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Salvicine, a novel DNA topoisomerase II inhibitor, exerting its effects by trapping enzyme–DNA cleavage complexes

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Abstract

Salvicine, a structurally modified diterpenoid quinone derived from *Salvia prionitis*, is a novel anticancer drug candidate. The compound has significant *in vitro* and *in vivo* activity against malignant tumor cells and xenografts, especially some human solid tumor models. This anticancer activity of salvicine is associated with its ability to induce tumor cell apoptosis. Salvicine was also found to have a profound cytotoxic effect on multidrug-resistant (MDR) cell lines by down-regulating the expression of MDR-1 mRNA of MDR cells. Salvicine acted as a topoisomerase II (Topo II) poison through its marked enhancement effect on Topo II-mediated DNA double-strand breaks as observed in the DNA cleavage assay. Strong inhibitory activity of salvicine against Topo II was observed in a kDNA decatenation assay, with an approximate IC_{50} value of 3 μM. A similar result was obtained by a Topo II-mediated supercoiled DNA relaxation assay. In contrast, no inhibitory activity was observed against the catalytic activity of Topo I. When the effects of salvicine on individual steps of the catalytic cycle of Topo II were dissected, it was found that the mechanism by which salvicine inactivates Topo II is different from that by other anti-Topo II agents. Salvicine greatly promoted Topo II–DNA binding and inhibited pre- and post-strand Topo II-mediated DNA religation without interference with the forward cleavage steps. In addition, salvicine was not a DNA intercalative agent, as demonstrated by DNA unwinding assays. The results of this study indicate that the inhibitory activity of salvicine against Topo II was derived from its ability to stabilize DNA strand breaks through interactions with the enzyme alone or with the DNA–enzyme complex. It is therefore postulated that salvicine acts on Topo by trapping the DNA–Topo II complex, which in turn produces anticancer effects.

Keywords: Salvicine; DNA cleavage; Catalytic inhibition; DNA topoisomerase II

1. Introduction

Salvicine is a novel diterpenoid quinone compound (Fig. 1) obtained by structural modification of a natural product lead isolated from *Salvia prionitis* Hance (Labiatae) [1–4]. The pharmaceutical activity of salvicine was evaluated *in vitro* against a panel of human tumor cells [5]. Compared to its moderate cytotoxicity on three leukaemia cell lines, salvicine exhibited potent activity against solid tumor cells, especially lung and gastric cancer cells [5]. Experiments *in vivo* indicated that salvicine possessed significant antitumor activity against murine S-180 sarcoma and Lewis lung cancer, as well as human lung adenocarcinoma xenograft models [6]. Additionally, the research demonstrated that the antitumor effect of salvicine was associated with its ability to induce tumor cell apoptosis with similar potency against both human leukaemia cells and gastric carcinoma cells, indicating its specific effect on solid tumor cells [7]. Furthermore, the cytotoxicity of salvicine was found not to be affected by the presence of P-glycoprotein in three multidrug-resistant (MDR) cell lines (K-562/A02, MCF-7/ADM, MKN28/VCR). The average resistance factor (RF) of salvicine on the three cell lines was 1.2, much lower than those of control anticancer drugs, including vincristine (VCR, RF 86.6), ADM (RF 233.2), and VP16 (RF 53.7). The main mechanism of circumventing MDR was related to inhibition of MDR-1 mRNA expression (Miao ZH, Tang T,

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Abbreviations: ADM, adriamycin; VP16, etoposide; Topo, topoisomerase; kDNA, knetoplast DNA; HCPT, hydrocamptothecin; SC, supercoiled form; RLX, relaxed form; LNR, linear form; and NC, nicked form.

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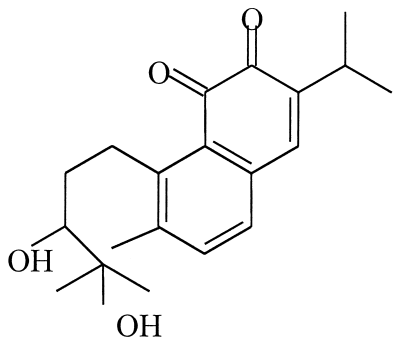

Fig. 1. Chemical structure of salvicine.

Ding J, unpublished data). Results from these experiments suggest that salvicine is a promising antitumor agent which is entering clinical trials in China.

Although the cytotoxic mechanism of salvicine has not yet been fully understood, it is believed that it acts through its effect on DNA–Topo II as observed in a preliminary study in our laboratory. The aim of this study was to explore the effect of salvicine on the activity of Topo II and its mode of action. Firstly, two assays were used to confirm its selective activity on Topo II. Secondly, the DNA cleavage assay was employed to determine whether salvicine is a Topo II "inhibitor" or a "poison." Thirdly, to gain insight into its action, effects of salvicine were characterised on the various steps of the catalytic cycle of the enzyme. Finally, unwinding assays were undertaken to observe whether salvicine could intercalate into DNA.

2. Materials and methods

2.1. Materials

Supercoiled plasmid pBR322 was isolated from *Escherichia coli* by a method of alkaline lysis [8]. Kinetoplast DNA (kDNA) was a gift from Dr. Toshiwo Andoh (Soka University, Japan). Proteinase K, EcoRI restriction endonuclease, T4 DNA ligase, and SDS were purchased from the Sino-American Biotechnology Company. Salvicine presenting tangerine yellow colour crystalloid was provided by the Phytochemistry Department of Shanghai Institute of Materia Medica. VP16 was obtained from Pudong Pharmaceutical Factory, HCPT from Feiyun Pharmaceutical Factory, and ADM from the Montedison Group. Salvicine and control drugs were solubilized at 10^4 μM in DMSO as stock solution. All stock solutions were stored at $-20°$ and diluted with double distilled water just before the test. HCPT, VP16, and ADM were used as positive controls at the concentrations referred to the previous report. Concentrations of salvicine were chosen according to the preliminary experimental results obtained from our laboratory. Two microliters of testing compound (or DMSO for control reactions or distilled water to control for the solvent's effect) was added in the reaction mixture. The concentration range of salvicine was 0–125 μM and the maximum final concentration of DMSO was 1.25% (v/v). All other chemicals were of analytical reagent grade.

2.2. Enzyme preparation and definition of enzymatic activity

Topoisomerases were extracted from rat Ehrlich ascites carcinoma cells from the peritoneal cavity 7 days after tumor innovation by the procedure of De Isabella *et al*. [9]. The extract was mixed with an identical volume of glycerol and stored at $-80°$. The protein concentration was immediately assayed with the method of Bradford. The enzyme contained the activity of Topo I and Topo II and was free of nuclease. Topo I activity was examined by the DNA relaxation reaction. One unit of Topo I activity was defined as the amount of enzyme required to fully relax 0.15 μg of supercoiled DNA under the conditions described below. Topo II activity was detected by the kDNA decatenation assay. One unit of Topo II activity was defined as the amount of enzyme that decatenated 0.2 μg kDNA to minicircles under the conditions described below. One mg of extract contained 2.56×10^5 units of Topo I activity and 1.02×10^6 units of Topo II activity.

2.3. Topo I- and Topo II-mediated supercoiled pBR322 relaxation

DNA relaxation assays were based on the procedure of Osheroff *et al*. [10]. Reaction buffer contained 10 mM Tris.HCl (pH 7.9), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/mL of bovine serum albumin (BSA), 1 mM ATP (ATP was omitted in Topo I-mediated DNA relaxation), 0.15 μg supercoiled pBR322, 1 unit of Topo I or 4 units of Topo II in a total of 20 μL. Relaxation was employed at 37° for 6 min and stopped by the addition of 3 μL of stop solution (100 mM EDTA, 0.5% SDS, 50% glycerol, 0.05% bromophenol blue). Electrophoresis was carried out in a 1% agarose gel in $0.5 \times$ TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA) at 4V/cm for 1 hr. DNA bands were stained with 0.5 μg/mL of ethidium bromide (E.B.) solution and photographed through a Gel Document System GDS8000 (UVP). The amount of DNA bands was quantified by Gel 1D Intermediate software.

2.4. kDNA decatenation assay

TOPO II activity was measured by the ATP-dependent decatenation of kDNA [11]. The standard reaction mixture consisted of 50 mM Tris.HCl (pH 7.7), 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, 50 μg/mL of BSA, 20 μg/mL of kDNA, and 1 unit of Topo II in a total volume of 10 μL. After incubation at 37° for 15 min, the reaction was stopped by addition of 1

μL of 5% SDS/50% glycerol/0.05% bromophenol blue. The DNA samples were subjected to electrophoresis under the same conditions as described above.

2.5. Topo II–DNA binding

It was employed by the gel mobility shift assay. All DNA binding reactions utilized 0.2 μg plasmid DNA and 1 unit of Topo II and were carried out in a total volume of 20 μL of cleavage buffer [10 mM Tris.HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 0.01 mM EDTA, and 2.5% glycerol]. Samples were incubated at 37° for 10 min, and then 2 μL of loading buffer (50% glycerol, 0.05% bromophenol blue) was added to each sample. Electrophoresis was carried out in 0.5 × TBE at 4V/cm for 1 hr.

2.6. Topo II-mediated DNA cleavage

DNA cleavage assays were performed as described by Marx *et al*. [12] with minor modifications. All DNA cleavage reactions employed 100 units of Topo II and 0.2 μg supercoiled pBR322 in total volume of 20 μL of cleavage buffer that contained 5 mM $MgCl_2$. In the overall cleavage reactions, 1 mM ATP was included. For post-strand passage DNA cleavage reactions, 1 mM adenyl-5′-ylβ,γ-imidodiphosphate (APPNHP), a non-hydrolyzable form of ATP, was added to the reaction mixtures. Samples were incubated at 37° for 6 min and stopped by the addition of 1 μL of 10% SDS and 1 μL of a 10 mg/mL solution of proteinase K. The reversibility of the compound-trapped cleavage complex was assayed by chelating metal irons with 2 μL of 100 mM EDTA and 0.6 μL of 5 M NaCl and a further incubation at 56° for 5 min before addition of SDS and proteinase K. The samples were incubated at 50° for 30 min to digest the enzyme. Final products were mixed with 2.5 μL of loading buffer, heated to 70° for 1 min and subjected to electrophoresis in 1% agarose containing 0.5 μg/mL of E.B. at 4V/cm for 1.5 hr. All DNA forms were separated and migrated as following: RLX, SC, LNR, and NC.

2.7. Pre- and post-strand passage Topo II-mediated DNA religation

Topo II-mediated DNA religations were undertaken according to previous reports [13,14]. Reactions contained 25 U Topo II and 0.2 μg supercoiled pBR322 in a total of 20 μL of cleavage buffer consisting 5 mM $CaCl_2$ or 1 mM APPNHP and 5 mM $MgCl_2$ for pre- and post-strand passage cleavage/religation respectively. Initial DNA cleavage/religation equilibria were established at 37° for 6 min. The testing samples were added to the reaction mixtures just before the initiation of DNA religation. For pre-strand passage religation, the TOPO II–DNA cleavage complex was trapped by the addition of EDTA (0.8 μL of a 250 mM solution) and NaCl (0.6 μL of a 5 M solution) was added to prevent recleavage of the DNA. The samples were placed on ice to slow the reaction rate. Religation was initiated by the addition of 1.8 μL cold $MgCl_2$ (8.5 mM final) and stopped by addition of 2 μL 10% SDS up to 2 min. For post-strand passage religation, samples were rapidly shifted from 37° to 55° to initiate the religation, and then the reactions were terminated by the addition of 2 μL 10% SDS up to 2 min. The samples were processed as described above.

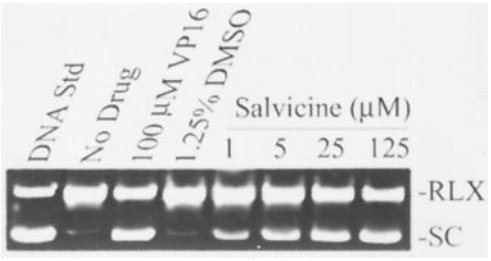

Fig. 2. Effect of salvicine on Topo II-mediated supercoiled pBR322 relaxation. Negatively supercoiled pBR322 (DNA Std) and relaxed pBR322 (no drug) are shown for reference. The positions of SC and RLX are indicated.

2.8. Unwinding assay

DNA unwinding effects of the compounds were assayed according to the method described previously [15]. Plasmid DNA was linearized by EcoRI restriction endonuclease and recovered by phenol extraction and ethanol precipitation. Reaction mixtures (20 μL) containing 30 mM Tris.HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, 0.1 μg of linearized DNA and testing compounds were equilibrated at 20° for 30 min, and then incubated with excessive amounts of T_4 DNA ligase at 20° for 30 min. The reactions were stopped by shifting the temperature to 55°. Afterwards, the compounds were removed from the reaction mixture by extraction with phenol. DNA bands were analyzed by 1% agarose gel electrophoresis.

Another unwinding assay was employed according to the Topo I-mediated supercoiled pBR322 relaxation except that the DNA–compound complexes were dissociated by extraction of the reaction mixture with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v). Then the samples were subjected to electrophoresis and visualized as described earlier.

3. Results

3.1. Inhibition of the activity of Topo II but not Topo I by salvicine

The effect of salvicine on the strand passage activity of Topo II was determined by the enzyme-mediated negatively supercoiled pBR322 relaxation. As shown in Fig. 2, salvicine displayed significant inhibition of this reaction in a concentration-dependent style. The inhibition rate in the

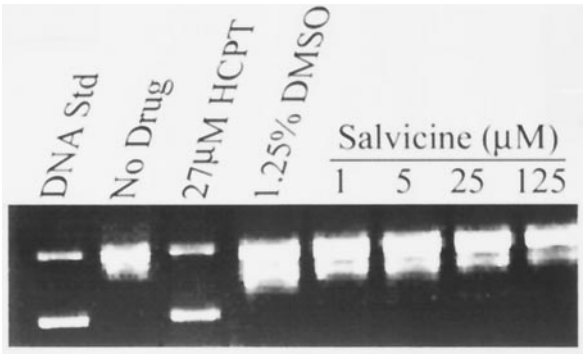

Fig. 3. Effect of salvicine on Topo I-mediated supercoiled pBR322 relaxation. The action was carried out in absence of ATP. Negatively supercoiled pBR322 (DNA Std) and relaxed pBR322 (no drug) are shown for reference. The positions of SC and RLX are indicated.

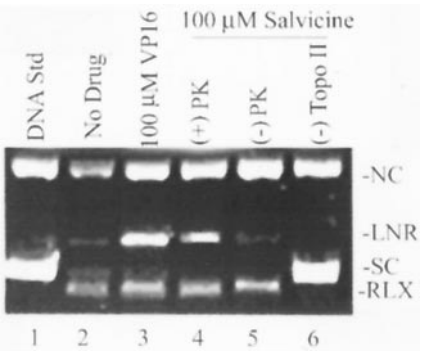

Fig. 5. Double-stranded DNA cleavage stimulated by salvicine was mediated by TOPO II. The reaction was carried out with 25 U TOPO II as described in Materials and methods. Proteinase K (PK) was omitted in lane 5. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of RLX, SC, LNR, and NC are indicated.

presence of 125 μM salvicine was 61.9% compared to 66.3% with 100 μM VP16. It appears that salvicine and VP16 are equipotent against Topo II in this reaction. To determine if salvicine is a selective inhibitor of Topo II, we tested its effect on the catalytic activity of Topo I. As shown in Fig. 3, no inhibitory effect was observed, even up to the concentration of 125 μM. On the other hand, obvious inhibition of the strand passage activity of Topo I was observed with 27 μM HCPT in the assay.

To further testify its effect on Topo II and exclude the possible contamination of Topo I in the relaxation assay, one more specific assay, Topo II-mediated kDNA decatenation, was assessed. kDNA is a massive network consisting of thousands of interlocked circular DNA molecules called "minicircles." Since a transient double-strand break is necessary to release a minicircle from the network, the decatenation of the kDNA is believed to be one of the highly specific assays of Topo II, the enzyme that catalyzes the double-strand passing reaction. As seen in Fig. 4, obvious inhibition of the strand passage activity of Topo II was observed. The result of semiquantification showed that the activity of the enzyme was inhibited by ~40% in the presence of 1 μM of salvicine and ~90% when the concentration was raised to 5 μM, with an IC_{50} of about 3 μM.

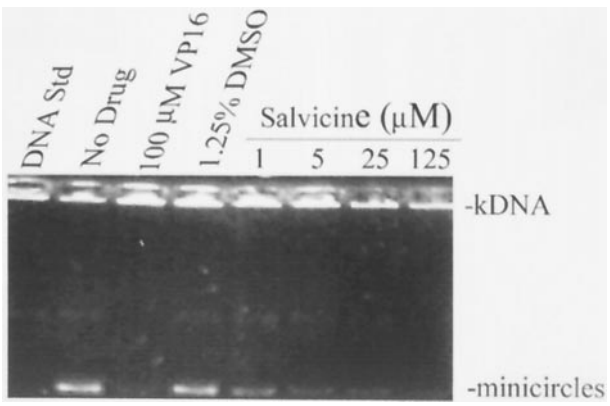

Fig. 4. Effect of salvicine on Topo II-mediated kDNA decatenation. The following control samples are shown: kDNA (DNA Std), minicircles (no drug). The Positions of kDNA and minicircles are indicated.

3.2. Induction of Topo II-mediated DNA cleavage by salvicine

Many agents that inhibit the catalytic activity of Topo II enhance the enzyme-mediated DNA cleavage [16]. A cleavage assay was employed to determine whether salvicine could trap the Topo II–DNA complex. As seen in Fig. 5, 100 μM of salvicine greatly enhanced the DNA cleavage (lane 4), and VP16 seemed to be even more potent, accounting for more linear DNA production at the same concentration (lane 3). Since salvicine is a new compound, it is important to ensure that DNA breakage was generated in the presence of Topo II. The data in Fig. 5 demonstrated that all DNA cleavage in the presence of VP16 or salvicine was covalently attached to the enzyme (lanes 3 and 4). Reaction products had to be digested with proteinase K in order for the cleaved DNA to be released. The protein-linked DNA break is a hallmark of Topo II. In the absence of Topo II, salvicine did not produce any DNA cleavage (lane 6).

Topo II-mediated DNA breaks resulting from trapping of the cleavage complex by some drugs have been shown to be reversible [17]. Our study confirmed this observation utilizing VP16, and experiments were performed to determine whether the cleavage complex trapped by salvicine could also be reversed under the same conditions. As shown in Fig. 6, if the reaction in the presence of salvicine was stopped by salt before addition of SDS, double-stranded DNA breaks were reversed. This result strongly suggests that the cleavage complex stabilized by salvicine formed between Topo II and DNA.

3.3. Effects of salvicine on Topo II catalytic cycle

The modes of action of the Topo II-targeting agents have been demonstrated to correlate with the abilities of these

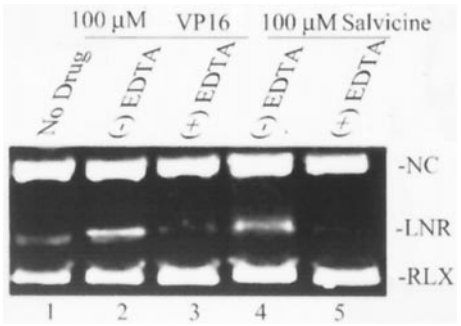

Fig. 6. Effect of removal of Mg^{2+} ions by EDTA/NaCl before protein denaturing by SDS. Lane 1: Topo II/DNA complex; lanes 2 and 3: enzyme/DNA complexes trapped by 100 μM VP16; lanes 4 and 5: enzyme/DNA complexes trapped by 100 μM salvicine; lanes 2 and 4: SDS was added before EDTA/NaCl addition; lanes 3 and 5: EDTA/NaCl was added before SDS addition. The positions of RLX, LNR, and NC are indicated.

drugs to stabilize the Topo II–DNA complex through interference of specific steps within the catalytic cycle of the enzyme, including enhancement of the forward cleavage or inhibition of religation. To determine the basis for salvicine action, the effects of this compound on the individual steps of Topo II were observed.

3.3.1. Topo II–DNA binding

The first step in the Topo II catalytic cycle is the noncovalent interaction of the protein with DNA. The influence of salvicine on this interaction was examined using gel mobility shift analysis. As determined by the upshift of bound DNA to the gel origin, salvicine greatly promoted the Topo II–DNA binding (Fig. 7). Decreasing of the supercoiled DNA displayed a concentration dependency in the presence of salvicine, and supercoiled pBR322 completely disappeared when 50 μM of salvicine was present. Decreasing of the supercoiled DNA was also observed in the presence of 50 and 100 μM VP16 (lanes 3 and 4), but the enhancement of binding affinity induced by salvicine was more obvious than that by VP16.

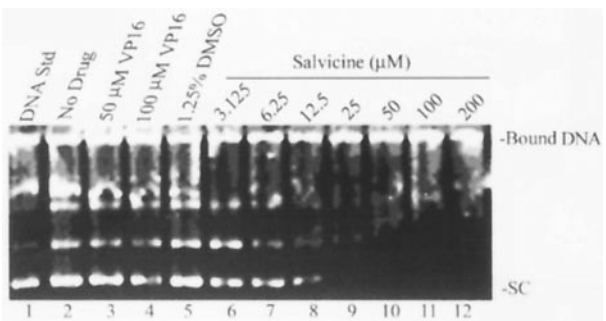

Fig. 7. Effect of salvicine on DNA–Topo II binding. The DNA–TOPO II binding was monitored using a gel mobility shift assay. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of SC and bound DNA (bound DNA) are indicated.

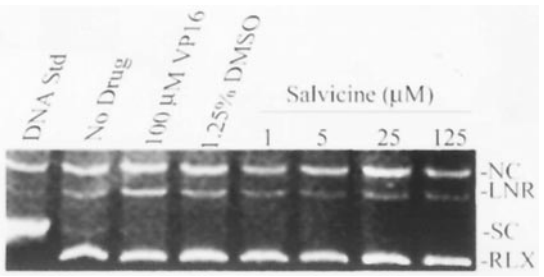

Fig. 8. Effect of salvicine on the pre-strand passage Topo II-mediated cleavage. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of RLX, LNR, SC, and NC are indicated.

3.3.2. Pre- and post-strand passage Topo II-mediated DNA cleavage

The step that immediately follows DNA binding is pre-strand passage DNA cleavage. The effect of salvicine on this step was monitored in the absence of a nucleoside triphosphate. As seen in Fig. 8, no obvious increase in linear DNA was detected by addition of salvicine, whereas a significant increase in the amount of linear molecule was observed under the same conditions by addition of 100 μM VP16. A similar result was obtained for post-strand passage cleavage, which was measured in the presence of a non-hydrolyzable ATP analogue (Fig. 9). These observations demonstrated that salvicine failed to influence the forward cleavage steps of Topo II.

3.3.3. Pre-strand passage Topo II-mediated DNA religation

Calcium-promoted DNA cleavage was induced by trapping covalent enzyme–DNA complex in active forms by addition of EDTA. The EDTA chelated all Ca^{2+}, leaving the enzyme covalently attached to the DNA but unable to religate. The samples were temperature-shifted to halt the forward cleavage reaction, and an excessive Mg^{2+} was added to allow religation. Effects of salvicine on pre-strand Topo II-mediated DNA religation in the catalytic cycle of Topo II are shown in Fig. 10. Lane 1 is the substrate pBR322 control; lane 2 demonstrates the cleavage–religation equilibrium; lane 3 shows Topo II-mediated DNA religation in the absence of the testing samples. In lane 4, an inhibition of DNA religation can be observed in the pres-

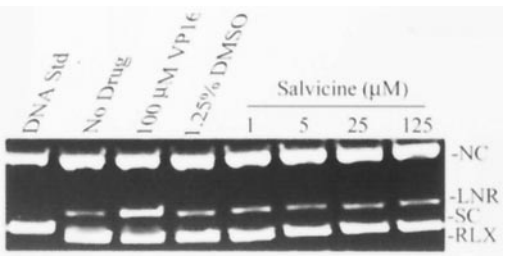

Fig. 9. Effect of salvicine on the post-strand passage Topo II-mediated cleavage. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of RLX, SC, LNR, and NC are indicated.

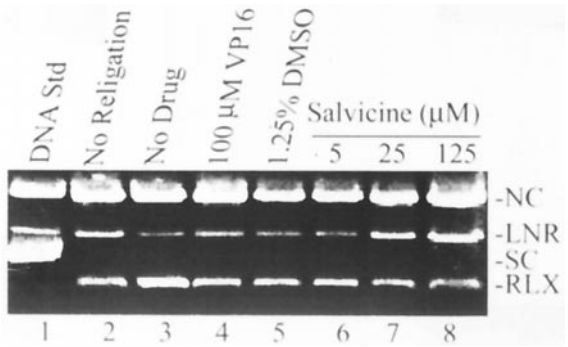

Fig. 10. Effect of salvicine on the pre-strand passage Topo II-mediated religation. The pre-strand DNA passage religation was monitored by the calcium assay. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of RLX, SC, LNR, and NC are indicated.

ence of 100 μM VP16. Compared to VP16, salvicine seems to be more active in stabilizing the pre-strand passage DNA cleavage complex of Topo II by decreasing the rate of religation. In lanes 7 and 8, an approximate 2- and 3-fold increase in linear DNA was detected in the presence of 25 and 125 μM salvicine, respectively, compared to that in the presence of 100 μM VP16.

3.3.4. Post-strand Topo II-mediated DNA religation

Since salvicine inhibited Topo II-mediated pre-strand passage DNA religation, its effect on the enzyme's post-strand passage DNA religation activity was evaluated. The heat-induced DNA religation assay was employed in the experiments. This assay takes advantage of the ability of Topo II to mediate DNA religation but not cleavage at 55°. The results of this experiment are shown in Fig. 11. Lane 1 is the plasmid standard; lane 2 is the vehicle control. When 100 μM VP16 was added to the reaction mixtures, the magnitude of the linear DNA rose about 4-fold compared to the vehicle control (lane 3). In the presence of different concentrations of salvicine, decrease in the linear DNA religation was observed. The intensity of the linear band increase to 2.5-fold in the presence of 125 μM salvicine was

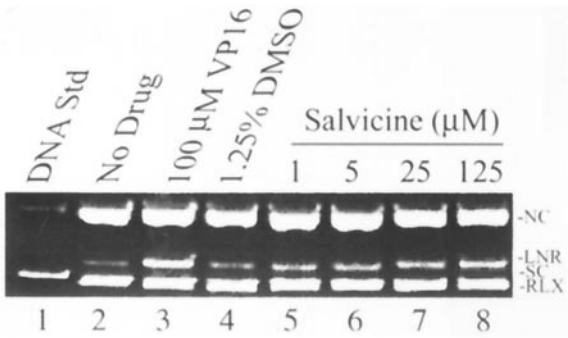

Fig. 11. Effect of salvicine on the post-strand passage Topo II-mediated DNA religation. The post-strand DNA passage religations were monitored by heat-induced religation assay. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of RLX, SC, LNR, and NC are indicated.

compared to DMSO control. The result demonstrated that both compounds impaired Topo II-mediated DNA religation following strand passage, whereas the effect of VP16 on this reaction was even more pronounced than that of salvicine.

3.4. Interactions between salvicine and DNA

Although VP16 and VM26 are classified as non-intercalative drugs, most of the antitumor drugs that can induce the cleavage complex with Topo II are DNA intercalators, such as amsacrine (m-AMSA), ADM, ellipticine, saintopin [18], and TAS-103 [19]. To investigate whether salvicine intercalates into DNA, an unwinding assay was performed using linearized pBR322 and T_4 DNA ligase. In this assay, DNA intercalators first unwind the linearized DNA, which results in a change in the twist of the duplex helix. Circulation of drug-bound DNA by T_4 DNA ligase freezes the linking number of the unwound DNA. Upon drug removal, the twist changes back to normal while the linking number remains constant, which causes the introduction of negative superhelicity into the DNA. Fig. 12 shows the separation of the products on an agarose gel. In lanes 3–5, an obvious inhibition of the activity of the T_4 ligase by ADM was observed, which was due to ADM's strong intercalation ability [20]. However, both the negative control (VP16) and salvicine had no influence on the DNA topoisomers, even at concentrations of 125 μM.

To verify the results, another unwinding assay was performed. In this assay, supercoiled DNA was relaxed by Topo I in the presence of the testing compounds. The intercalation of the compounds into a closed circular DNA reduced the twisting number of the DNA. Upon Topo I addition, the linking number was adjusted to the twist of the intercalated DNA. Following relaxation, samples were phenol-extracted to remove the intercalator and the twisting number restored thus leaving the DNA in a new supercoiled state. As shown in Fig. 13, ADM converted the topoisomers to a less relaxed form at a low concentration of 5 μM, and to a complete supercoiled form at a higher concentration of 25 μM, whereas both VP16 and salvicine had no effect. From these two experiments, we concluded that salvicine is a non-intercalative agent.

4. Discussion

Topo II is an essential enzyme which plays an important role in DNA replication, repair, transcription and chromosome segregation [21,22]. In addition to its critical functions, Topo II has been identified as an important antitumor target [23]. A number of the most active and widely prescribed antineoplastic drugs are targeted at Topo II [24]. In addition, there has been continuous interest in studying and developing new anti-Topo II agents and several potent compounds are currently in clinical trial, including DACA (*N*-

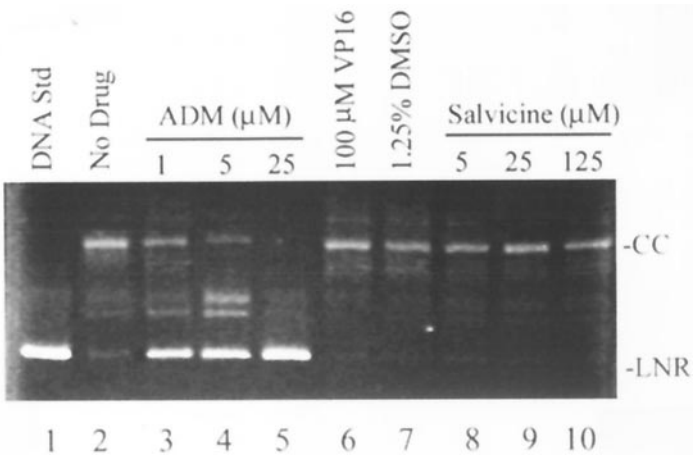

Fig. 12. Effect of salvicine on the DNA unwinding assay with T4 DNA ligase. In this assay, linearized plasmid DNA was incubated with T4 DNA ligase in the presence of testing compounds. Linear DNA is shown for reference. The positions of LNR DNA and circularized DNA (CC) are indicated.

[2-(dimethylamino) ethyl]acridine-4-carboxamide) [25], intoplicine [26], fostriecin [27], S16020-2 [28], and TAS-103 [19]. This study demonstrated that salvicine is a new Topo II inhibitor. It brought about a marked inhibition in the mammalian Topo II-mediated decatenation of kDNA. On the contrary, salvicine had no effect on Topo I-mediated pBR322 relaxation, suggesting that salvicine has a highly selective effect on Topo II.

Drugs that block the overall catalytic activity of Topo II can be divided into two classes. Topo II poisons trap the Topo II–DNA cleavage complex, generating high levels of DNA breaks, such as VP16, aminoaridines, and ellipticine. The other class is Topo II inhibitors, which inhibit the overall catalytic activities without inducing DNA breaks, such as merbarone and bisdioxopiperazines. These two classes of drugs have different mechanisms of action as well as their clinical schemes. As presented in this study, salvicine is a novel Topo II poison which induces protein-linked DNA breaks.

The catalytic cycle of Topo II can be divided into six discrete steps [29] (Fig. 14). In order to determine the level at which the process was influenced by salvicine, various steps in the catalytic cycle were probed. Results of these studies revealed that salvicine trapped the cleavage complex mainly by inhibiting pre-strand Topo II-mediated DNA religation and a relatively weak inhibition of post-strand Topo II-mediated DNA religation, but not by enhancing forward DNA cleavage. Furthermore, salvicine greatly enhanced the binding affinity of Topo II to pBR322 plasmid. To our limited knowledge, only covalent attachment of ethidium to DNA was reported to promote Topo II–DNA binding [12], indicating the different mechanism of salvicine from other

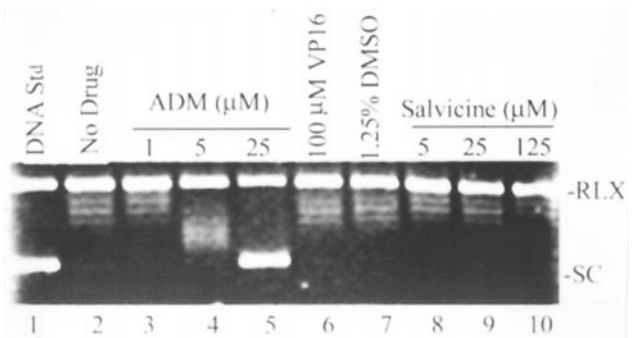

Fig. 13. Effect of salvicine on the DNA unwinding assay with DNA Topo I. In this assay, supercoiled plasmid DNA was incubated with Topo I. Negatively supercoiled pBR322 (DNA Std) is shown for reference. The positions of SC and RLX are indicated.

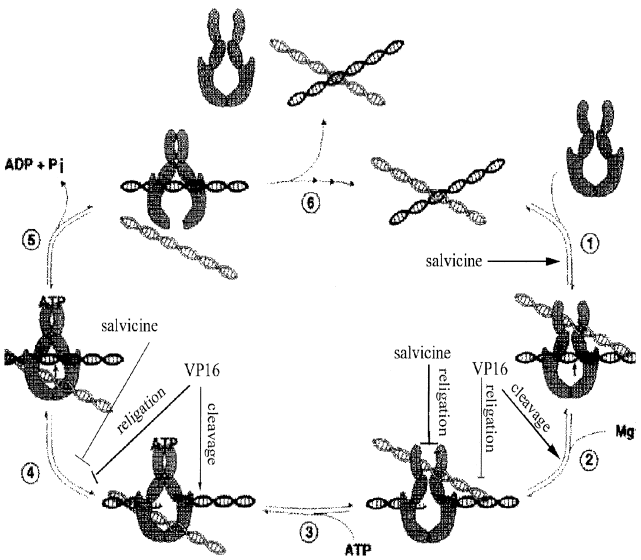

Fig. 14. Effects of salvicine and VP16 on the catalytic cycle of Topo II. The catalytic cycle was diagrammed according to Fortune *et al*. [29]: 1) binding of Topo II to DNA; 2) double-stranded cleavage of the DNA, which is accompanied by the subsequent covalent attachment of the enzyme to the cleaved 5′ termini of the DNA; 3) double-stranded passage through the break, which is dependent on ATP binding to the enzyme; 4) religation of the cleaved DNA; 5) ATP hydrolysis; and 6) enzyme turnover. Salvicine acts mainly on DNA binding and DNA religation, while VP-16 mainly inhibits the enzyme-mediated DNA religation and enhances pre- and post-cleavage. ⊣: inhibition; →: promotion.

compounds. However, the pathway by which the non-covalent complex is assembled is yet to be determined. There are three possibilities for the complex formation: the compound binds to the Topo II–DNA complex, or DNA, or the enzyme alone. Results obtained from this study provide a plausible mechanism for the increase in DNA cleavage by Topo II in that the presence of salvicine results in enhanced binding affinity for the DNA and a concomitant alteration of the Topo II-mediated DNA religation.

A number of Topo II poisons stabilize the cleavage complex by disturbing the gross structure of DNA [30]. It is important to determine whether salvicine is able to intercalate into DNA. Our results with two independent DNA unwinding assays showed that salvicine failed to intercalate into DNA in the desired concentration range. Salvicine therefore belongs to the class of non-intercalative Topo II poisons. In this class, besides VP16 and VM26, few new compounds have been described, indicating its specificity. The result that salvicine is a DNA non-intercalator provides an important clue that salvicine seems to exert its effects on Topo II catalytic activity by a specific interaction with either enzyme or enzyme–DNA complex. Previous studies have shown that ellipticine [31] and VP16 [32] can bind to Topo II in the absence of the DNA. However, the site(s) of drug binding have not been determined. A number of studies reveal that the Topo II mutations which induce drug resistance cluster in two regions, one located near the ATP-binding site and the other around the catalytic tyrosine [33]. On the other hand, differential resistance to VP16, m-AMSA, and quinolones of the Topo II mutants had different amino acid mutations [16]. A recently described mutation that confers hypersensitivity to CP115,953 fails to alter the sensitivity of VP16 [34]. The question whether agents from different structural classes share a common interaction domain on the enzyme is hence raised. Results of the present work indicate that salvicine and VP16 probably share overlapping, but not identical, binding sites on Topo II. This conclusion was based on three lines of evidence: firstly, they both trapped the cleavage complex mainly by inhibiting Topo II-mediated DNA religation, indicating a common (or at least overlapping) site of drug action; secondly, salvicine was more potent than VP16 in promoting DNA–Topo II binding and inhibiting pre-strand Topo II-mediated DNA religation; and thirdly, VP16 enhanced forward DNA cleavage, but this was not the case with salvicine.

In summary, the present study characterized the mechanistic basis for the antitumor activity of salvicine. Based on the findings described above, we consider that the compound should be classified as a Topo II poison. An attempt to further understand the mechanism of its action in cultured tumor cells is being undertaken in our laboratory. This information might provide insight into rational designing of salvicine-like anti-Topo II agents and thus the development of anticancer drugs.

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