IN VITRO CYTOTOXICITY OF CPT-11 (SN-38) ALONE AND IN COMBINATION WITH CISPLATIN ON OVARIAN CANCER CELLS

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Abstract : We investigated the *in vitro* cytotoxic effects of CPT-11, a new derivative of camptothecin, and its metabolizable form *in vivo*, SN-38, on cisplatin-resistant (SHIN-3) and-sensitive (MN-1) ovarian cancer cell lines using the MTT assay. We also attempted to identify the optimal schedule of administration for cisplatin combined with low-dose continuous exposure to CPT-11 (3 μg/ml) or SN-38 (5 ng/ml). With either CPT-11 or SN-38 alone, a marked schedule-dependent inhibition of growth was obtained with exposure for over 40 hours to concentrations of those agents applicable to clinical use (CPT-11 : <7.58 μg/ml, SN-38 : <72 ng/ml), even in a cisplatin-resistant cell line. Increasing the assay AUC of these agents only by dose escalation did not enhance cytotoxity with both MN-1 (CPT-11 : 3-50 μg/ml, SN-38 : 2.16-72 ng/ml) and SHIN-3 (CPT-11 : 5-50 μg/ml, SN-38 : 21.6-720 ng/ml) cell lines up to 72 hours of exposure. Pretreatment of SHIN-3 cells with either CPT-11 (3 μg/ml) or SN-38 (5 ng/ml) for 48 hours before the administration of cisplatin (4-20 μg/ml) appeared to produce an inhibition which exceeded that produced by either a longer (96 hours) or shorter (0 hour) pretreatment with them. Flow cytometric analysis showed that treatment with CPT-11 and SN-38 in SHIN-3 cell line was associated with a peak in G_2/M phase at 24 hours and an increase in the G_0/G_1 phase fraction for up to 96 hours. It is thus suggested that the continuous administration of a low dose of CPT-11 or of SN-38 may be useful clinically. The *in vitro* cytotoxicity of cisplatin could be increased by pretreatment with either CPT-11 or SN-38.

Index Terms

CPT-11, SN-38, cisplatin, combination chemotherapy, overian cancer

The abbreviations used here are : MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide ; AUC, area under the curve ; CPT-11, 7-ethyl-10 [4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin ; SN-38, 7-ethyl-10-hydroxy-CPT ; DMSO, dimeth-

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yl sulfoxide.

INTRODUCTION

While podophyllotoxin was identified as a cytostatic agent in the 1940s, the available derivatives were highly toxic. Etoposide, a more recent glycosidic derivative of podophyllotoxin, exhibits marked clinical activity against a wide variety of neoplasms, including lung cancer, lymphoma, and gynecologic malignancies[1,2]. This agent may exert its cytotoxic effects, in part, by interfering with the scission-reunion reaction of topoisomerase II[3]. Breakage of DNA strands are detected at concentrations of etoposide significantly below those required to affect cell kinetics and DNA synthesis[4]. Etoposide-related inhibition of topoisomerase II is reversible, and DNA strand breaks are repaired rapidly after the drug is discontinued[5].

Etoposide is one of the few antineoplastic agents that is effective as a second-line treatment[6] ; it may be synergistic with cisplatin[7]. The schedule dependency of etoposide was first demonstrated in early preclinical investigation[8]. Clinically, patients with small-cell lung cancers given etoposide as five consecutive daily infusions showed a response rate of 89%, being superior to results obtained by giving a continuous intravenous infusion for 24 h. This suggests the importance of a longer duration of etposide administration at concentrations above 1 μg/ml in obtaining efficacy[9].

Camptothecin (CPT), a plant alkaloid derived from *Camptotheca acuminatum*, was isolated by Wall et al. in 1966[10]. While this agent demonstrated a good spectrum of activity against numerous cancer models, its clinical use was limited by severe toxicity. Miyasaka et al. then developed CPT-11 (7-ethyl-10- [4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) as an alternative to CPT[11]. CPT-11 is less toxic and shows increased tumor activity as compared to CPT. It is also water-soluble. CPT-11 exhibits significant antitumor activity against various experimental tumor models[12]. In a clinical stuby of 39 various tomors obtained from patients, CPT-11 produced an overall response rate of 44%. A particularly good response (56%) was observed in ovarian cancer[13]. In a phase II study conducted in Japan, monotherapy with CPT-11 induced a clinical response in 35% of the patients with advanced ovarian cancer treated with this agent[14].

The cytotoxic mechanism of CPT-11 has not been established, although inhibition of the nuclear enzyme topoisomerase I appears to be involved[15]. It therefore seemed likely that the combination of CPT-11 whth cisplatin would exhibit effects on cisplatin-resistant cancer cells similar to those achieved by the combination of etposide (another topoisomerase inhibitor) and cisplatin.

We inventigated the *in vitro* effects of CPT-11 and its active metabolite, SN-38, on ovarian cancer cells as well as the effects of combined treatment with cisplatin and CPT-11, and of cisplatin and SN-38. We also attempted to identify the optimal schedule for administering cisplatin in combination with CPT-11.

MATERIALS AND METHODS

Ovarian cancer cell lines

We investigated the effects of CPT-11 on SHIN-3 cells, ovarian serous cystadenocarcinoma cells which we had obtained from a patient who developed resistance to cisplatin[16]. In addition, we studied results in the MN-1 cell line developed from a patient with mucinous cystadenocarcinoma responsive to cisplatin-based chemotherapy[17].

Both cell lines were incubated in Dalbecco's minimum essential medium (DMEM) supplemented with 10% FCS (GIBCO, Grand Inland, NY), 100 units/ml penicillin, and 0.3 mg/ml glutamine at 37℃ in a humidified atmosphere of 5% CO_2 in air.

For the cytotoxicity assay, 5×10^3 to 1×10^4 cells per 50 μl of culture medium were plated onto 96-well multiplates and incubated for 24 hours before the addition of drugs.

Drugs

CPT-11 (provided by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) was reconstituted in distilled water at a concentration of 20 mg/ml and SN-38 (Daiichi) was reconstituted in a sterile dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. These substances were diluted with FCS containing DMEM to final concentration immediately before use. Cisplatin (provided by Nippon Kayaku Co., Ltd., Tokyo, Japan) was diluted in distilled water to a concentration of 0.5 mg/ml. Diluted CPT-11 and cisplatin were each stored at 4℃, while SN-38 was stored at −20℃. The final concentration of DMSO in culture medium was adjusted to less than 1.0 % (V/V), which was confirmed in preliminary studies to have no antiproliferative effect on either cell line (data not shown).

MTT assay

The MTT assay was performed as previously described[18]. In brief, the number of cells plated onto 96 wells was determined after preliminary studies of cell growth using the MTT assay (data not shown) so that the untreated cells were in the exponential growth phase during the experiment. CPT-11 or SN-38 (150 μl of 4/3 fold concentration of drug diluted in medium) was added to the incubated cells (5×10^3 to 1×10^4cells/well in 50 μl of medium) in the multiplates. FCS concentration in each sample was adjusted at 10% (V/V). We studied the effects of 10 different concentrations of CPT-11 and of SN-38, covering a 4-log range, that spanned the 50% inhibitory concentration, as determined by preliminary assays (data not shown). After cells were cultured, 13 μl (5 mg/ml) of MTT (Sigma Chemical Co., St. Louis, MO) diluted with phosphate-buffered saline (PBS, pH 7.6) was added to each well and incubated at 37℃ for an additional 4 hours. The medium was then aspirated from each well using an automatic plate washer (Eimax 601, Fujirebio Inc., Shizuoka, Japan). To solubilize the formazan, 150 μl of DMSO was added to each well using a multichannel pipette, and the plates were placed on a plate shaker (Timer mixer MB-1, Torika Corp., Tokyo, Japan) for 2 minutes. Absorbance was read immediately at 540 nm on a scanning multiwell spectrophotometer (Eimax A-4, Fujirebio Inc., Shizuoka, Japan).

Assay of cytotoxicity of monotherapy with CPT-11 or SN-38

Concentrations of 0.3 to 300 μg/ml of CPT-11 and 21.6 to 36000 ng/ml of SN-38 were added to samples of both cell lines. The SHIN-3 cell line was incubated for 24 to 96 hours, while the MN-1 cell line was incubated for 20 to 64 hours. The number of cells present after treatment

was determdned by the MTT assay. Additional control experiments used culture medium alone. Fractional absorbance after treatment was expressed as the percentage of control absorbance : that is, the absorbance levels from drug-treated cells were corrected by the untreated contral absorbance values, from which we defined the schedule- and concentration-dependent growth inhibition curves. The inhibitory concentration$(IC)_{50}$ was defined as a 50% reduction of absorbance as determined by the MTT assay.

The AUC (area under the curve) value of the assay expressed as μg/ml•hour was determined using the drug stability data described by Hildebrand-Zanki et al. [19]. The assay AUC was calculated by multiplying the concentration of drug by the duration of assay. We determined the relation between the growth inhibition induced by CPT-11 or SN-38, and the assay AUC.

Combination of CPT-11 or of SN-38 with cisplatin

The IC_{10} values of CPT-11 or SN-38 after 72-hour incubation with the SHIN-3 cell line, determined from the growth inhibition curves, were selected as those to be used in combination with cisplatin treatment. IC_{10} values were 3 μg/ml for CPT-11 and 5 ng/ml for SN-38.

CPT-11 or SN-38 were continuously administrated for 6 consecutive days. Cisplatin was added on day 1 (group I), day 3 (group II), or day 5 (group III) for 24 hours. Doses of 4, 10, and 20 μg/ml cisplatin were administrated to each group (Fig. 1). Additional cell samples were treated with cisplatin alone on days 1, 3, or 5 (group I', II', or III'), or with CPT-11 or SN-38 alone. Untreated samples were used as controls. The number of cells after treatment was determined by the MTT assay and expressed as the relative absorbance (% of control absorbance). The effectiveness of the administration schedule for cisplatin (relative rate of inhibition) was determined by the following formula :

Relative rate of inhibition (%) = $[(A-B)\div A]\times 100$

where A is % of control absorbance in the group treated with cisplatin alone, and B is % of control absorbance in the group treated with combination therapy

The culture medium was changed at the same time in all groups to equalize the decrease in

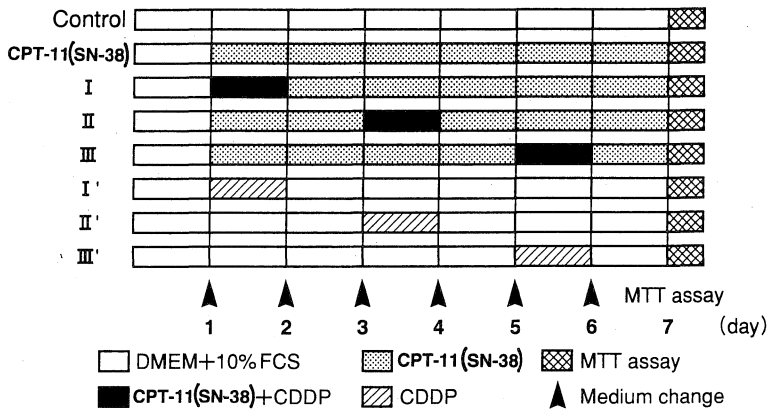

Fig. 1. Drug administration schedule for evaluating relative rate of growth inhibition. CPT-11 (3 μg/ml) or SN-38 (5 ng/ml) plus cisplatin (4 , 10 and 20μg/ml), or cisplatin alone, was added to SHIN-3 cell line. Duration of preincubation before administering each agent was 12 hours.

cell number, the change in pH, and the effect of G_0 phase arrest that may occur when the medium is changed.

Flow cytometry

SHIN-3 cells at a concentration of 5×10^5 were plated in 75-cm^2 culture flasks and incubated for 24 hours. Next, 3 μg/ml of CPT-11 or of 5 ng/ml of SN-38 was added to the cells. After culture for 24 to 96 hours, the control, CPT-11-treated, or SN-38-treated cells were harvested by trypsinization. Cells were filtered through a 60 μm metal mesh, treated with 0.01 % RNase (Type 1A, Sigma Chemical Co., St. Louis, Mo) and then stained with propidium iodine (50 μg/ml). The amount of nuclear DNA in the stained samples was measured by FACStar flow cytometer (Nippon Becton Dickinson Co., Tokyo, Japan) with CELLFIT program.

The change in cell cyclic phase fraction during drug exposure was determined.

Statistical methods

Data points represent the mean and one SD of 8 wells in monotherapy with CPT-11 or SN-38, and of 16 wells in combination treatment. Statistical significance was determined by using the paired Student's t-test to compare the relative rates of inhibition in each group. A P value less than 0.05 was considered statistically significant.

RESULTS

Cell culture

Cells were routinely subcultured by trypsin/EDTA treatment with a split of 1 : 4. Under the conditions of our experiment, the logarithmic growth phase took about 140 hours in 96-well plates following inoculation of 5×10^3 cells/well. The doubling time of the SHIN-3 cells (27 hours) and of MN-1 cells (23 hours) did not differ in each passage.

Cytotoxic effect of CPT-11 or of SN-38 on SHIN-3 and MN-1 cell lines

Exposure of SHIN-3 cells to CPT-11 resulted in IC_{50} values of 280 μg/ml at 24 hours, 150 μg/ml at 48 hours, 130 μg/ml at 72 hours, and 18 μg/ml at 94 hours (Fig. 2). No IC_{50} was achieved, even after 94 hours, when cells were cultured with a peak plasma CPT-11 concentration (PPC)

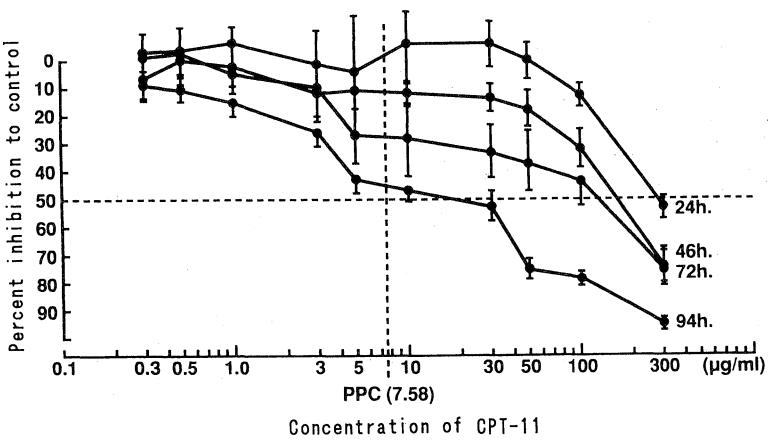

Fig. 2. Effect of CPT-11 on SHIN-3 cell line. Cell were incubated for 24 to 94 hours. PPC : peak plasma concantration.

of 7.58 μg/ml. The PPC of CPT-11 was determdned as a result of administering the maximal tolerated dose in a phase II clinical trial[14]. The response curve indicated a concentration- and schedule-dependent effect over PPC. Exposure of SHIN-3 cells to SN-38 resulted in IC_{50} values of 3000 ng/ml at 24 hours, 1500 ng/ml at 46 hours, 500 ng/ml at 72 hours, and 150 ng/ml at 94 hours, all of which exceeded the PPC of SN-38. The response appeared to be concentration-dependent over 720 ng/ml. However, a schedule-dependent effect was observed at and below the PPC (Fig. 3).

IC_{50} values were not observed during 20-hour exposure of MN-1 cells to CPT-11 or SN-38. A concentration-dependent effect was observed after 40 hours of exposure, resulting in an IC_{50} value of 2.6 μg/ml at 40 hours for CPT-11, and 2.5 ng/ml for SN-38. These IC_{50} values were below the clinically determined PPC. After exposure for 64 hours, IC_{50} values were 0.4 μg/ml for CPT-11 and below 0.72 ng/ml for SN-38 (Figs. 4,5). Response curves revealed that the MN-1 cell line exhibited a greater sensitivity to CPT-11 and to SN-38 than did the SHIN-3 cell line.

Correlation between growth inhibition and AUC induced by CPT-11 and SN-38

There were no significant differences in the effects of CPT-11 at doses of 1.0 to 100 μg/ml on MN-1 cells with treatment given for 20 hours. The degree of inhibition decreased at concentrations under 0.5 μg/ml. AUC-dependent cytotoxicity was observed after exposure for 40 hours, but was not apparent after exposure for 64 hours at concentrations above 1.0 μg/ml (Fig. 6). The same tendency was observed with SN-38. A significant AUC-dependent inhibition of growth was also observed with treatment given over 20 hours ; however, it was saturated above a concentration of 2.16 ng/ml over 64 hours (Fig. 7).

CPT-11 was associated with an AUC-dependent inhibition of growth of SHIN-3 cells. The steepest response curves were seen at concentrations of 5 to 50 μg/ml. No significant AUC-dependent inhibition of growth was observed at concentration below 3 μg/ml (Fig. 8). AUC-dependent growth inhibition was observed with concentrations of SN-38 below 720 ng/ml. Concentrations of 7.2 to 72 ng/ml were associated with a growth inhibition below 50%, while concentrations of 216 ng/ml with growth inhibition of about 50% with treatment for 94-hour.

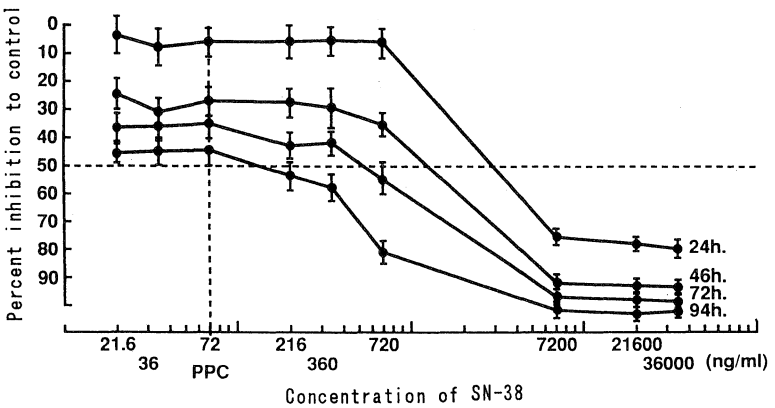

Fig. 3. Effect of SN-38 on SHIN-3 cell line. Cell were incubated for 24 to 94 hours. PPC : peak plasma concentration.

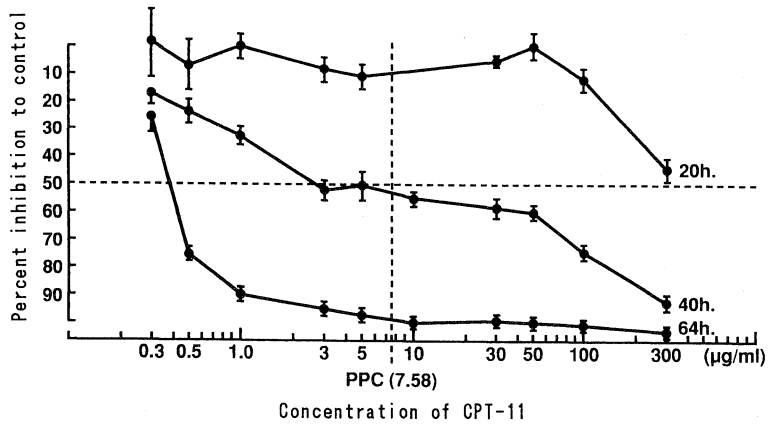

Fig. 4. Concentration and schedule-dependent response curve of CPT-11 to MN-1 cells. Cells were incubated for 20 to 64 hours. PPC : peak plasna conceotration.

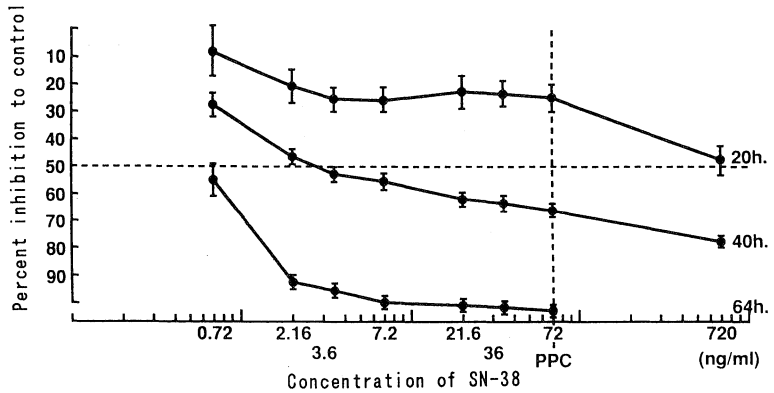

Fig. 5. Concentration and schedule-dependent response curve of SN-38 to MN-1 cells. Cells were incubated for 20 to 64 hours. PPC : peak plasma concentration.

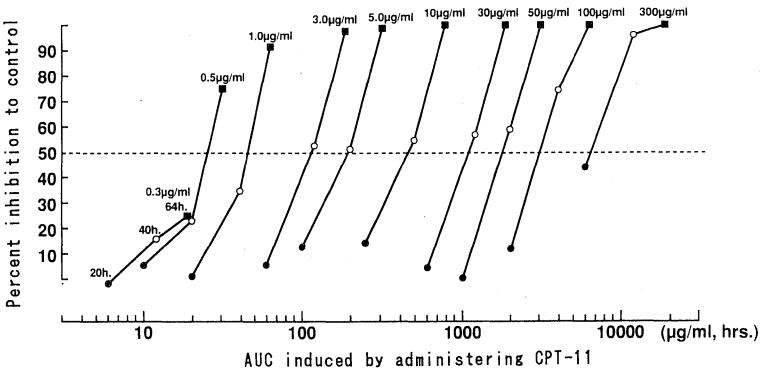

Fig. 6. Relation between growth inhibition and AUC induced by CPT-11 in MN-1 cells. CPT-11 concentrations were ranged from 0.3 to 300 μg/ml and cells were incubated for 20 to 64 hours.

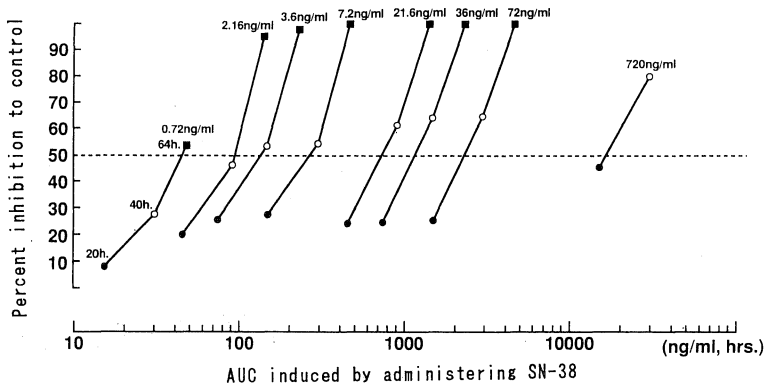

Fig. 7. Relation between growth inhibition and AUC induced by SN-38 in MN-1 cells. SN-38 concentrations were ranged from 0.72 to 720 ng/ml and cells were incubated for 20 to 64 hours.

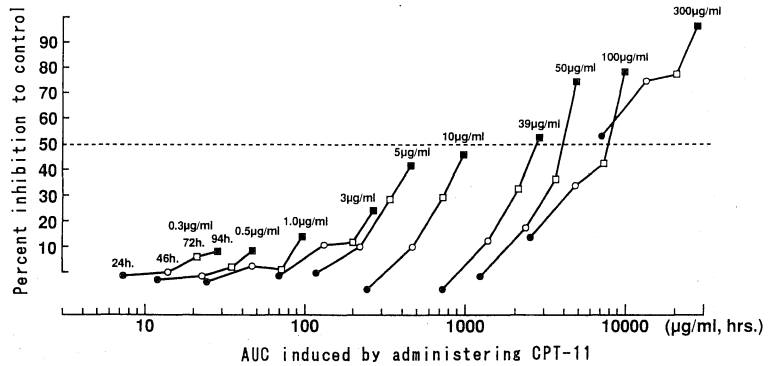

Fig. 8. Relation between growth inhibition and AUC induced by CPT-11 in SHIN-3 cells. CPT-11 concentrations were ranged from 0.3 to 300 μg/ml and cells were incubated for 24 to 94 hours.

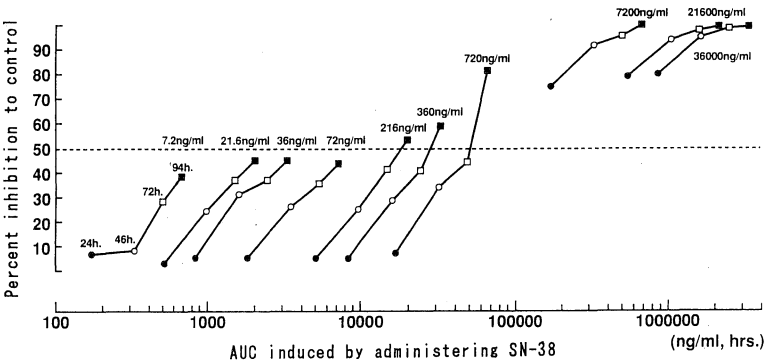

Fig. 9. Relation between growth inhibition and AUC induced by SN-38 in SHIN-3 cell line. SN-38 concentrations were ranged from 7.2 to 36000 ng/ml and cells were incubated for 24 to 94 hours.

Only a schedule depencency was prominent over 46 hours. No significant dependency on the AUC was observed with doses over 7200 ng/ml (Fig. 9).

Combination CPT-11 or SN-38 with cisplatin

The relative rate of inhibition tended to increase in association with an increase of CPT-11 preincubation time in the groups exposed to combination therapy compared with those given cisplatin alone. In combination with 4 to 20 μg/ml of cisplatin, the inhibitory rates were maximum as 42.9±4.2 ($P<0.005$ to groups I-I' and II-II') and 77.8±15.6 % ($P<0.005$ to groups II-II', $P<0.10$ to groups I-I') between group III and III'. The maximum rate of 60.5± 13.6 % at a concentration of 10 μg/ml of cisplatin was observed in groups II and II'($P<0.05$

Table 1. Relative rates of inhibition (I'-I/I', II'-II/II', III'~III/III') determined by %control of absorbance in groups treated with cisplatin alone (I', II', III') and with combination therapy of CPT-11 and CDDP (I, II, III)(mean±SD)

	CDDP		
	4 μg/ml	10μg/ml	20μg/ml
% control of absorbance			
I'	46.5±8.6	19.2±4.0	5.9±3.5
I	40.4±3.8	10.7±1.8	2.2±2.4
II'	61.7±9.5	63.3±14.1	17.1±5.2
II	43.9±7.2	25.0±6.0	5.9±2.6
III'	99.9±6.2	94.8±13.7	87.9±16.9
III	56.5±9.2	66.8±15.9	19.5±3.7
Relative rate of inhibition			
I'-I/I'	15.0±10.6[a]	55.9±9.4[d]	59.1±48.4[g]
II'-II/II'	28.8±5.5[b]	60.5±13.6[e]	65.2±18.8[h]
III'-III/III'	42.9±4.2[c]	29.5±3.6[f]	77.8±15.6[i]

$p<0.005$: a to b and c, b to c, d to f, e to f, h to i,
$p<0.05$: d to e. $p<0.10$: g to i.
N. S. : g to h. (Student's t - test)

Table 2. Relative rates of inhibition (I'-I/I', II'-II/II', III'-III/III') determined by % control of absorbance in groups treated with cisplatin alone (I', II', III') and with combination therapy of SN-38 and CDDP (I, II, III)(mean±SD)

	CDDP		
	4 μg/ml	10μg/ml	20μg/ml
%control of absorbance			
I'	16.5±8.3	41.0±3.1	7.8±1.0
I	57.8±8.0	55.6±23.3	6.8±3.3
II'	85.1±10.0	79.2±7.3	64.7±4.1
II	67.3±7.9	53.9±6.3	34.3±13.3
III'	89.1±10.7	84.9±6.6	82.2±10.7
III	81.6±5.2	68.9±5.4	56.7±7.2
Relative rate of inhibition			
I'-I/I'	6.9±4.2[a]	2.4±1.7[d]	12.4±2.1[g]
II'-II/II'	20.9±4.1[b]	32.0±3.1[e]	57.4±2.6[h]
III'-III/III'	10.1±5.5[c]	18.8±3.5[f]	28.6±3.4[i]

$p<0.005$: a to b, b to c, d, to e and f, g to h and i, h to i.
N. S. : a to c (Student's t-test)

Table 3. Changes in cell cyclic piase fractions after CPT-11 (3 μg/ml)or SN-38 (5 ng/ml) treatment in SHIN-3 cell line

	CPT-11			SN-38		
Hours	%G0/G1	%S	%G2/M	%G0/G1	%S	%G2/M
0	12.1	57.7	30.2	12.1	57.7	30.2
24	14.7	27.0	58.3	20.0	25.2	54.9
48	22.9	32.1	45.0	25.5	38.2	36.3
72	20.1	32.4	47.4	34.6	39.5	25.9
96	27.0	26.7	46.3	36.1	31.7	32.2

to groups III-III', $P<0.5$ to groups I-I') (Table 1).

In combination therapy with SN-38, the maximum relative rate of inhibition was indicated between groups II and II' with the preincubation time for 48 hours. They were 20.9±4.1 % in the combination with 4 μg/ml of cisplatin, 32.0±3.1 % at 10 μg/ml, 57.4±2.6 % at 20 μg/ml ($P<0.005$ to groupsI-I' and groups II-II') (Table 2).

Flow cytometric analysis

The greatest extent of G2/M phase arrest was achieved just after exposing the SHIN-3 cells to 3 μg/ml CPT-11 for 24 hours. The percentage of cells in the S phase decreased from 57.7 to 27.0 % after 24-hour exposure ; the G_0/G_1 phase fraction increased gradually from 12.1 to 27.0 % (Table 3).

The same tendency was observed with the 5 ng/ml dose of SN-38. However, the number of cells in the S pahse exceeded that in the G_2/M phase after 48 hours (Table 3).

DISCUSSION

Although significant advances have been made in treating advanced ovarian cancer, especially with the introduction of cisplatin about 15 years ago, less than 20% of the patients experience a long-term disease-free survival after cisplatin-based treatment.

CPT-11, developed in Japan, is a new derivative of the plant alkaloid CPT[11]. CPT and most of its derivatives, such as 7-ethyl-CPT (SN-22), 10-hydroxy-CPT, and 7-ethyl-10-hydroxy-CPT (SN-38), are insoluble in water in the forms used clinically. CPT-11 exhibited greater anti-tumor activity and less toxicity than CPT[12]. In an experimental study, CPT-11 demonstrated the apparent cure of a variety of susceptible murine tumors including S-180, Meth-A fibrosarcoma, Lewis lung carcinoma, Ehrlich carcinoma, MH-134 hepatoma, mammary carcinoma of C3H/HeN mice, L1210 leukemia, and P388 leukemia, suggesting that it has a broad spectrum of activity[12].

By subrenal capsule assay, CPT-11 demonstrated an overall chemo sensitivity rate of 44% in 39 samples of various tumors, with a statistically significant response of 56% observed in 10/18 ovarian cancer samples[13].

A phase I clinical stuby demonstrated that the maximum tolerated dose of CPT-11 was 250 mg/m^2 (q 3-4w)[20]. An early phase II study suggested that the optimal dose schedule was 200 mg/ml every 3 to 4weeks[14]. CPT-11 is believed to be changed into SN-38, via hepatic metabolism[12].

Most antineoplastic agents used to treat gynecologic malignancies act as DNA intercalators.

Agents such as etoposide, a topoisomerase II inhibitor, and taxol have different mechanisms of action and have been scown effective in patients with platinum-resistant ovarian cancer[21]. A combined regimen of low-dose continuous etoposide and cisplatin has been proposed as second -line therapy for such cases[22].

In a previous study, we determined the optimal schedule for administering cisplatin in combisation with low-dose etoposide by determining its cytotoxic effect *in vitro*[23]. In the present study, we used the same method to investigate the effects of CPT-11.

We investigated the effects of CPT-11 in two lines of human ovarian cancer cells, SHIN-3, which is cisplatin-resistant, and MN-1, which is cisplatin-sensitive. In previous experiments, the SHIN-3 cell line demonstrated a weak response to 8.0 μg/ml of cisplatin after 72-hour exposure as the IC_{50} value to untreated control ; the MN-1 cell line demonstrated a response to 1.8 μg/ml of cisplatin after 48-hour exposure[23].

A single dose of CPT-11 or of SN-38 exerted a strong cytotoxic effect over PPC on MN-1 cells. When cells were exposed to CPT-11 or SN-38 for over 40 hours, IC_{50} values were easily achieved even when drug concentrations were below the clinically determined PPC. AUC-dependent growth inhibition was also observed at the same condition of exposure. In treating ovarian cancer, it may be advantageous to administer CPT-11 by a continuous infusion over 40 hours to treat cisplatin-sensitive overian cancers in the clinical setting. The SHIN-3 cell line showed cross-resistance to CPT-11 and SN-38, as indicated by the absence of an IC_{50}, even after 96 hours of exposure at concentrations below the PPC. It is possible that only a schedule -dependent program of administration would exhibit anti-tumor efficacy in treating cisplatin -resistant ovarian cancer. When AUC was accelerated by increasing the dose of CPT-11 or SN -38 to the clinically applicable concentration, growth inhibition did not rise significantly over prologed exposure.

In our previous study, the number of viable cancer cells treated with cisplatin gradually decreased even after the washout of cisplatin, indicating that the cytocidal effect of cisplatin gradually appeared *in vitro* for over 24 hours[23]. Therefore, it was not possible to compare cytotoxic effects among groups I, II, and III in the present study because of differences in the duration until MTT assay. We therefore evaluated differences in growth inhibition between groups treated with cisplatin only and in those treated with the combination therapy, with results expressed as relative rate of inhibition according to the formula indicated in "Methods." IC_{10} values of CPT-11 and SN-38 in these combinations were selected voluntarily, as growth inhibiton of combination therapy be limited within evaluable range in their growth inhibition curves, even after 6 days of treatment (Fig. 1).

The rate of growth inhibition was greatest in SHIN-3 cells that had been pretreated for 48 hours with SN-38 (groups II-II') compared with a longer or shorter pretreatment period (groups I-I' and III-III') over the groups treated with cisplatin alone.

Pretreatment with CPT-11 for 96 hours appeared to be useful in combination with 4 and 20 μg/ml doses of cisplatin and in combination for 48 hours at 10 μg/ml doses of cisplatin. Pretreatment with CPT-11 or SN-38 in conjunction with this concentration of cisplatin failed to produce a lesser degree of cytotoxicity.

Although no information is available concerning the relation of CPT-11 or SN-38 to the change in cell cycle phase, our results showed that G_2/M phase peak appeared at 24 hours and

the G_0/G_1 phase fraction increased gradually up to 96 hours. These changes may be involved in the cytocidal mechanism of CPT-11. Exposure of SHIN-3 cells to 1 μg/ml etoposide significantly increased the G_0/G_1 phase fraction at 100 hours, and the peaks of G_2/M and S phase at 20 hours[23]. We found similar results with CPT-11 or SN-38, except for the motion of the S phase fraction.

Although the *in vitro* pharmacological effects of CPT-11 should be studied using SN-38, its metabolizable form, our results may be useful in determining the schedule for administering CPT-11 in the clinical setting. Our findings showed that low-dose continuous treatment of ovarian cancer cells with CPT-11 or SN-38 for over 40 hours had beneficial effects. Pretreatment with these agents in rather low concentration for over 48 hours before the administration of cisplatin appeared to be the most effective combination regimen.

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