical Factory (Hubei, China). Salvicine (tangerine yellow color crystalloid, the purity was above 99.3 %) was provided by Phytochemistry Department of Shanghai Institute of Materia Medica. The drugs were solubilized at 10 mmol/L in dimethyl sulfoxide as stock solution and diluted to desired concentrations with normal saline before use. All other chemicals were of analytical reagent grade.

Yeast strains The *S cerevisiae* strains used were the generous gifts of Dr Neil Osheroff (School of Medicine, Vanderbilt University, USA). The parental yeast strain used in this study was *S Cerevisiae* JN394, whose genotype was *ura3-52, leu2, trp1, his7, ade1-2, ISE2, rad52::LEU2*^[7]. Three gene-modified strains derived from JN394 were also employed: JN394top1⁻, which had a chromosomal deletion of the topoisomerase I (Topo I) gene^[7]; JN394t2-1, in which the wild Topo II gene was replaced with the temperature-sensitive top2-1 mutant allele^[8] and JN394t2-5, in which the wild Topo II was replaced with the drug-resistant top2-5 mutant allele^[9]. These three strains were isogenic to JN394 in all other respects.

Relaxation of supercoiled pBR322 by Topo II Topo II activity was measured by the ATP-dependent relaxation of supercoiled pBR322. The standard reaction mixture consisted of Tris-HCl 50 mmol/L (pH 7.7), KCl 50 mmol/L, MgCl₂ 5 mmol/L, ATP 1 mmol/L, dithiothreitol 0.5 mmol/L, edetic acid 0.5 mmol/L, BSA 50 mg/L, 0.15 μg of supercoiled pBR322 and 1 unit of enzyme in a total volume of 10 μL. One unit of enzyme activity was defined as the amount of enzyme that relaxed supercoiled DNA 0.15 μg to relaxed DNA under

the conditions described here. After incubation at 37 °C for 6 min, the reaction was stopped by addition of 1 μL of 5 % SDS/50 % glycerol/0.05 % bromphenol blue. The DNA samples were subjected to electrophoresis. The DNA bands were stained and photographed with Gel Document System GDS8000 (UVP, USA).

Determination of the growth inhibition of yeast strains JN394, JN394top1⁻, JN394t2-1, and JN394t2-5 by salvicine were determined as previously described^[10]. Briefly, cells were cultured in YPDA media or synthetic complete media in the case of JN394top1⁻ at 25 °C. In some experiments as indicated, cells also were cultured at 30 °C. After logarithmically growing cells were adjusted to a titer of 1 × 10⁹/L, the drugs or vehicle control were added to the cultures. Cells were incubated with drugs for 24 h, then diluted into drug-free YPDA media, and plated in triplicate onto YPDA media or synthetic complete media solidified with 1.5 % bacto-agar. Plates were incubated at 25 °C, and drug sensitivity was determined by counting the number of surviving colonies 3–4 d later. The relative survival was calculated as: surviving colonies in the presence of drug/surviving colonies of vehicle control × 100 %.

RESULTS

Salvicine inhibited the catalytic activity of eukaryotic Topo II in cell-free system The effect of salvicine on the catalytic activity of Topo II was assessed by a DNA strand passage assay. This assay monitored the ability of the enzyme to relax the supercoiled DNA. As shown in Fig 1, the supercoiled form (SC) of DNA turned to be relaxed form (RLX) completely at the presence of 1 U of Topo II. When the samples were incubated with either salvicine 100 μmol/L or VP16 100 μmol/L (a well-known Topo II inhibitor), the SC form remained almost unchanged. The phenomenon demonstrated that both salvicine and VP16 inhibited the strand passage activity of Topo II.

Topo I was not the cellular target of salvicine Previous work in our laboratory showed that salvicine failed to affect the activity of Topo I in cell-free system^[5]. JN394top1⁻ which completely lacked Topo I was employed to prove the result on cellular level. This yeast strain was constructed taking the advantage of the fact that TOP1 gene was not essential in yeast. As shown in Fig 2A, the parent cells (JN394) were hypersensitive to CPT which was a famous Topo I poison. The viability of this yeast strain was reduced nearly to zero in the presence of CPT 6.25 μmol/L.

However, CPT failed to reduce the growth of the mutant cells (JN394top1⁻) at the same concentration (Fig 2A). In contrast, no resistance was observed when top1⁻ cells were treated with salvicine. The sensitivity was similar in the case of parent cells and the mutant cells (Fig 2B). This finding provided strong evidence that Topo I was not the cellular target of salvicine.

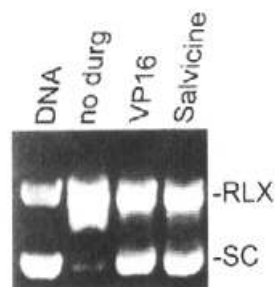


Fig 1. Salvicine inhibited Topo II mediated supercoiled pBR322 relaxation. Negatively supercoiled pBR322 (DNA) and relaxed pBR322 (no drug) are shown for reference. The concentration of salvicine and VP16 was 100 $\mu\text{mol/L}$. The positions of SC (supercoiled form) and RLX (relaxed form) are indicated.

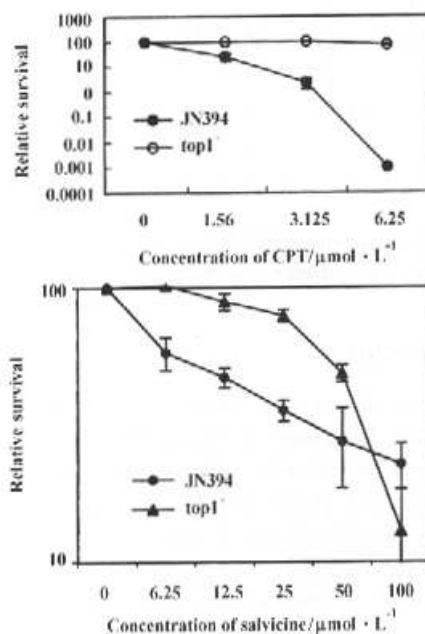


Fig 2. Sensitivities of JN394 and JN394top1⁻ to CPT and salvicine. JN394 and JN394top1⁻ were exposed to CPT or salvicine for 24 h. Percentage survival rate relative to the vehicle control was determined. $n = 2$ separate experiments. $x \pm s$.

Salvicine acted as a novel Topo II "poison"

Since it has been excluded that Topo I is the target of salvicine *in vitro* and *in vivo*, three other possibilities exist for the physiological mechanism of salvicine's cytotoxicity. The first is that Topo II is the primary target responsible for the drug, and salvicine acts as a Topo II poison to kill cells by trapping the enzyme mediated DNA cleavage. The second is salvicine functions through Topo II, but its cytotoxicity correlates with the ability to block the overall catalytic activity of the enzyme. The third possibility is that Topo II is not the physiological target predominantly responsible for cytotoxicity. The possibilities described above can be distinguished by utilizing a yeast strain JN394t2-1. This yeast strain expresses the temperature-sensitive top2-1 mutant in place of the wild type TOP2 gene. The top2-1 protein showed wild type activity at 25 $^{\circ}\text{C}$, while its activity was reduced to about 5 %-10 % of the wild type at the semipermissive temperature of 30 $^{\circ}\text{C}$. Therefore, if salvicine functions as a Topo II "poison," a reduction in enzyme activity should greatly diminish salvicine-induced cell death. Conversely, if the cytotoxicity of salvicine correlates with the ability to impair the catalytic function of the enzyme, cells with decreased levels of Topo II activity should become hypersensitive to salvicine. Finally, if salvicine's action is mediated by target other than this enzyme, reduced levels of Topo II should have little influence on drug sensitivity at the semipermissive temperature.

Salvicine was a potent toxic agent toward JN394t2-1 yeast cells at 25 $^{\circ}\text{C}$, at which Topo II activity was similar to the wild type. The relative survival of t2-1 cells reduced to 74.8 % in the presence of salvicine 12.5 $\mu\text{mol/L}$, and to 31.6 % when the concentration of salvicine rose to 50 $\mu\text{mol/L}$. About 90 % of the cells were killed following a 24-h incubation with salvicine 100 $\mu\text{mol/L}$ (Fig 3A). It seemed that VP16 was more potent to induce cell death than salvicine at the same concentration. No survival clone was observed in the presence of VP16 100 $\mu\text{mol/L}$. However, a marked different result was obtained when the cytotoxicity of salvicine was examined in 30 $^{\circ}\text{C}$, at which enzyme activity was greatly reduced. Salvicine displayed weak cytotoxicity toward JN394t2-1 at semi-permissive temperature. In fact, at a concentration of 6.25 $\mu\text{mol/L}$, a 1.5-fold increase in cell proliferation was observed. Similar results were got in the case of VP16. The lack of drug toxicity did not result from the slow growth rate of JN394t2-1 cells at the elevated temperature.

Actually, at 30 °C this yeast strain was still highly sensitive to CPT⁽⁸⁾, which required cell proliferation and DNA replication for its cytotoxic action. Finally, resistance to salvicine or VP16 was not due to the difference in the cellular efflux or metabolism of salvicine because the sensitivity of JN394 cells was identical at 25 °C and at 30 °C (Fig 3B). The lack of salvicine-induced toxicity toward cells that contain decreased levels of Topo II activity strongly suggests that Topo II is the primary physiological target for salvicine and the salvicine's cytotoxicity results from the ability to enhance enzyme-mediated DNA breaks.

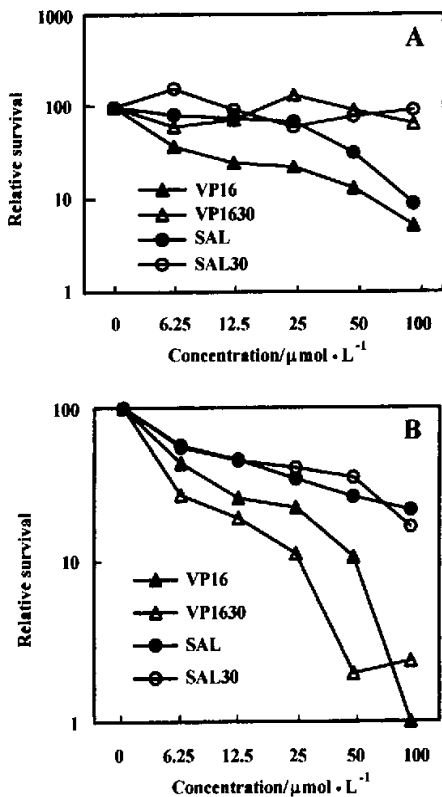


Fig 3. Effects of salvicine and VP16 on survival of JN394t-1 cells (A) and JN394 (B). Effects of the two drugs at 25 °C (●, ▲) and at 30 °C (○, △) were assessed. Each value represents the average for 3 separate experiments.

Salvicine may share similar action site(s) on Topo II with VP16 To investigate the domain of Topo II that may be important in the interaction with

Topo II-targeted drugs, a yeast strain JN394t2-5 that expresses top2-5 mutant allele has been established. The top2-5 enzyme is temperature sensitive and loses its activity at the temperature of 30 °C. At the permissive temperature of 25 °C, it is fully active but highly resistant to a number of Topo II poisons, such as amsacrine and VP16⁽⁹⁾. In this experiment, this yeast strain was utilized to explore whether salvicine shared the similar action site(s) on Topo II with other anti-Topo II agents. The viability of JN394t2-5 cells did not reduce in both salvicine-treated and VP16-treated groups even at a high concentration of 100 μmol/L (Fig 4). This result demonstrated that the top2-5 mutation conferred high levels of resistance to both salvicine and VP16, suggesting these two drugs may share some common action locus on Topo II.

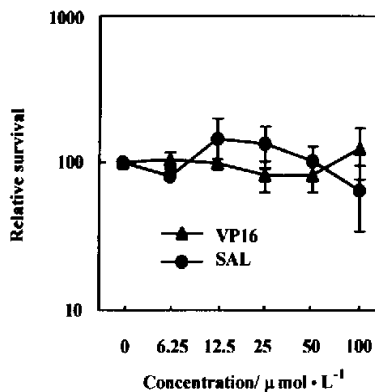


Fig 4. Sensitivity of JN394t2-5 mutants to salvicine and VP16. *n* = 3 separate experiments. $\bar{x} \pm s$.

DISCUSSION

Topo II is involved in DNA replication, recombination, and chromosome segregation⁽¹¹⁾. In addition, it also plays important roles in chromosome structure. Beyond its critical physiological functions, Topo II is the primary target for some of the most active drugs currently used for the treatment of human cancers⁽¹²⁾. Salvicine is a new Topo II inhibitor displaying significant antitumor activity. Previous experiments demonstrated that the drug trapped the Topo II-DNA cleavage complex in cell-free system, and the superior antitumor action of salvicine was believed to be attributed to this effect. However, direct evidence that salvicine-induced Topo II mediated DNA breaks lead to cell death has not been delineated.

The present study in which a *S cerevisiae* system was employed to allow the manipulation of Topo II activity on cellular level is to prove the previous experimental results in cell-free system.

The results showed that cells with non-functional activity of Topo I were as sensitive to salvicine as the parent cells, suggesting salvicine did not kill yeast cells by disturbing the activity of Topo I. On the other hand, the susceptibility to salvicine was related to the activity of Topo II in yeast cells. Cells having higher Topo II activity were hypersensitive to salvicine, while those having lower Topo II activity had enhanced resistance. The different effects of salvicine on JN394t2-1 yeast cells at 25 °C and at 30 °C are in agreement with the proposed action mode of the drug. Since salvicine is capable of trapping the Topo II-DNA complex, cells with higher Topo II activity would be sensitive to salvicine; while cells with lower enzymatic activity would be resistant to salvicine. Taken together, the results support the notion that salvicine poisons Topo II by stabilizing Topo II-DNA covalent complex. It is also consistent with the action of salvicine observed in mammalian cells. Salvicine induced DNA breaks in HL-60 leukemia cell and the DNA cleavage might trigger HL-60 cell death (unpublished data).

Cells carrying the top2-5 mutant have been demonstrated to be highly resistant to anti-Topo II agents such as amsacrine and VP16⁽⁹⁾. The drug resistance of top2-5 is due to its inherent drug resistance rather than a decrease in Topo II activity. The mutations occur in a region that is important for eukaryotic Topo II function. Results in this study showed that JN394t2-5 yeast cells were hyper-resistant to both salvicine and the control drug VP16, suggesting salvicine might share similar action site (s) with VP16 on Topo II. The study in our laboratory has confirmed that salvicine and VP16 share a number of mechanistic features. For example, both drugs increase levels of enzyme-generated DNA breaks by inhibiting the religation reaction of Topo II⁽⁵⁾. The recessive activity of salvicine to JN394t2-5 further supports that Topo II is the primary cellular target of salvicine.

In summary, the present study proved that salvicine inhibited the catalytic activity of Topo II. Using a yeast model system, we demonstrated that Topo II was the primary cellular target of salvicine and salvicine killed yeast cells mainly by trapping the Topo II-DNA cleavage complex. The information constructed the basis for salvicine cytotoxicity and provided useful clues for rational designing of salvicine analogues.

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DNA 拓扑异构酶 II 是 salvicine 作用于酿酒酵母的主要靶点¹

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关键词 salvicine; DNA 拓扑异构酶; 酿酒酵母

目的: 确定 DNA 拓扑异构酶 II (Topo II) 是否为 salvicine 在酿酒酵母细胞内的主要作用靶点及其作用方式. **方法:** 用 Topo II 介导的超螺旋 pBR322 解旋反应检测 salvicine 在无细胞体系中对 Topo II 催化活力的影响; 用克隆形成实验检测 salvicine 对四种酵母细胞系生长的作用. **结果:** salvicine 能明显抑制无细胞体系中 Topo II 对超螺旋 pBR322 的解旋作用. salvicine 对 JN394 母系细胞及 Topo I 缺失的

JN394top1⁻ 细胞毒作用相似, 验证了 Topo I 不是其作用靶点. 在 25 ℃ 时, salvicine 对温度敏感型 JN394t2-1 细胞具有良好的细胞毒作用; 但在 30 ℃ 时, Topo II 的活力大为降低, 在有意义的浓度范围内 salvicine 对此类细胞的生长无明显抑制作用. 另外, Topo II 发生突变的 JN394t2-5 细胞显示出对 salvicine 和 etoposide (VP16) 高度的耐受性. **结论:** Topo II 是 salvicine 细胞内主要作用靶点; salvicine 通过捕获 Topo II-DNA 断裂复合物而杀死细胞. 此外, salvicine 与 VP16 在 Topo II 上有相似的作用位点.

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