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NOTE

Inhibition of DNA Topoisomerase I by Cryptotanshinone from *Salvia miltiorrhiza*

LEE, DONG-SUN AND SOON-DUCK HONG*

Department of Microbiology, College of Natural Science, Kyungpook National University, Taegu 702-701, Korea

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Abstract Cryptotanshinone induced topoisomerase I-mediated DNA cleavage *in vitro* as strongly as camptothecin, whereas topoisomerase II-mediated DNA cleavage was not induced by this agent. In DNA relaxation assay using calf thymus DNA topoisomerase I and supercoiled pBR322 DNA, cryptotanshinone inhibited topoisomerase I-mediated DNA relaxation in a dose-dependent manner. In unwinding assay, cryptotanshinone (50 μM) did not shift the topoisomers of DNA. These results suggest that cryptotanshinone exerted a preferential inhibition of topoisomerase I without intercalating into DNA.

Key words: Cryptotanshinone, inhibitor of DNA topoisomerase I

DNA topoisomerases are enzymes that regulate the superhelical density of DNA by transiently breaking and rejoining DNA strands [10]. They are essential in nucleic acid metabolism including DNA replication, RNA transcription, and chromosomal segregation in mammalian cells [9, 10] and have also been proposed as intracellular targets for cancer chemotherapy [5]. While there are many potent antitumor agents acting on topoisomerase II (teniposide, adriamycin, ellipticines, anthracyclines, and epipodophyllotoxins), few agents have been found to act on topoisomerase I (camptothecin and its derivatives) [1–3, 5, 8].

We found that a cryptotanshinone isolated from the powdered rhizomes of *Salvia miltiorrhiza* is a radical-producing antibiotic which inhibits the growth of *Bacillus subtilis* (our unpublished results). In the course of our continuning reserch, we found that cryptotanshinone is a potent inhibitor of topoisomerase I. In this study, we describe the effect and the mode of action of cryptotanshinone on calf thymus topoisomerase I.

To determine whether cryptotanshinone stimulates the enzyme-linked DNA breakage as camptothecin, supercoiled pBR322 DNA was incubated with topoisomerase I (derived from calf thymus, Amersham, Arlington Heights, IL, U.S.A.) in the presence of cryptotanshinone.

In the relaxation and cleavage reaction with topoisomerase I [11, 12], 17 μl of reaction buffer (35 mM Tris-HCl (pH 7.5), 75 mM KCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM spermidine, and 100 μg/ml bovine serum albumin), 1 μl of plasmid pBR322 DNA (0.1~0.3 μg), 1 μl of drug dissolved in dimethyl sulfoxide/methanol (2 : 3), and 1 μl of topoisomerase I in storage buffer were mixed in this order on ice bath. For the DNA relaxation assay, reaction mixtures were incubated at 37°C for 30 min, terminated by adding 6× loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 15% Ficoll), and analyzed by agarose gel electrophoresis as described below. For DNA cleavage, reactions were terminated by the addition of 2 μl of a solution containing 5% SDS and 2.5 mg/ml proteinase K (Sigma, St. Louis, MO, U.S.A.) and incubated for an additional 30 min at 37°C. After an appropriate volume of 6× loading buffer was added, samples were run onto 1.2% agarose gel (in the presence of 0.5 μg/ml ethidium bromide) in 89 mM Tris-borate (pH 8.3)/2 mM EDTA buffer containing 0.1% SDS at 2 V/cm overnight. Gels were stained with ethdium bromide and washed in large amounts of water. The increase of nicked DNA was estimated as drug-induced topoisomerase I mediated DNA cleavage.

DNA topoisomerase II reactions were performed in 20 μl of reaction buffer (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 15 μg/ml BSA, and 1 mM ATP) supplemented with 0.2 μg of pBR322 DNA, and 1 μl of DNA topoisomerase II

*Corresponding author
Phone: 053-950-5374; Fax: 053-955-5522;
E-mail: biochom@kyungpook.ac.kr

(Amersham) [12]. Reaction mixtures were incubated for 15 min at 30°C.

In the DNA cleavage assay, cryptotanshinone induced the formation of nicked circular DNA which resulted from topoisomerase I-mediated single strand cleavage (Fig. 1, lane c). The activity of cryptotanshinone in inducing cleavages was similiar to that of camptothecin (Fig. 1, lane d).

Topoisomerase I-mediated DNA cleavage is represented by the conversion of closed circular DNA into slower migrating nicked DNA on an agarose gel containing 0.5 μg/ml ethidium bromide.

Cryptotanshinone was found to be active against topoisomerase I-mediated DNA relaxation in vitro (Fig. 2). As shown in Fig. 2, treatment with 2.5 μM cryptotanshinone afforded minimal inhibitory activity (lane d) but, at 5 μM (lane e) formation of relaxed DNA was significantly reduced and the reaction was almost completely inhibited at 25 μM (data not shown); the potency of cryptotanshinone in inhibiting relaxation was almost the same as that camptothecin (lanes g to j).

In a separate experiment, cryptotanshinone was tested for the inhibition of topoisomerase II. However, cryptotanshinone exerted no significant inhibition of topoisomerase II activity even at 100 μM (data not shown). Thus, it appeared that cryptotanshinone exerted a preferential inhibition of topoisomerase I.

The effects of the cryptotanshinone on the inhibition of topoisomerase I were examined by increasing the amount of enzyme or substrate in the reaction mixture (Fig. 3). Inhibition of topoisomerase I caused by cryptotanshinone was prevented by increasing the amount of enzyme (1 to 6 units; lanes d to f). By contrast, no recovery of enzyme activity was observed when the amount of DNA was increased to 0.2 μg (lanes g to I). It is observed that the migration of DNA on the agarose gel was not affected by cryptotanshinone (100 μM, data not shown). These results suggest that cryptotanshinone doesn't interact with DNA (Fig. 3) but inhibits topoisomerase I activity in does-dependent manner (Fig. 2).

It is known that cleavable complex formation with antitumor drugs results in inhibition of the catalytic activity of topoisomerase [11, 12]. The time course of DNA relaxation by topoisomerase I in the presence or absence of drugs is shown in Fig. 4. Under the conditions used in this study, 2 units of topoisomerase I relaxed supercoiled DNA in a time-dependent manner and little supercoiled DNA remained unchanged after 30

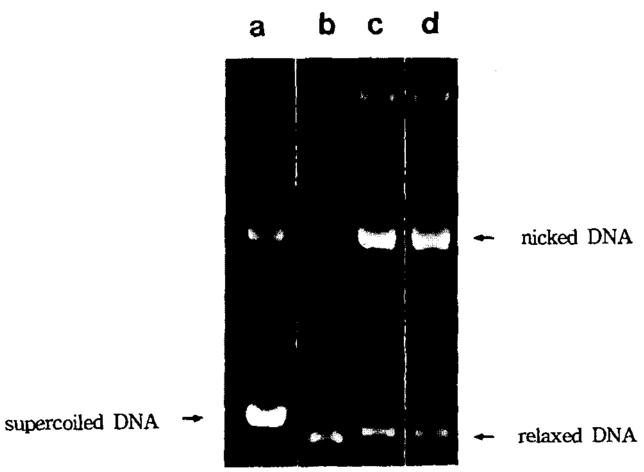

Fig. 1. Mammalian DNA topoisomerase I-mediated DNA cleavage activities of cryptotanshinone.
Plasmid DNA (pBR322 DNA, 0.3 μg) was incubated with 60 units of topoisomerase I in the presence of drugs (lane c~d, 50 μM) followed by SDS/proteinase K treatment, and then analyzed on an agarose gel containing 0.5 μg/ml ethidium bromide. Lane a, pBR322 DNA; lane b, no drug; lane c, cryptotanshinone; lane d, camptothecin.

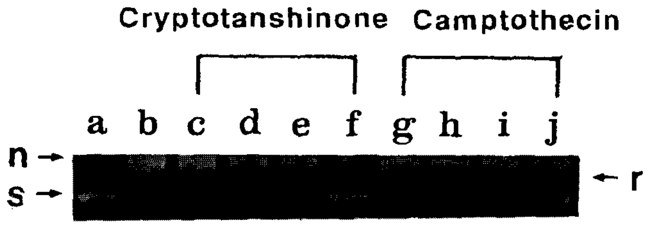

Fig. 2. Effect of cryptotanshinone on relaxation activity of DNA topoisomerase I.
Plasmid DNA (pBR322, 0.2 μg) was treated with 1 unit of topoisomerase I in the presence of drug (lanes c to j) and analyzed on an agarose gel (1%): lane a, pBR322 DNA control; lane b, no drug; lanes c to f, cryptotanshinone; lanes g to j, camptothecin. Drug concentrations were as follows: lanes c and g, 1 μM; lanes d and h, 2.5 μM; lanes e and i, 5 μM; lanes f and j, 10 μM. "n", "r", and "s" denote nicked, relaxed, and supercoiled DNA, respectively.

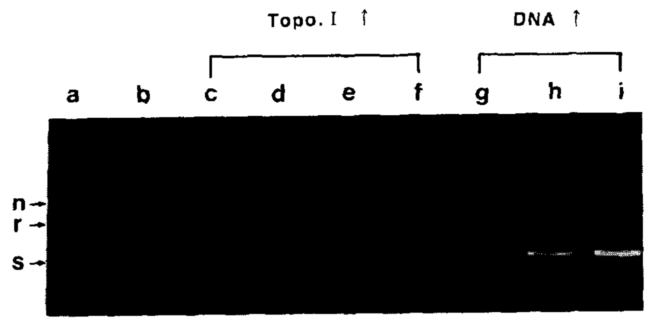

Fig. 3. Effect of the enzyme and substrate DNA concentrations on inhibition of topoisomerase I action by cryptotanshinone.
Lane a, 0.1 μg pBR322 DNA alone; lane b, 0.1 μg pBR322 DNA was incubated with 1 unit of topoisomerase I in 20 μl of the reaction mixture; lane c, 25 μM of cryptotanshinone was added to the reaction mixture; lanes d to f, enzyme concentration was increased to 2, 4, and 6 units in the same reaction mixture as lane c; lanes g to i, DNA concentration was increased to 0.1, 0.15, and 0.2 μg in the same reaction mixture as lane c. "n", "r", and "s" denote nicked, relaxed, and supercoiled DNA, respectively.

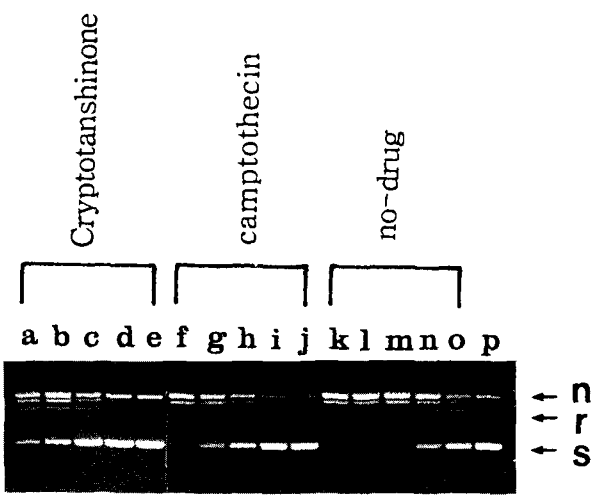

Fig. 4. Time course of DNA relaxation by topoisomerase I with cryptotanshinone.
Supercoiled pBR322 DNA (lane p) was reacted with 2 units of topoisomerase I in the absence (lanes k to o) or presence of the drugs at a concentration of 25 μM: lanes a to e, cryptotanshinone; lanes f to j, camptothecin. Time intervals in relaxation reaction were as follows: lanes a, f, and k, 90 min; lanes b, g, and l, 60 min; lanes c, h, and m, 30 min; lanes d, i, and n, 10 min; lanes e, j, and o, 2 min. "n", "r", and "s" denote nicked, relaxed, and supercoiled DNA, respectively.

min or more. The presence of cryptotanshinone or camptothecin, at 25 μM, resulted in a decrease in the velocity of the relaxation. Cryptotanshinone was slightly more efficient than camptothecin with respect to the inhibition of DNA relaxation, which correlates with the ability to induce enzyme-mediated DNA cleavage and relaxation (See Fig. 1 and 3). The lack of DNA relaxation observed for cryptotanshinone was due to the inhibition of topoisomerase I and was not due to drug-induced DNA unwinding, since supercoiled DNA was relaxed in the presence of cryptotanshinone at 25 μM when higher amounts of topoisomerase I (> 10 units) were used.

Although camptothecin, VP-16, and terpentecin are classified as nonintercalative drugs, most of the antitumor drugs which can induce the cleavable complex with topoisomerase II are DNA intercalators, such as m-AMSA, adriamycin, ellipticines, and saintopin [6, 7, 11]. To investigate whether cryptotanshinone intercalates into DNA, an unwinding assay was performed using linearized pBR322 DNA and T4 DNA ligase according to the method described by Yamashita *et al.* [11]. In this assay, cryptotanshinone (50 μM) was not shifted by the topoisomers of DNA (data not shown). This result indicates that cryptotanshinone is a non-intercalator.

In previously study, we found result that dibutyl phthalate [4] is a poison of topoisomerase I and II like saintopin [12]. However, cryptotanshinone induced single-strand cleavages of DNA in the presence of topoisomerase I as potently as camptothecin. Cryptotanshinone was found to introduce DNA single-strand breaks in association with topoisomerase I but not with topoisomerase II.

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