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ORIGINAL ARTICLE

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A lymphoma cell line resistant to 4-piperidinopiperidine was less sensitive to CPT-11

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Abstract CPT-11 is a promising new anticancer drug in which 4-piperidinopiperidine is a side-chain structure. In the present studies, we examined the role played by 4-piperidinopiperidine in the pharmacological activity of CPT-11. When T-cell lymphoma RVC cells were incubated with 4-piperidinopiperidine at concentrations higher than 50 μg/ml, the cells underwent apoptosis with a nucleosomal ladder of chromosomal DNA on agarose gels in a dose-dependent manner. We then established a cell line resistant to 4-piperidinopiperidine (4-pp-R), which was about 20-fold more resistant to 4-piperidinopiperidine than the parent RVC cells. Moreover, 4-pp-R cells showed coresistance to CPT-11. However, the growth rate and cell cycle population of 4-pp-R cells were not different from those of the parent RVC cells, and there were no differences between the two cell lines with regard to their drug transport system, CPT-11-metabolizing activity, their activity and amount of topoisomerase I, or their sensitivity to either SN-38 or etoposide, suggesting that the cytotoxicity of CPT-11 is not a consequence of the activity of its metabolite SN-38. The present studies suggested that resistance to CPT-11 is in part due to insensitivity to 4-piperidinopiperidine and its metabolites, since 4-piperidinopiperidine was cytotoxic and 4-pp-R cells were less sensitive to CPT-11.

Key words 4-Piperidinopiperidine · CPT-11 · Apoptosis · Topoisomerase · Drug resistance

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Abbreviations *CPT* Camptothecin · *CPT-11* 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-CPT · *DAPI* 4′,6-diamidino-2-phenylindole · *HPLC* high-performance liquid chromatography · *PAGE* polyacrylamide gel electrophoresis · *PMSF* phenylmethylsulfonyl fluoride · *SDS* sodium dodecyl sulfate · *SN-38* 7-ethyl-10-hydroxy-CPT

Introduction

Camptothecin, an alkaloid isolated from *Camptotheca acuminata* [1], has an antitumor effect on a variety of experimental tumors [2], but has had no favorable results in clinical investigations because of its severe and unpredictable toxicity [3]. SN-38, a derivative of camptothecin, has also been shown to have a potent antitumor effect by inhibiting topoisomerase I activity through stabilizing DNA-topoisomerase I cleavable complexes. SN-38 is less toxic than camptothecin, but is impractical for clinical use because of its insolubility. Camptothecin and SN-38 contain a closed lactone ring in their structure. The sodium salts of these compounds with an open lactone ring are soluble, but they exhibit weaker activity than the closed ring compounds [4]. CPT-11, was derived from SN-38 and 4-piperidinopiperidine since it is water soluble, but does not have an open lactone ring [5]. CPT-11 itself has only a marginal antiproliferative effect, but in vivo it is converted to 4-piperidinopiperidine, and the presumed active product, SN-38, which is cytotoxic [6, 7]. However, it has been reported that CPT-11 is cytotoxic to a cell line that is resistant to SN-38 [8]. In that cell line, there is no difference in the uptake of CPT-11 and SN-38, or in their activity in inhibiting topoisomerase I. These findings suggest that the antitumor effect of CPT-11 cannot be explained only by the inhibition of topoisomerase I through SN-38. To examine whether the cytotoxicity of CPT-11 could be due, in part, to its 4-piperidinopiperidine moiety, we studied the effect of

4-piperidinopiperidine on the growth of lymphoma RVC cells, and established a 4-piperidinopiperidine-resistant cell line (4-pp-R) which showed crossresistance to CPT-11.

Materials and methods

Drugs and chemicals

CPT-11 and SN-38 were kindly provided by Yakult Co. (Tokyo, Japan), 4-piperidinopiperidine was purchased from Aldrich Chemical Co. (Milwaukee, Wis.), DAPI was obtained from Sigma Chemical Co. (St. Louis, Mo.), and etoposide was a generous gift from Nippon Kayaku Co. (Tokyo, Japan). Cell culture materials were obtained from GIBCO Laboratories (Grand Island, N.Y.), supercoiled pBR322 DNA was from Takara Shuzo Co. (Tokyo, Japan), and kinetoplast DNA and antitopoisomerase I antibody were from TopoGEN (Columbus, Ohio). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell lines and culture conditions

The cell line used in this study was a radiation leukemia virus-induced leukemia line, RVC cell line [9], provided by Dr. T. Tadakuma (National. Defense Medical College, Saitama, Japan). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 m*M* 2-mercaptoethanol, 10 m*M* HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator in an atmosphere containing 5% CO_2. The 4-pp-R cell line was established by treating RVC cells with a sublethal concentration of 4-piperidinopiperidine (50 μg/ml) and then gradually increasing the drug concentration to 500 μg/ml. The 4-pp-R cells were maintained in the presence of 500 μg/ml 4-piperidinopiperidine.

Cell growth and cell viability

The number of viable cells were assessed by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide method described by Scudiero et al. [10], using a microplate reader model 450 (BioRad, Hercules, Calif.) with a test wavelength of 450 nm and a reference wavelength of 650 nm.

DNA electrophoresis and quantitation of DNA fragmentation

Electrophoretic analysis and a fragmentation assay of genomic DNA were performed as described previously [11]. DNA fragmentation is expressed as the ratio of the DNA content in the detergent-soluble supernatant (fragmented DNA) to that in the pellet plus supernatant (total DNA) expressed as a percentage.

Analysis of cell cycle population

Cells (1×10^5) were stained with propidium iodide solution (0.05 mg/ml) in 0.1% sodium citrate as described by Krishan [12]. Samples were kept at 4 °C in the dark, and the cell cycle population was analyzed with a CytoAce-150 apparatus (Jasco, Tokyo).

Preparation of nuclear extract

All procedures were carried out at 4 °C. Cells were homogenized with a Dounce homogenizer in 5 m*M* potassium phosphate buffer (pH 7.0) containing 2 m*M* $MgCl_2$, 1 m*M* PMSF, 1 m*M* 2-mercaptoethanol, 0.5 m*M* dithiothreitol, 0.1 m*M* EDTA, and 0.1% Nonident-P40. Nuclei were collected by centrifugation at 2000 *g* for 10 min, and were suspended in 20 m*M* Tris-Cl, pH 8.0, containing 1 m*M* PMSF, 10 m*M* 2-mercaptoethanol and 4 m*M* EDTA. The nuclear proteins were then extracted by adding NaCl to a final concentration of 0.5 *M*. The supernatant was obtained by centrifugation at 100 000 *g* for 30 min to remove chromosomal DNA, and then subjected to ammonium sulfate fractionation. The precipitate obtained by ammonium sulfate saturation of between 30% and 60% was dialyzed against 20 m*M* Tris-HCl (pH 8.0) containing 1 m*M* PMSF, 10 m*M* 2-mercaptoethanol and 10% glycerol at 4 °C for 24 h with one change of buffer. This dialyzed sample was used for the topoisomerase assay and Western blot analysis.

Assay for topoisomerase I and II activities

Topoisomerase I activity was determined by measuring the relaxation of supercoiled plasmid DNA using a HPLC system as described previously [13]. One unit of activity was defined as the amount of topoisomerase I that relaxed half the amount of the substrate, pBR322 DNA, for 30 min. Topoisomerase II activity was determined by measuring the decatenation of kinetoplast DNA [14]. One unit of activity was defined as the amount of topoisomerase II needed to decatenate 50% of the kinetoplast DNA under the experimental conditions used.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blotting were performed as described previously [15]. After the nuclear proteins were electrophoresed on a 7.5% polyacrylamide separation gel, proteins were visualized by silver staining [16] or transferred to the nitrocellulose membrane. The blotted membrane was incubated first with a human antitopoisomerase I antibody and then with a horseradish peroxidase-conjugated antihuman IgG (Cappel, Durham, N.C.). Topoisomerase I bound to the membrane was visualized with enhanced chemiluminescence (ECL) solution (Amersham, UK), used according to the manufacturer's instructions.

Determination of intracellular CPT-11 and SN-38 contents

RVC cells and 4-pp-R cells (8×10^6 cells) were exposed to 50 μ*M* CPT-11 for various lengths of time, and washed once with ice-cold phosphate-buffered saline (pH 7.4). Intracellular CPT-11 and SN-38 were extracted with ice-cold 0.15 *M* KCl containing 0.1% Triton X-100, and the concentrations of these drugs were then determined by HPLC as described previously [6, 7]. The samples were applied to a TSK gel ODS 80TM column (4.6 × 250 mm, TOSO, Tokyo), and the column was eluted with 0.001 *N* HCl and CH_3CN/H_2O (2:1 v/v) at a flow rate of 0.5 ml/min at 50 °C. The concentration of the compounds was quantified using a fluorescence spectrophotometer F-1000 (Hitachi, Tokyo) with an excitation wavelength of 380 nm, and an emission wavelength of 430 nm for CPT-11 and 540 nm for SN-38.

Results

Effect of 4-piperidinopiperidine on cell growth in lymphoma cells and establishment of the 4-pp-R cell line

When RVC cells were incubated with 4-piperidinopiperidine for 24 h, cell viability decreased dose-dependently (Fig. 1A). At 25 µg/ml, the cells did not grow but were alive, but at 500 µg/ml after a 24-h incubation, almost all the cells had died. The DNA from the treated cells was extracted after a 24-h incubation and analyzed by agarose gel electrophoresis. Internucleosomal DNA fragmentation, a biochemical characteristic of apoptosis, was observed to have occurred dose-dependently in these cells (Fig. 1B). DNA fragmentation in the cells treated with 4-piperidinopiperidine at 50, 100, and 200 µg/ml was 7.2%, 17.7%, and 33.2%, respectively, after the 24-h incubation. In the absence of 4-piperidinopiperidine, DNA fragmentation was less than 1.7%. These results indicated that 4-piperidinopiperidine was cytotoxic to the RVC cells and induced apoptosis at concentrations exceeding 50 µg/ml. We then established the 4-pp-R cell line by increasing the concentration of 4-piperidinopiperidine in the medium from 50 µg/ml to 500 µg/ml. The established cell line was viable even in the presence of 500 µg/ml 4-piperidinopiperidine, indicating that the 4-pp-R cells were about 20-fold more resistant to 4-piperidinopiperidine than the parent RVC cells (Fig. 1A). In the 4-pp-R cells, DNA fragmentation was not observed (less than 2%) after a 24-h incubation in the presence of 4-piperidinopiperidine at 500 µg/ml (Fig. 1B). The growth rate and cell cycle population of the 4-pp-R cells were almost the same as those of the parent cells as shown in Fig. 2, indicating that the 4-pp-R cells had growth properties similar to those of the parent cells.

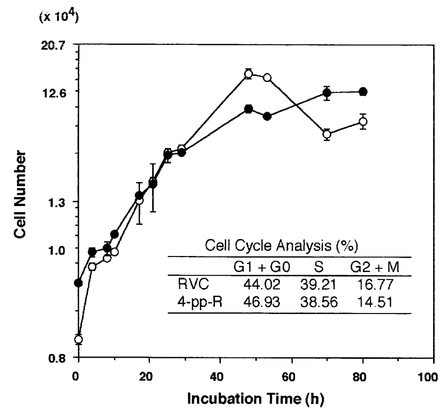

Fig. 2 Cell growth curves of RVC cells and 4-pp-R cells. RVC cells (○) and 4-pp-R cells (●) were incubated and the cell number was determined. *Vertical bars* represent standard errors. The cell cycle population analysis is also shown

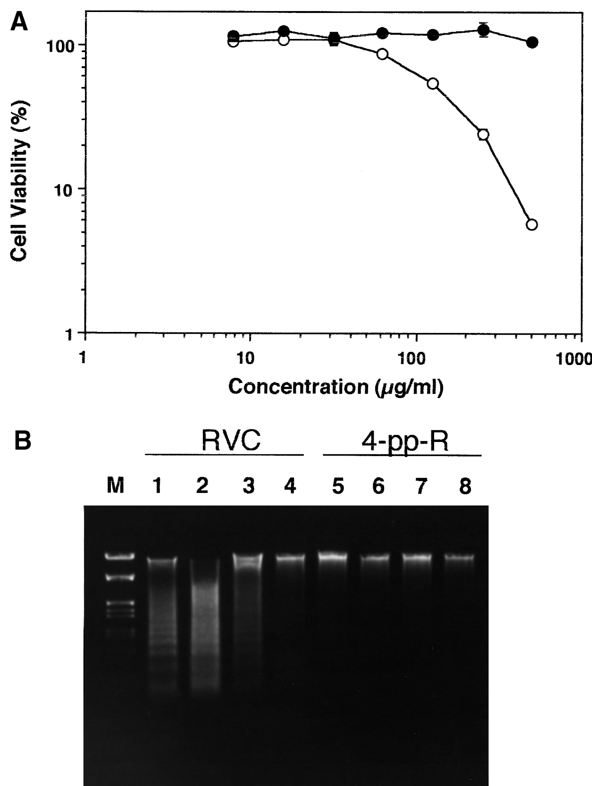

Fig. 1 A Effects of 4-piperidinopiperidine on cell viability. RVC cells (○) and 4-pp-R cells (●) were incubated with 4-piperidinopiperidine for 24 h. Values are percentages of the viability at the initiation of culture. *Vertical bars* represent standard errors. **B** Electrophoretic analysis of chromosomal DNA ladder formation. After incubation with 4-piperidinopiperidine for 24 h, chromosomal DNA was extracted and analyzed by electrophoresis on a 1.5% agarose gel. The concentrations of 4-piperidinopiperidine used were 200 µg/ml (*lanes 1 and 5*), 100 µg/ml (*lanes 2 and 6*), 50 µg/ml (*lanes 3 and 7*), and no drug (control, *lanes 4 and 8*). The amount of each DNA sample applied was approximately 2 µg. *Lane M* is λDNA double digested by *Eco*RI and *Hin*dIII

Effects of CPT-11, SN-38 and etoposide on cell growth

When RVC cells were incubated with CPT-11, SN-38, or etoposide, apoptosis was induced. After incubation with CPT-11 at 31.3, 62.5, 125, and 250 µ*M* for 24 h, the viability of RVC cells was 38.1%, 16.4%, 13.0%, and 11.8%, respectively, whereas that of the 4-pp-R cells was 104.1%, 81.2%, 55.1%, and 28.5%, respectively (Fig. 3A). The IC_{50} value (drug dose required to reduce the initial cell viability to 50%) in the RVC and 4-pp-R cells was 25 m*M* and 150 µ*M*, respectively, indicating that the 4-pp-R cells had about sixfold more resistance to CPT-11 than the parent cells. Nevertheless, RVC cells and 4-pp-R cells showed no difference in sensitivity to SN-38 and etoposide (Fig. 3B, C).

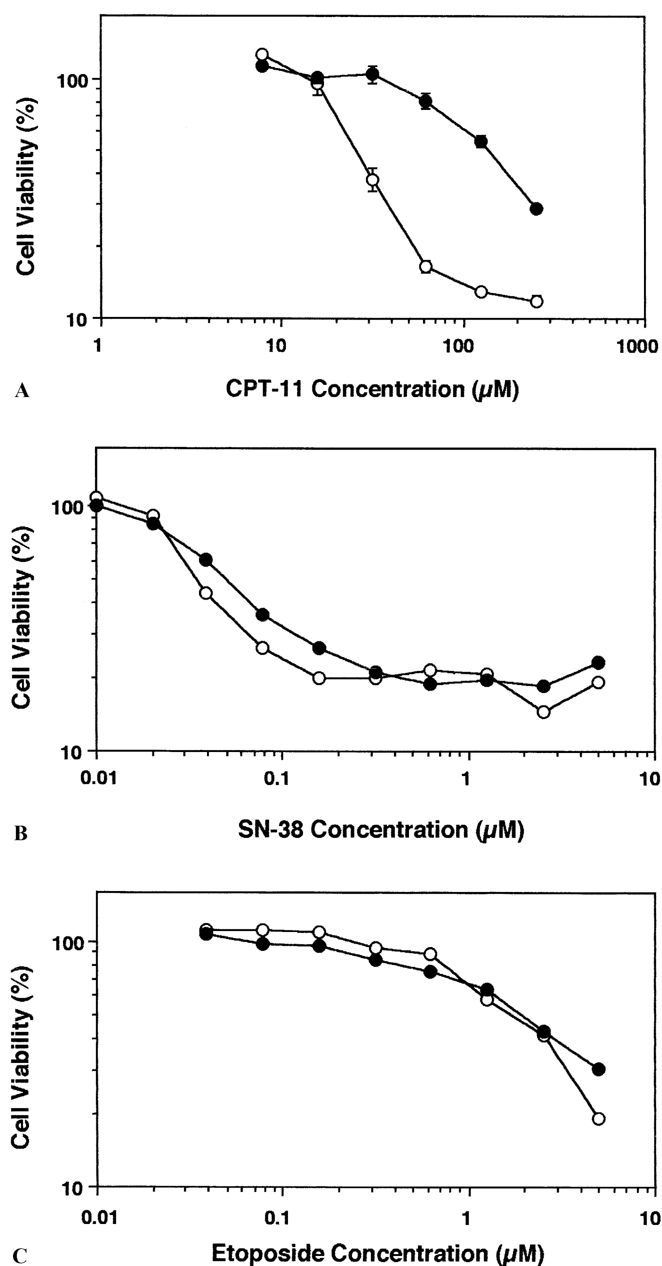

Fig. 3A–C Effects of CPT-11, SN-38 and etoposide on viability of the parent cell line, RVC, and the 4-piperidinopiperidine-resistant cell line, 4-pp-R. RVC cells (○) and 4-pp-R cells (●) were incubated in the presence of CPT-11 (**A**), SN-38 (**B**), or etoposide (**C**) at the concentrations indicated 24 h, and cell viability was determined. Data are mean values of triplicate determinations and *vertical bars* represent standard errors

Intracellular concentrations of CPT-11 and SN-38 in RVC and 4-pp-R cells

The 4-pp-R cells were resistant to CPT-11, but were as sensitive to SN-38 and etoposide as the RVC cells. We then measured the intracellular concentration of CPT-11 and SN-38 in the parent and 4-pp-R cells after incubation with CPT-11 for periods of 30 min to 4 h. The intracellular concentrations of CPT-11 and SN-38 were at a similar level in both RVC and 4-pp-R cells (Table 1). These results indicate that the resistance of 4-pp-R cells to CPT-11 was not due to an alteration in drug uptake or excretion, or to a change in the activity in converting CPT-11.

Topoisomerase activity in RVC and 4-pp-R cells

To investigate whether the resistance to CPT-11 in the 4-pp-R cells was a result of a decrease in the amount of topoisomerase or its activity, we analyzed the expression of topoisomerase I by Western blotting, and measured the activities of topoisomerase I and II. No significant difference in the expression of topoisomerase I was observed between RVC and 4-pp-R cells, as shown in Fig. 4. Both the specific activity of topoisomerase I and II and the activity per cell were at similar levels in these two cell lines (Table 2). Further, the topoisomerase I partially purified from both cell lines exhibited similar sensitivity to SN-38 and CPT-11 (Table 2). These results suggest that the 4-pp-R cells exhibited neither a qualitative nor a quantitative alteration in topoisomerase I. 4-Piperidinopiperidine did not inhibit the activity of topoisomerase I (Table 2). There was no difference in the activity of topoisomerase II in the two cell lines (Table 2), and both cell lines were sensitive to etoposide. Thus, it is unlikely that the resistance to CPT-11 in 4-pp-R cells is a result of alterations in topoisomerases.

Discussion

The topological state of DNA plays a crucial role in cell growth and differentiation through the regulation of basic processes such as DNA replication, transcription, recombination, and repair by topoisomerases [17–19]. Thus, it is likely that the inhibition of topoisomerase activity is lethal for cells, and DNA topoisomerases appear to be a principal target for several antitumor drugs [20]. CPT-11 is a derivative of camptothecin and is converted to SN-38 and 4-piperidinopiperidine by the action of esterase within the cells. The functions of SN-38 have been studied in depth to elucidate the cytotoxicity of CPT-11, and SN-38 has been shown to inhibit topoisomerase I [7]. However, the effect of 4-piperidinopiperidine on tumor cells has not been elucidated. In the present study, we showed that 4-piperidinopiperidine induced apoptosis in RVC cells, although relatively high doses were required. We have observed that the compounds also induces apoptosis in mouse thymocytes and spleen cells at concentrations of less than 5 μg/ml, suggesting that 4-piperidinopiperidine is capable of inducing apoptosis in a variety of cells.

Table 1 Intracellular concentration of CPT-11 and SN-38. RVC cells and 4-pp-R cells (each of 8×10^6) were incubated at the presence of 50 μ*M* CPT-11 for the indicated times. Then the intracellular concentrations of CPT-11 and SN-38 were analyzed as described in Materials and methods (values are means ± SD of three experiments)

Incubation time (h)	RVC		4-pp-R	
	CPT-11 (pmol/10^6 cells)	SN-38 (fmol/10^6 cells)	CPT-11 (pmol/10^6 cells)	SN-38 (fmol/10^6 cells)
0.5	0.843 ± 0.025	5.22 ± 1.82	0.889 ± 0.031	5.10 ± 0.97
1	0.839 ± 0.012	4.13 ± 0.34	0.810 ± 0.003	3.71 ± 0.28
2	0.513 ± 0.009	2.63 ± 0.18	0.480 ± 0.006	3.35 ± 0.20
4	0.352 ± 0.013	2.25 ± 0.19	0.517 ± 0.132	2.53 ± 0.44

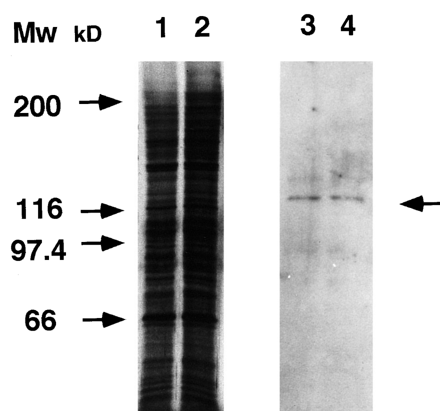

Fig. 4 Western blot analysis of DNA topoisomerase I. The extract from RVC cells (*lanes 1 and 3*) and that from 4-pp-R cells (*lanes 2 and 4*) were electrophoresed on 7.5% polyacrylamide gel. *Lanes 1 and 2* were visualized by silver staining. *Lanes 3 and 4* were transferred to the nitrocellulose membrane and visualized using anti-topoisomerase I human antibody. Migrations of molecular weight standards are as indicated in the left column. *Arrow on the right* shows the position of topoisomerase I

Relatively high doses of 4-piperidinopiperidine and CPT-11 were required for cytotoxicity against both RVC and 4-pp-R cells. However, the intracellular content of 4-piperidinopiperidine was too low to be detected by gas-liquid chromatography and that of CPT-11 was also extremely low even in the presence of CPT-11 at high concentrations (Table 1). These results suggest that these drugs are hardly incorporated into the cells. The 4-pp-R cells showed a reduced sensitivity to CPT-11 (Fig. 3A), but were still sensitive to SN-38 and etoposide (Fig. 3B, C). Many camptothecin-resistant cell lines have been established. These cell lines exhibit various characteristics including a decrease in the amount of topoisomerase I protein [21], a decreased enzyme activity resulting from a mutation in the topoisomerase I gene [22, 23], and low intracellular drug concentration resulting from alterations in drug transport systems [24]. The 4-pp-R cells showed no significant decrease in topoisomerase I protein (Fig. 4), no change in the activity of topoisomerase I (Table 2), and no significant alteration in intracellular drug concentration (Table 1). Thus, it is unlikely that either the uptake or excretion of the drug, or the esterase activity were altered in 4-pp-R cells. The resistance to CPT-11 in the 4-pp-R cells is probably brought about by a yet-unknown mechanism. Takeda et al. reported a human pancreatic tumor cell line sensitive to CPT-11, but resistant to SN-38, and suggested different action mechanisms of CPT-11 and of SN-38 [8]. The present studies showed that 4-piperidinopiperidine exerted cytotoxicity and 4-pp-R cells were less sensitive to CPT-11. These results suggest that the antitumor effect of CPT-11 cannot be explained only by the inhibitory action of SN-38 on topoisomerase I. 4-Piperidinopiperidine or its metabolite may contribute in part to the cytotoxicity of CPT-11, although the cytotoxic dose of 4-piperidinopiperidine did not appear relevant to cytotoxicity of CPT-11. However, we could not measure the exact concentration of

Table 2 Characterization of topoisomerases of RVC cells and 4-pp-R cells. The topoisomerase I and topoisomerase II values are the means ± SD from three separte experiments, and total protein values are the mean of two experiments (*4-pp* 4-Piperidinopiperidine, *Topo* topoisomerase)

	RVC Mean ± SD (ratio)	4-pp-R Mean ± SD (ratio)
Topo I (U/μg protein)		
Contro	7.80 ± 1.14 (1.00)	8.77 ± 0.79 (1.00)
5 μ*M* SN-38	3.03 ± 0.82 (0.39)	3.76 ± 0.83 (0.43)
50 μ*M* CPT-11	2.27 ± 0.28 (0.29)	2.77 ± 1.23 (0.32)
2 μg/ml 4-pp	8.42 ± 3.84 (1.08)	
20 μg/ml 4-pp	8.00 ± 0.42 (1.03)	
200 μg/ml 4-pp	9.08 ± 3.28 (1.16)	
Topo II (U/μg protein)	0.301 ± 0.043	0.321 ± 0.128
Total protein (μg/10^7 cells)	139.5	140.5

4-piperidinopiperidine or its metabolite in the 4-piperidinopiperidine-treated and CPT-11-treated cells because of its low concentration. Further studies of CPT-11 and 4-piperidinopiperidine in addition to SN-38 should be considered to elucidate the antitumor activity of CPT-11 and the mechanism of cell death induced by 4-piperidinopiperidine.

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