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Inhibition of DNA Topoisomerase I by Cyclo(L-Prolyl-L-Phenylalanyl) Isolated from *Streptomyces* sp. AMLK-335

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Abstract Cyclo(L-prolyl-L-phenylalanyl) [cyclo(pro-phe)] was isolated from *Streptomyces* sp. AMLK-335 and found to inhibit DNA topoisomerase I activity. In a DNA relaxation assay using supercoiled pBR322 DNA, cyclo(pro-phe) inhibited the DNA topoisomerase activity more strongly than camptothecin, a known topoisomerase inhibitor. However, at a concentration of 10 μM, cyclo(pro-phe) produced a lower degree of DNA relaxation than camptothecin, therefore, the inhibition of topoisomerase I activity by cyclo(pro-phe) was also found to be dose dependent. Accordingly, the current results suggest that cyclo(pro-phe) may be a novel inhibitor of topoisomerase I.

Key words: *Streptomyces* sp. AMLK-335, cyclo(L-prolyl-L-phenylalanyl), topoisomerase I

DNA topoisomerases regulate the superhelical structure of DNA by transiently breaking and rejoining DNA strands [3]. These processes are essential for the metabolism of nucleic acid in mammalian cells during DNA replication, therefore, they have been proposed as intracellular targets for cancer chemotherapy [1, 4, 8]. Eukaryotic cells have two types of topoisomerase, topoisomerases I and II. Topoisomerase I translocates the chromosomal linkage in a single step and does not require any cofactor, whereas topoisomerase II accomplishes the process in two steps and requires ATP [2, 14].

In recent years, the inhibition of topoisomerase I has been considered as an attractive target for cancer treatment [16, 17, 19, 20, 25, 25]. The expression of topoisomerase I is enhanced in several types of leukemia, lymphomia, and colon carcinoma cells. Thus, topoisomerase I-targeted drugs, such as the plant alkaloid camptothecin (CPT) and its derivatives, including topotecan, 9-amino-CPT, and CPT-1, are currently used in cancer chemotherapy [6, 10, 17]. CPT inhibits topoisomerase I, thereby reducing the cleaving of the DNA and isomerase complex [5, 6].

Recently, cryptotanshinone, β-Lapachone, and diospyrin have also been reported as topoisomerase I inhibitors [9, 11, 19]. Accordingly, these findings prompted the current authors to screen for topoisomerase I inhibitors in soil bacteria, and isolated cyclo(pro-phe) from *Streptomyces* sp. AMLK-335 [20]. Cyclo(pro-phe) was found to exhibit anti-VRE (vancomycin-resistant enterococci) activity against two VRE strains. Therefore, the present study was undertaken to investigate the possible antitumor activity of cyclo(pro-phe), based on the supposition that cyclo(pro-phe) may have an inhibitiory effect on topoisomerase I.

The DNA topoisomerase I was purchased from TAKARA SHUZO, Ltd. (Tokyo, Japan). The supercoiled pBR322 DNA, purified from *Escherichia coli* using the method described by Maniatis *et al.* [13], was purchased from Promega (Madison, WI, U.S.A.). Camptothecin (lactone form) isolated from *Camptotheca accuminata* wood using the method of Liu *et al.* [12], was obtained from Sigma Chemical Co. (St. Louis, U.S.A.), and the cyclo(pro-phe) was isolated from *Streptomyces* sp. AMLK-335 [20]. The structure of cyclo(pro-phe) is shown in Fig. 1.

The topoisomerase I activity was determined by a DNA relaxation assay carried out in 17 μl of a reaction buffer contaning 35 mM Tris-HCl (pH 7.5), 75 mM KCl, 5 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM spermidine, 100 μg/ml

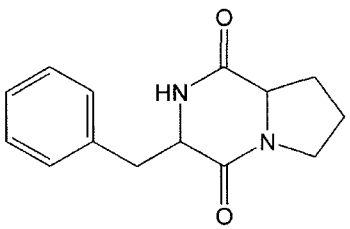

Fig. 1. Chemical structure of the isolated compound, cyclo(L-prolyl-L-phenylalanyl).

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bovine serum albumin, 2 μl of supercoiled pBR322 DNA (0.2-0.6 μg), 1 μl of the drug to be tested dissolved in dimethylsulfoxide/methanol (2:3), and topoisomerase I (1 unit). One unit of topoisomerase I activity was defined as the amount of the enzyme required to completely relax 0.4 μg of supercoiled DNA. In some experiments, the relaxation mixtures were supplemented with cyclo(pro-phe) or CPT [1 μl in dimethylsulfoxide (DMSO)]. After incubation at 37°C for 30 min, the reaction was terminated by 5 μl of a stop buffer containing 5% SDS, 50 mM EDTA, 20% Filcoll, 0.1 mg/ml bromophenol blue, and 0.1 mg/ml of xylene cyanol, and the DNA samples were then electrophoresed in a 0.7% agarose gel. The gels were stained with ethidium bromide (5 μg/ml) and photographed. The DNA relaxation activity of topoisomerase II was assayed as described above, except that APT (1 mM) and topoisomerase II were added to the reaction mixture. The quantity of DNA was measured by scanning the negatives using a Shimazu scanning densitometer. The inhibition of the topoisomerase I catalytic activity by cyclo(pro-phe) and camptothecin, a known topoisomerase I inhibitor included as a positive control, was assessed. As shown in Fig. 2, only pBR322 DNA was represented (lane a), and treatment with 2 units of topoisomerase I resulted in the formation of a relaxed form of DNA (lane b). Cyclo(pro-phe) at 10 μM inhibited this relaxation more effectively than camptothecin (10 μM) (lanes c, d). Also, cyclo(pro-phe) had no effect on the plasmid DNA conformational topology (lane e). Cyclo(pro-phe) was also found to be active against DNA topoisomerase

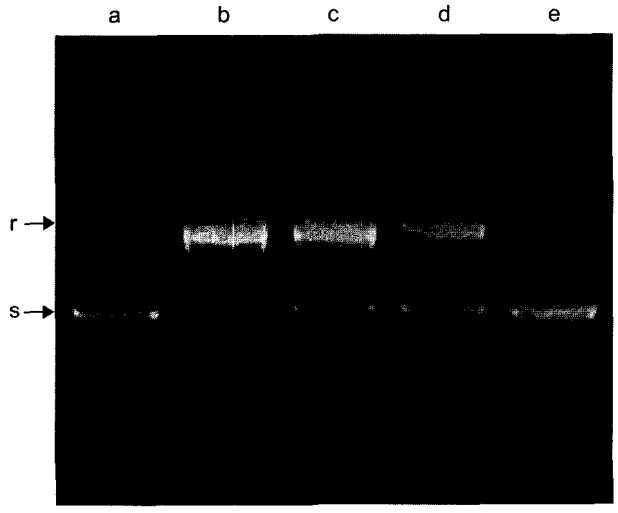

Fig. 2. Inhibitory activity of cyclo(pro-phe) against DNA topoisomerase I.

Lane a: pBR322 DNA; lane b: pBR322 DNA+topo I; lane c: pBR322 DNA+topo I+camptothecin (10 μM); lane d: pBR322 DNA+topo I+ cyclo(pro-phe) (10 μM) purified from isolated strain AMLK-335; lane e: pBR322 DNA+cyclo(pro-phe) (10 μM). "r" and "s" denote relaxed and supercoiled DNA, respectively.

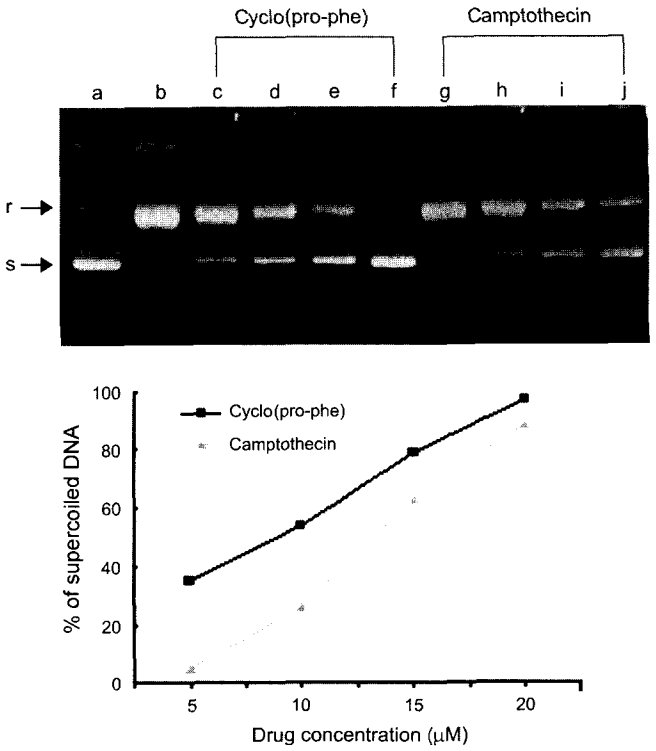

Fig. 3. Effect of cyclo(pro-phe) on relaxation activity of DNA topoisomerase I.

The plasmid DNA (pBR322, 0.4 μg) was treated with 2 units of topoisomerase I in the presence of cyclo(pro-phe) (lanes c to f) and analyzed on an agarose gel (1.2%): lane a, pBR322 DNA control; lane b, cyclo(pro-phe); lanes c to f, cyclo(pro-phe); lanes g to j, camptothecin. The cyclo(pro-phe) and camptothecin concentrations were as follows: lanes c and g, 5 μM; lanes d and h, 10 μM; lanes e and i, 15 μM; lanes f and j, 20 μM. "r" and "s" denote relaxed and supercoiled DNA, respectively.

I-mediated DNA relaxtion *in vitro* (Fig. 3). As shown in Fig. 3, treatment with 5 μM (lane c) cyclo(pro-phe) afforded minimal inhibitory activity, yet 15 μM (lane e) resulted in a significant reduction in the DNA relaxation, which became almost completely inhibited at 20 μM (lane f). Cyclo(pro-phe) was more potent in inhibiting the DNA relaxation than camptothecin (compare lanes c-f with lanes g to j). Furthemore, the IC_{50} value of cyclo(pro-phe) was approximately 13 μM, whereas the topoisomerase I activity was inhibited by CPT with an IC_{50} of about 17 μM under the same conditions (data not shown), in agreement with a previous report [7]. Furthermore, cyclo(pro-phe) inhibited topoisomerase I more strongly than cryptotanshinone, which had previously been reported as a topoisomerase I inhibitor [9]. To investigate whether mammalian DNA topoisomerase II was also inhibited by cyclo(pro-phe), human topoisomerase IIα was tested in an *in vitro* DNA relaxation assay. However, cyclo(pro-phe) did not inhibit the DNA relaxation by the human topoisomerase II at the concentration of 20 μM nor the restriction enzyme *Eco*RI

(lata not shown), indicating that cyclo(pro-phe) isolated f om *Streptomyces* sp. AMLK-335 specifically inhibited c ıly topoisomerase I activity.

In summary, it would appear that cyclo(pro-phe) exhibits ɛ ıtitumor activity. Indeed, cyclo(pro-phe) inhibited DNA t ɪpoisomerase more strongly than CPT, a known topoisomerase I inhibitor. The inhibition of DNA topoisomerase I by c ʏclo(pro-phe) was also found to be dose dependent and ʂ gnificantly reduced the DNA relaxation. The lack of I ꞌNA relaxation with cyclo(pro-phe) was due to the i ıhibition of topoisomerase I rather than drug-induced I ꞌNA unwinding, as the supercoiled DNA became relaxed i ı the presence of 20 μM cyclo(pro-phe), when larger ɛ nounts of topoisomerase I (>10 units) were used, thereby ʂ ıggesting that cyclo(pro-phe) was more effective as a I ꞌNA topoisomerase I inhibitor. Our recent suggestion that c ʏclo(pro-phe) can be used as both an anti-VRE and ɛ ıtimicrobial agent [20], together with other reports that t ıe cyclic dipeptide of cyclo(pro-phe) exhibits antiviral, ɛ ntibiotic, and antitumor properties [14, 16, 23], strongly ı ecessitate further research on the inhibition mechanism of c ʏclo(pro-phe) on DNA topoisomerase I.

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