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Enhancement of Tumor Radio-response by Irinotecan in Human Lung Tumor Xenografts

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We investigated the ability of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) to increase tumor radio-response *in vivo* using human lung tumor xenografts. The xenografts were treated with (1) CPT-11 (10 mg/kg) intraperitoneally on days 1, 5 and 9, (2) single dose radiation (10 Gy/leg) on day 1, or (3) a combination regimen of both treatments in which radiation was given 1 h after the first dose of CPT-11. DNA flow cytometry studies were performed to define the cell cycle changes following treatment for 1 to 12 h with 0, 0.5, 2.0 or 8.0 ng/ml SN-38, the major active metabolite of CPT-11. In both small cell lung cancer (MS-1) and small cell/large cell carcinoma (LX-1) xenografts, combination treatment resulted in significant tumor regression compared with the use of CPT-11 ($P=0.0005$, 0.0053) or radiation treatment ($P=0.00221$, 0.0035) alone. Neither severe body weight loss nor enhanced skin reaction was observed following the combined treatment. In flow cytometry studies, the proportion of cells in G_2/M-phase, the most radio-sensitive phase, increased after 1 h exposure to the lowest dose of SN-38 (0.5 ng/ml). These findings suggest that CPT-11 is a potent radiosensitizing agent, and that its activity is related to the cell cycle. This is the first report to indicate that CPT-11 serves as a radiosensitizer *in vivo*.

Key words: CPT-11 — Radiosensitization — Lung cancer — Xenograft

7-Ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11, irinotecan) is a semisynthetic inhibitor of topoisomerase I. This agent has strong antitumor activity against a variety of common cancers.[1,2] In lung cancer patients, phase I and II studies of this agent have yielded excellent results, indicating that CPT-11 may become a key drug in lung cancer chemotherapy.

Combined modality treatment is highly effective and important in non-metastatic inoperable non-small cell lung cancer,[3] and limited disease small cell lung cancer.[4] However, more effective anticancer agents with radiosensitizing potential are still needed. Furthermore, it is essential to evaluate the optimal schedule of combined chemoradiotherapy.

In the present study we investigated whether CPT-11 enhances the radiosensitivity of human lung tumor xenografts, and whether such enhancement is related to the cell cycle.

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MATERIALS AND METHODS

Mice and tumors We used male athymic BALB/c (*nu/nu*) mice (Japan SLC Inc., Shizuoka), maintained in a laminar air-flow room at a constant temperature (24°C) and humidity of 30–50%. The mice were 7–8 weeks of age at the beginning of the experiments and were housed 2–3 per cage. The tumors were small cell lung cancer, designated MS-1[5] and mixed small cell/large cell carcinoma, designated LX-1.[6] Solitary tumors were generated subcutaneously in the right thigh of the mice by inoculation of 1.0×10^7 viable tumor cells. Cell viability was in the range of 85–90% as determined by trypan blue exclusion and phase-contrast microscopy.

CPT-11 CPT-11 was supplied by Daiichi Pharmaceutical Co., Ltd. (Tokyo). When tumors reached 15 mm in diameter (day 1), CPT-11 was administered intraperitoneally at a dose of 5, 10, 15, or 20 mg/kg body weight, and was re-administered on days 5 and 9.

Irradiation When tumors reached 15 mm in diameter (day 1), the tumor-bearing legs were locally irradiated with single dose γ-irradiation (10, 20, or 30 Gy) delivered from a dual-source ^{137}Cs irradiator at a dose rate of 5 Gy/min. During irradiation, mice were anesthetized

with subcutaneous administration of 25 mg/kg pentobarbital sodium, and were fixed on an acrylic board to center the tumor in a 3-cm diameter irradiation field.

Combined modality treatment After selecting an optimal dose of either CPT-11 or irradiation which did not cause considerable cell death, we initiated experiments to examine the combined effect of CPT-11 and radiation. We set up three groups: 1) CPT-11 treatment group, 2) radiation treatment group, and 3) combined modality group. Each treatment group comprised 7–8 mice and the experiment was repeated 3 times. In the combined modality group, irradiation was performed 1 h after CPT-11 administration.

Evaluation of antitumor activity Tumor growth was determined twice a week using sliding calipers. The tumor volume (TV)[7] was calculated by use of the formula $TV=(a^2\times b)/2$, where b is the largest diameter and a is the diameter perpendicular to b. %T/C[7] was calculated by means of the formula $\%T/C=V_{t(21)}/V_{c(21)}\times 100$, where $V_{t(21)}$ is the average tumor volume at day 21, and $V_{c(21)}$ is that of the control. Tumor growth delay (GD)[8] was calculated by using the formula $GD=DT_t-DT_c$, where DT_t is the tumor doubling time of the treatment group, and DT_c is that of the control. The weight of the mice was measured twice a week to evaluate the toxicity of the therapy. In the combined modality treatment study, three BALB/c (*nu/nu*) non-tumor-bearing mice were also maintained under the same conditions and weighed.

DNA flow cytometry MS-1 cells suspended in RPMI1640 medium were exposed to various concentrations of SN-38 (0, 0.5, 2.0 or 8.0 ng/ml). 7-Ethyl-10-hydroxycamptothecin was donated by Daiichi Pharmaceutical Co., Ltd. CPT-11 is a pro-drug that undergoes deesterification to yield SN-38, the major active metabolite, which is 1000-fold more potent than the parent compound *in vitro*. After 1 or 12 h exposure to SN-38, each cell suspension was immediately fixed for 1 h in 70% ethanol at 4.0°C and stored in a freezer at −80°C. Staining and DNA flow cytometry were performed as described previously[9] with the assistance of *SRL* Co., Ltd., Tokyo.

Statistics Daily differences in TV among the groups and the GD of each group were analyzed using Student's unpaired t test. All P values reported are two-tailed, and differences were considered significant when the P value was less than 0.05.

RESULTS

To determine an optimal dose which did not cause considerable cell death, single doses of 5, 10, 15 and 20 mg/kg CPT-11 were administered to six mice bearing MS-1 tumors. At 5 to 15 mg/kg, CPT-11 did not induce statistically significant tumor regression in comparison with the untreated control, and no toxic death occurred within 2 weeks after irradiation. In contrast, a dose of 20 mg/kg resulted in significant tumor regression, but also caused one death due to toxicity.

Single doses of radiation (10, 20 and 30 Gy) were also administered to five mice bearing MS-1 tumors. A dose of 10 Gy did not induce statistically significant tumor

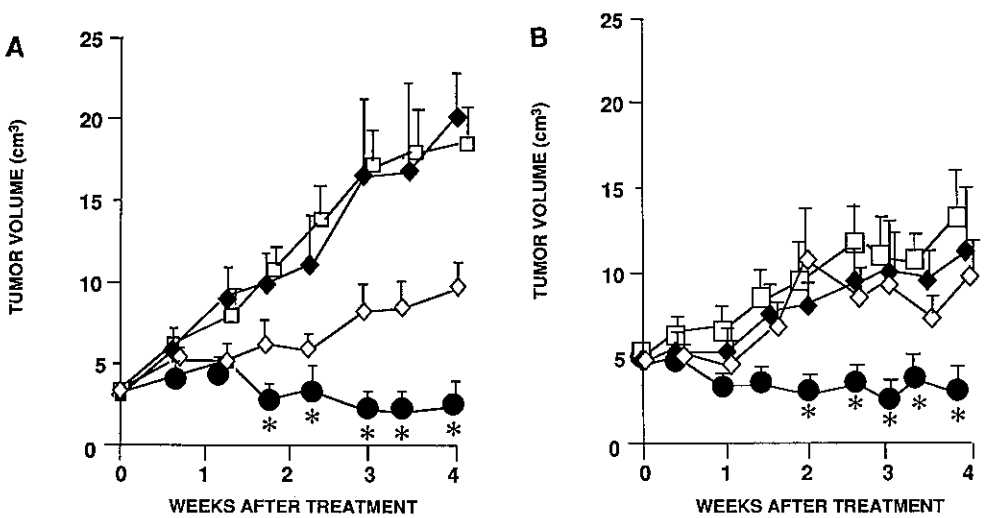

Fig. 1. Tumor volume (TV) in small cell carcinoma (MS-1) (A) and small and large cell carcinoma (LX-1) (B) xenografts. Combination treatment resulted in significant tumor regression in comparison with the control, although CPT-11 alone and single radiation did not. Volume values are given as the mean±SE. CPT-11 10 mg/kg+RT 10 Gy (●); RT 10 Gy (◇); CPT-11 10 mg/kg (◆); control (□). * $P\leq 0.01$.

Table I. Effects of CPT-11, Single Radiation and Combination Therapy in MS-1

Drug	Dose/Schedule		TV±SD[a] (cm^3)	%T/C[b] (%) (range)	DT[c] (days)	GD[d] (days) (range)	P-value[e]
CPT-11	10 mg/kg day 1,5,9	A	15.7±11.9	99.3 (75–101)	5.12	−1.43 (−3.9–1.6)	NS[f]
RT	10 Gy/body day 1	B	7.66±5.20	48.5 (37–66)	18.5	12.0 (11–12)	NS
CPT-11+RT	(A+B)	C	1.95±2.24	12.4 (1.5–28)	35.9	29.3 (29–32)	0.0001

A vs. C; $P=0.0005$, B vs. C; $P=0.00221$.
Combination treatment resulted in significant tumor regression in comparison with the control, CPT-11 treatment alone or single-dose radiation treatment.
a) TV±SD, mean of tumor volumes with standard deviation at 3 weeks after treatment.
b) %T/C, percentage of tumor volume against control at 3 weeks after treatment. ( ), 95% confidence interval.
c) DT, doubling time.
d) GD, delay in growth until doubling of the tumor volume. ( ), 95% confidence interval.
e) Each treatment modality was compared with non-treated tumor-bearing mice.
f) NS, not significant.

Table II. Effects of CPT-11, Single Radiation and Combination Therapy in LX-1

Drug	Dose/Schedule		TV±SD[a] (cm^3)	%T/C[b] (%) (range)	DT[c] (days)	GD[d] (days) (range)	P-value[e]
CPT-11	10 mg/kg day 1,5,9	A	8.76±4.75	92.8 (64–100)	16.1	2.35 (−0.1–7.6)	NS[f]
RT	10 Gy/body day 1	B	8.01±1.04	89.5 (66–81)	18.9	5.15 (2.5–6.2)	NS
CPT-11+RT	(A+B)	C	1.96±2.00	20.8 (−12–23)	47.9	34.2 (27–36)	0.0007

A vs. C; $P=0.0053$, B vs. C; $P=0.0035$.
Combination treatment resulted in significant tumor regression in comparison with the control, CPT-11 treatment alone or single-dose radiation treatment.
a) TV±SD, mean of tumor volumes with standard deviation at 3 weeks after treatment.
b) %T/C, percentage of tumor volume against control at 3 weeks after treatment. ( ), 95% confidence interval.
c) DT, doubling time.
d) GD, delay in growth until doubling of the tumor volume. ( ), 95% confidence interval.
e) Each treatment modality was compared with non-treated tumor-bearing mice.
f) NS, not significant.

regression in comparison with the control, and no death occurred. In contrast, irradiation at doses of 20 and 30 Gy induced significant tumor regression in comparison with the control, but each dose also caused 2 deaths.

As similar results were observed in LX-1, we considered the optimal dose of CPT-11 to be 10 mg/kg, and that of irradiation to be 10 Gy for combined treatment of both MS-1 and LX-1.

The response of MS-1 to chemoradiotherapy is shown in Fig. 1A. CPT-11 alone and single-dose irradiation resulted in no significant tumor regression in comparison with the untreated control (Table I). In contrast, combination treatment resulted in significant tumor regression ($P=0.0001$). This synergistic effect also resulted in significant tumor regression in comparison with CPT-11 alone ($P=0.0005$) and single-dose irradiation ($P=0.00221$).

To clarify the efficacy of combined modality treatment, the same experiment was performed using LX-1 (Fig. 1B). In the LX-1 xenografts, CPT-11 alone and single-dose irradiation also resulted in no significant tumor regression in comparison with the control (Table II), whereas combination treatment produced significant tumor regression in comparison with the control ($P=0.0007$), CPT-11 alone ($P=0.0053$) and single-dose irradiation ($P=0.0035$).

The body weight change in each group after the treatment is shown in Fig. 2, A and B. Severe body weight loss exceeding 10% of pre-treatment weight was not observed in any treatment group within 4 weeks. Also, no body weight change exceeding 10% of the body weight of BALB/c (*nu/nu*) non-tumor bearing mice was observed, except in untreated tumor-bearing mice (control).

No severe skin reaction or pulmonary toxicity was seen.

In order to elucidate the underlying mechanism of the combined modality treatment, we performed flow cytometry studies. Fig. 3A shows the percentages of MS-1

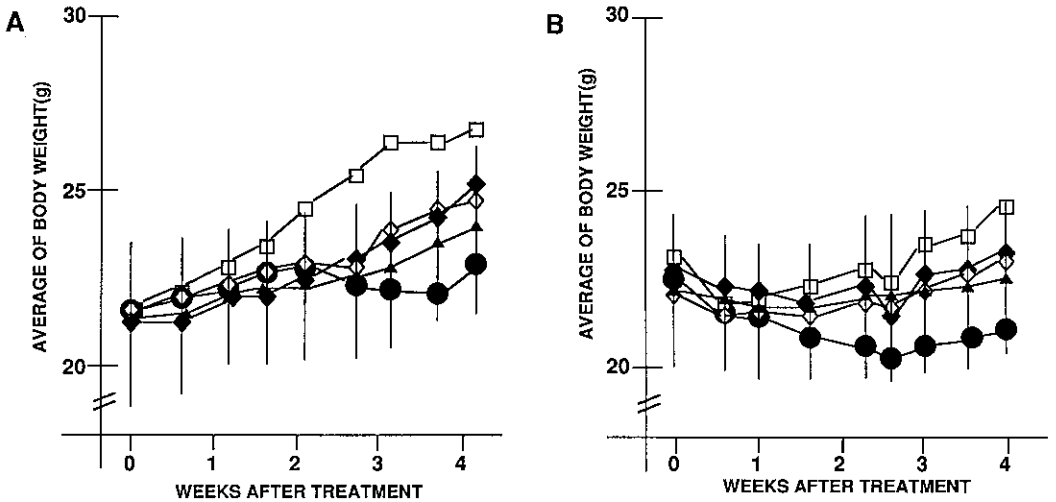

Fig. 2. Average body weight change in mice bearing small cell carcinoma (MS-1) (A) and small and large cell carcinoma (LX-1) (B) xenografts. No body weight change exceeding 10% of the body weight of BALB/c (*nu/nu*) non-tumor-bearing mice was observed, except in untreated tumor-bearing mice (control). CPT-11 10 mg/kg+RT 10 Gy (●); RT 10 Gy (◇); CPT-11 10 mg/kg (◆); untreated tumor-bearing mice (control) (□); BALB/c (*nu/nu*) non-tumor-bearing mice (▲). Vertical lines indicate the 10% range of body weight of BALB/c (*nu/nu*) non-tumor-bearing mice.

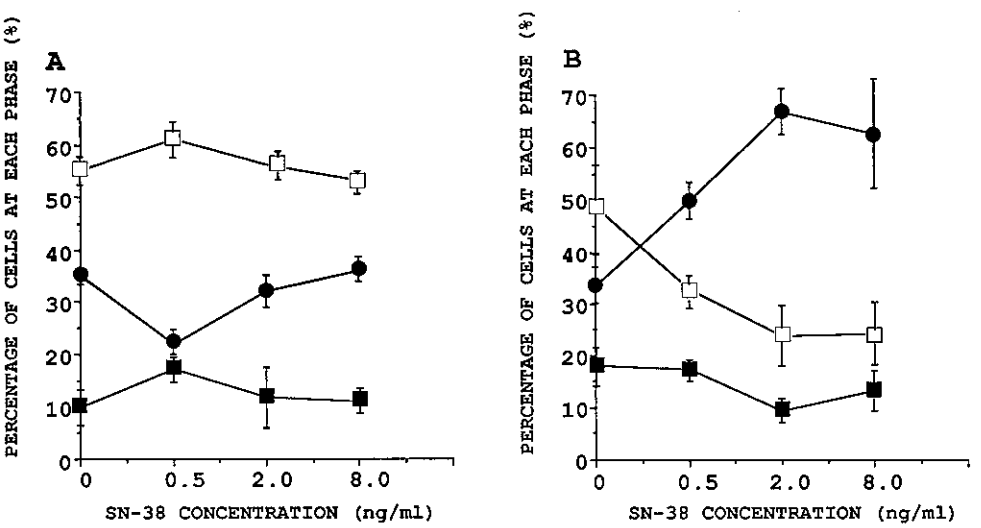

Fig. 3. The percentage of cells at each phase after 1 (A) or 12 h (B) exposure of the small cell carcinoma cell line MS-1 to SN-38 at 0.5, 2.0 or 8.0 ng/ml. After 1 h exposure to a low dose of SN-38, the percentage of cells in G_2/M-phase increased and that of cells in the S-phase decreased, compared with the control or high-dose SN-38 groups. The ratio of each phase is given as the mean±SE. G_0/G_1-phase (□); S-phase (●); G_2/M-phase (■).

cells at each cell-cycle phase after 1 h exposure to SN-38, the major active metabolite of CPT-11. In previous studies, the concentration which produced a 50% cell growth inhibition (IC_{50}) in MS-1 was 2.0 ng/ml. After 1 h exposure to a low dose of SN-38 (0.5 ng/ml), the percentage of G_2/M-phase cells increased and the percentage of S-phase cells decreased, compared with the untreated control or cells that received high doses of SN-

38 (above 2.0 ng/ml). Twelve-hour exposure to a low dose of SN-38 (Fig. 3B) produced no marked changes in the ratio of G_2/M-phase cells and maintained a relatively low ratio of S-phase cells in comparison with the high-dose treatment.

DISCUSSION

Several anticancer drugs have been examined for synergistic effects with radiation, including cyclophosphamide, cisplatin, mitomycin C, 5-fluorouracil, doxorubicin, and taxol.[10–14] Camptothecin and topotecan, a topoisomerase I inhibitor, have also been reported to potentiate the lethal effects of ionizing radiation *in vitro*[15–18] and *in vivo*.[19] CPT-11 is a derivative of camptothecin, and is also a topoisomerase I inhibitor which has strong antitumor activity against lung cancer. It is important to evaluate whether this drug acts synergistically with radiation.

In the present study, using lung cancer tumor xenografts, we have shown for the first time that the combination of CPT-11 treatment with radiation resulted in significant tumor regression compared to the use of either treatment alone. These findings suggest that CPT-11 may have a radiosensitizing effect.

Boothman *et al.*[16] reported that a low dose of camptothecin was optimal for greatly enhancing the radiosensitivities of melanoma cell lines, and Musk and Steel[15] reported that a low dose of irradiation produced greater synergistic antitumor effects when combined with camptothecin. In the present study, in order to evaluate whether CPT-11 does, in fact, act synergistically with radiation, we chose doses of CPT-11 and radiation which produced no significant tumor regression when each was employed as a single modality. It was found that the low-dose combination therapy greatly enhanced the tumor radiosensitivity, and further, the effect was superior to that of the maximum tolerated dose of CPT-11 (15 mg/kg) alone (data not shown) or radiation (10 Gy/body) alone.

It has long been recognized that cells at G_2/M-phase are more radiosensitive than cells at other cell-cycle phases.[20–23] Drugs, including vinca alkaloids, that are capable of arresting the cell cycle, have been investigated as potential radiosensitizers.[24] Furthermore, recent studies have demonstrated that taxol-treated tumor cells exhibit enhanced radiosensitivity only when irradiation is performed during the G_2/M-phase.[14, 25] These data imply that G_2/M block is necessary for radiosensitization.

Several authors have discussed the radiosensitizing effect of topoisomerase I inhibitors in terms of the cell cycle.[26–31] Our flow cytometry studies showed that brief exposure (1 h) to a low dose of SN-38 (0.5 ng/ml), 25% of the IC_{50}, induced an initial cell cycle block in the G_2/M-phase. Li *et al.*[26] also reported the induction of a similar initial cell cycle change by a lower concentration of CPT-11. These data suggest that a low dose of CPT-11 can potentially alter cell cycle progression and make cells more radiosensitive.

On the other hand, several investigators have reported the induction of a synergistic effect through a cell cycle delay in S-phase.[30, 31] The S-phase specificity of the lethal action of topoisomerase I inhibitors may cause impairment of the efficient repair of DNA damage caused by radiation.[26–29]

In the present study, we showed that CPT-11 produced a radiosensitizing effect when applied 1 h before irradiation *in vivo*, and that 1 h exposure to a low dose of SN-38 increased the number of cells in the radiosensitive phase. However, the optimum timing of topoisomerase I inhibitor treatment (pre-, concurrent or post-irradiation) for maximizing the radiosensitizing effect remains controversial. Boothman *et al.*[16] and Kim *et al.*[19] reported that in *in vitro* studies, topotecan radiosensitized cells specifically for 4–6 h after irradiation. Mattern *et al.*,[18] however, reported that the radiosensitizing effect of topotecan was dependent on the presence of the drug during the first few (<30) minutes after irradiation *in vitro*. In *in vivo* studies, Boscia *et al.*[17] demonstrated the radiosensitizing effect of camptothecin or topotecan when applied at 15 min prior to irradiation. Although our present data might be useful for clarifying the optimal timing of CPT-11 treatment combined with irradiation, additional comparative studies and pharmacokinetic studies *in vivo* should be done.

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