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Effect of CPT-11 in combination with other anticancer agents in lung cancer cells

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To determine the optimal combination of commonly used anticancer agents with 7-ethyl-10-hydroxy-camptothecin (SN-38), an active metabolite of 7-ethyl-10-[4(1-piperidino)-1-piperidino] carbonyloxy camptothecin (CPT-11), for chemotherapy of lung cancer, we studied the effects of SN-38 in combination with six representative anticancer agents on the human small cell lung cancer (SCLC) cell line, NCI N417, and the non-small cell lung cancer (NSCLC) cell line, PC-9. The anticancer activity was evaluated by MTT assay and the effects of drug combinations on ID_{50} were analyzed by an improved isobologram method. In the SCLC cell line, supra-additive effect was observed for SN-38 in combination with cisplatin, etoposide (VP-16) and paclitaxel (Taxol). An additive effect was observed for its combination with bleomycin. Sub-additive and protective effects were found in combination with adriamycin (ADR) and 5-fluorouracil (5-FU). In the NSCLC cell line, supra-additive and marginal supra-additive effects were found for SN-38 in combination with VP-16, ADR, 5-FU and bleomycin. The others showed additive effects with SN-38. No drug showed sub-additive and protective effects with SN-38. These results suggest that all the drugs we selected can be used with SN-38 simultaneously for NSCLC, while for SCLC, cisplatin, VP-16 and Taxol are the most suitable for combination with SN-38.

Key words: Anticancer agents, combination chemotherapy, CPT-11, *in vitro*, lung cancer.

Introduction

Lung cancer is the leading cause of cancer deaths in human beings. Combination chemotherapy is universally accepted as primary treatment for small cell lung cancer (SCLC) because nearly all patients present with occult distant metastases. Even for non-small cell lung cancer (NSCLC), new anticancer agents and combinations are also necessary since chemotherapy is rather ineffective. In the last few years, a number of new anticancer drugs have been developed which have definite activity in this disease. Among them are 7-ethyl-10-[4(1-piperidino)-1-piperidino] carbonyloxy camptothecin (CPT-11) and paclitaxel (Taxol) two promising anticancer agents in chemotherapy.[1]

CPT-11, a newly semisynthesized water-soluble derivative of camptothecin (CPT), is a potent antitumor drug with a broad spectrum of antitumor activity. It induces the formation of protein-linked DNA strand breaks. These breaks are single stranded and result from stabilization of covalent cross-links between genomic DNA and a separate nuclear enzyme, topoisomerase 1 (Topo 1). Replication fork arrest or fork breakage resulting from drug-stabilized Topo 1–DNA cleavable complexes is lethal to proliferating cells.[2] Upon removal of CPT-11, these drug-induced breaks also reseal. Nonetheless, a fraction of the cells, especially those cells in S phase at the time of drug exposure, proceed to die.[1,3]

It has been reported that CPT-11 or 7-ethyl-10-hydroxy-camptothecin (SN-38) (an active metabolite of CPT-11 *in vivo*)-based combination therapy is effective in some tumor cell lines.[1,3] However, since different tumor cell types respond to different combinations and schedules, universally accepted combinations and schedules for lung cancer chemotherapy have not been found.

In this study, we evaluated the *in vitro* effect of SN-38 in combination with Taxol and other commonly used anticancer agents, including an alkylating agent (cisplatin); an antimetabolite [5-fluorouracil (5-FU)]; antibiotics [bleomycin (BLEO) and adriamycin (ADR)] and a plant alkaloid [etoposide (VP-16)], against two human lung cancer cell lines (NSCLC and SCLC). These agents were selected because they represent six distinct categories of antineoplastic mechanisms. The MTT assay was used to measure the cytotoxicity of the combination and data were analyzed using an improved isobologram method developed for the evaluation of drug combinations.

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Materials and methods

Cell cultures and chemicals

Human pulmonary adenocarcinoma cells (PC-9) and small cell lung cancer cells (NCI N417) were maintained in RPMI-1640 medium containing 5% fetal bovine serum (FBS; CC Laboratories, Cleveland, OH). MTT was bought from Sigma (St Louis, MO). SN-38 was kindly provided by Yakult (Tokyo, Japan). The other agents kindly provided were; Taxol (Bristol-Myers Squibb, Tokyo, Japan), BLEO, cisplatin and VP-16 (Nippon Kayaku, Tokyo, Japan), 5-FU and ADR (Kyowa Hakko, Tokyo, Japan). SN-38 and Taxol were dissolved in DMSO at a concentration of 1 mM and aliquots were frozen at −80°C until use. The drugs were diluted with RPMI 1640 immediately before their use. All other drugs were dissolved in RPMI 1640.

MTT assay

The MTT assay was carried out as described elsewhere.[4,5] Briefly, cell suspensions (100 μl) were dispensed into individual wells of a 96-well tissue culture plate. Each plate had one 8-well control column containing medium alone and one control column containing cells but no drugs. Solutions (100 μl) of SN-38 and the other drug at different concentrations were then added. The plate was subsequently incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 4 days, 0.1 mg (50 μl of 2 mg/ml) MTT was added and the plate was incubated at 37°C for 4 h and subsequently centrifuged at 800 g for 10 min, and the media were removed. MTT formazan crystals were then solubilized by adding 200 μl DMSO and absorbance was measured using an automated microplate reader at a wavelength of 540 nm (Easy Reader EAR 340; SLT-Labinstruments, Austria). The dose–response curves were plotted on a semilog scale as a percentage of the absorbance of the control, which was obtained from samples with no drug exposure that were processed simultaneously.

Isobologram analysis

The effect of SN-38 in combination with other anticancer agents at ID_{50} were analyzed by an improved isobologram method described previously.[6,7] Based on the dose–response curves of SN-38 and the combined drug, three isoeffect curves (mode 1, mode 2a and mode 2b) were drawn (Figure 1). Mode 1: when the dose of SN-38 was chosen, an increment in effect remained to be produced by the combined drug. The calculation of the addition was performed by taking the increment in doses, starting from zero, that produced log survivals that added up to ID_{50} (hetero-addition). Mode 2a: when the dose of SN-38 was chosen, an increment in effect remained to be produced by the combined drug. The calculation of the addition was performed by taking the increment in doses, starting from the point on the dose–response curve of SN-38 where the effect of its dose had ended, that produced log survivals that added up to ID_{50} (iso-addition). Mode 2b: similarly, when the dose of combined drug was chosen, an increment in effect remained to be produced by SN-38. The calculation of the addition was performed by taking the increment in doses, starting from the point on the dose–response curve of the combined drug where the effect of its dose had ended, that produced log survivals that added up to ID_{50} (iso-addition). Since we do not know whether the combined effect of two drugs will be iso-additive, hetero-additive or intermediate, all possibilities should be considered. When the data points of the drug combination fall within the area surrounded by the three lines (envelope of additivity) (Pb), this combination is regarded as additive. When the data points fall to the left of the envelope (Pa), the two drugs have

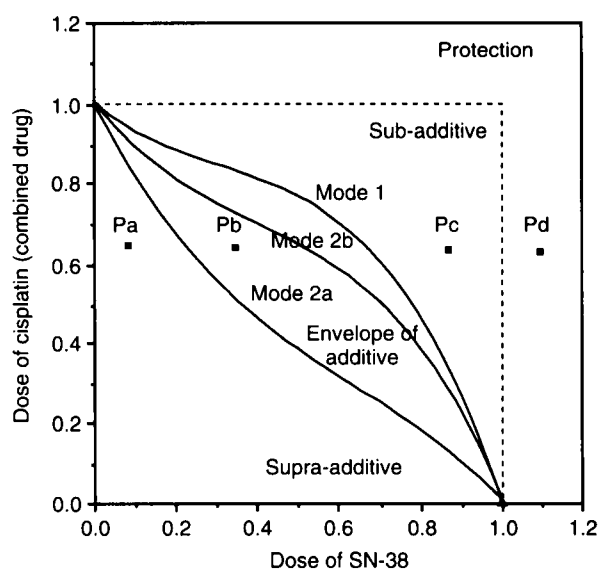

Figure 1. An envelope of additivity constructed from the dose–response curves of two drugs (SN-38 and a combined drug). Data points Pa, Pb, Pc and Pd show supra-additive, additive, sub-additive and protection, respectively.

supra-additive interaction (synergism). When the data points fall to the right of the envelope, but within or on the square dot line (Pc), the two drugs have sub-additive interaction. When the points are outside the square (Pd), this combination is regarded as protective interaction. Both sub-additive and protective interaction are considered to be antagonism.

Experiments were repeated at least three times. In each experiment, the dose–response curves of SN-38 and the combined drugs were slightly different, but a similar tendency was observed. Representative dose–response curves and isobolograms are shown.

Results

Cytotoxicity of SN-38 and other anticancer agents *in vitro*

The cytotoxicity of anticancer agents singly administrated was assayed against PC-9 and NCI N417 (Table 1). SN-38 and Taxol showed a significant cytotoxicity at extremely low concentrations. Except for cisplatin and Taxol, NCI N417 was more sensitive to anticancer agents than PC-9 did.

Combined effects of SN-38 with other agents on NCI N417 cells

Representative dose–response curve of SN-38 in combination with VP-16 against NCI N417 cells is presented in Figure 2. Isobolograms were made based on the dose–response curves obtained from all combined agents (Figure 3). In NCI N417 cells, for simultaneous and continuous exposure to SN-38 and cisplatin, the combined data points fell on the left side of the envelope. This was regarded as a supra-additive effect produced by simultaneous exposure to SN-38 and cisplatin. Similar tendencies

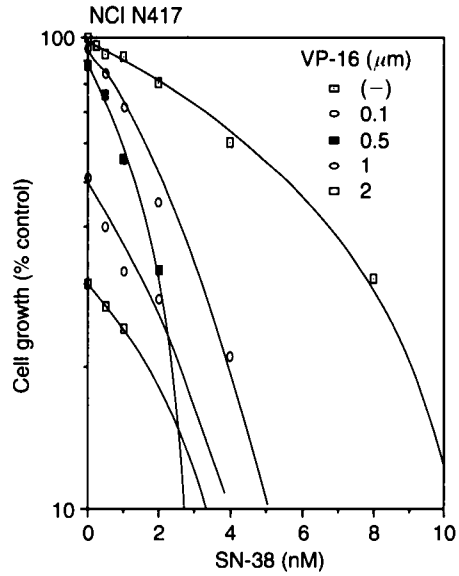

Figure 2. Dose–response curve of SN-38 in combination with VP-16 in NCI N417 cells. Each assay was repeated at least three times and cell growth number was plotted as a percentage of the control. The concentrations of combined drugs for each symbol are shown in the upper right. SN-38 concentrations are shown on the abscissa.

were revealed for combining with VP-16 and Taxol. In the combination of SN-38 with BLEO, the data points fell within the envelope of additivity. However, when these cells were simultaneously and continuously exposed to SN-38 and either ADR or 5-FU, all the data points fell in the area of sub-additivity and protection.

Combined effects of SN-38 with other agents on PC-9 cells

The combined effects of SN-38 with other agents in PC-9 cells differed from those in NCI N417 cells. Supra-additive effects were found in the combination

Table 1. ID_{50}[a] of the cell lines following continuous exposure to anticancer agents

Cell line	IC_{50} of anticancer drugs						
	Taxol (nM)	SN-38 (nM)	VP-16 (μM)	ADR (μM)	Cisplatin (μM)	5-FU (μM)	BLEO (μM)
NCI N417	1.85	5.60	1.01	0.38	5.00	3.45	4.20
PC-9	1.75	45.00	12.00	0.84	2.00	7.20	8.50

[a] ID_{50} values were obtained graphically as the dose of drug causing a 50% reduction of the control values. Each value represents the average of four to six separate experiments.

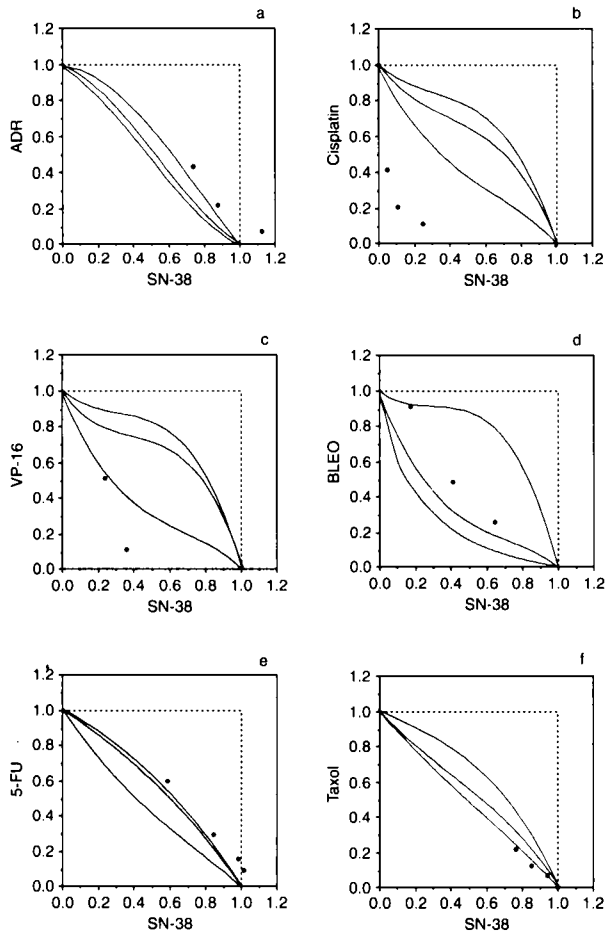

Figure 3. Isobologram of SN-38 in combination with (a) ADR, (b) cisplatin, (c) VP-16, (d) BLEO, (e) 5-FU and (f) Taxol in NCI N417 cells.

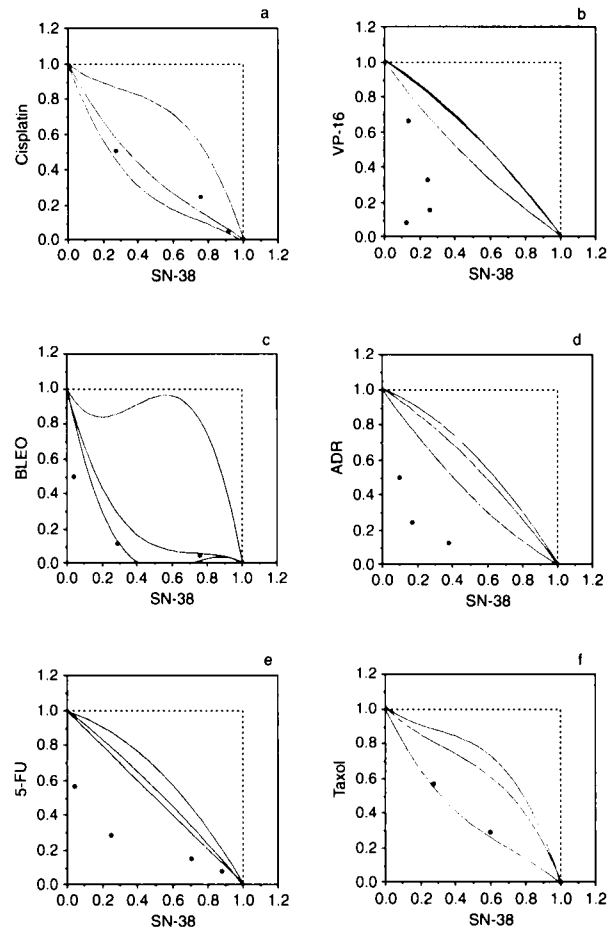

Figure 4. Isobologram of SN-38 in combination with (a) cisplatin, (b) VP-16, (c) BLEO, (d) ADR, (e) 5-FU and (f) Taxol in PC-9 cells.

of SN-38 with VP-16, 5-FU and ADR (Figure 4). When combined with cisplatin and Taxol, respectively, data points fell within the envelope of additivity. In the combination of BLEO, the data points fell both in the area of supra-additivity and the envelope of additivity. No drug showed sub-additive and protective effects with SN-38 on this cell line.

Discussion

CPT-11, a DNA Topo 1 inhibitor, has an unprecedented antitumor activity against a broad spectrum of experimental tumor models.[1] Phase I and II studies of this agent in patients with lung cancer have yielded excellent results.[8–10] Since the combined use of anticancer agents may be more effective than the use of a single one, it is very important to find which agent is useful and effective in combination with CPT-11, and which should be avoided. Although many authors have done research in this field and made great progress, because the tissue-specific factors may modulate the cytotoxicity effect of the drug, different tumor cells may respond to anticancer agents differently.[11,12] There is still a long way to go to get a universally accepted combination and schedule.

The present study evaluated the cytotoxicity of simultaneous combination with the Topo 1 inhibitor, SN-38, and other representative anticancer agents including an alkylating agent (cisplatin), an antimetabolic agent (5-FU), an antibiotic (BLEO), and Topo 2 inhibitors (ADR, VP-16 and Taxol). In the comparison of cytotoxic effects of a single agent, SCLC was more sensitive to these agents than NSCLC, except for cisplatin and Taxol. Furthermore, we found SN-38 and Taxol, two promising new drugs, significantly inhibited proliferation of the tumor cells at low

concentrations, which was consistent with previous reports and suggested their significant cytotoxicity on lung cancer cells.[1,3]

In the present study, we demonstrated supra-additive (synergistic) effects of combination of SN-38 with VP-16 on both PC-9 and NCI N417 cells, while combined with ADR, a supra-additive effect was observed for PC-9; however, a protective effect was obtained for NCI N417 cells.

Topo 2 inhibitors including intercalative (ADR) and non-intercalative anticancer agents induce the stabilization of cleavable double-strand DNA–Topo 2 complexes that are converted to double-strand DNA breaks.[13] Elevated expression of DNA Topo 2 and decreased expression of DNA Topo 1 was found in CPT-resistant human tumor cells. Topo 2 compensates for the decrease of Topo 1 activity. So co-administration of both topoisomerase inhibitors should effectively prevent the development of resistance and cause complete cell killing.[4,5] However, the addition of Topo 2-directed drugs to CPT-11 (or SN-38) yielded mixed results. When they were administrated simultaneously *in vitro*, antagonism was observed in human colon carcinoma cells[16] and human leukemia cell lines.[17] The reason that antagonism arises may be that CPT-11 or its analogs inhibit the nucleic acid synthesis that is required to convert Topo 2–DNA adducts into cytotoxic lesions.[18] On the other hand, simultaneous administration of Topo 1 and Topo 2 inhibitors may also cause synergistic cytoxicity *in vitro* and *in vivo*.[7,19] Furthermore, CPT-11 was effective against pleiotropically drug-resistant tumors (including an ADR-resistant tumor) *in vitro* and *in vivo*.[20] A phase I study of CPT-11 and VP-16 in patients with NSCLC also suggested that the combination is effective and encouraging.[21] Different sensitivities of S phase cells in myelogenous versus lymphocytic leukemia lines to both DNA Topo 1 and 2 inhibitors emphasized the tissue-specific factors that modulate the cytotoxic effects of these inhibitors.[11] CPT-induced replication fork-associated double-strand DNA breaks in different cells also have a different fate (chemosensitivity).[12] In the present study, it is not surprising that discrepant results were obtained on two different cell lines in the combination of SN-38 with ADR.

Our data showed that when combined with cisplatin, SN-38 had a supra-additive effect for NCI N417 cells and an additive effect for PC-9, since the improved isobologram method is, in general, stricter for defining supra-additive (synergism) than other methods.[22] This combination would be synergistic when the other methods are used for evaluation. In this method, an additive effect also indicates marked superiority of the combination to a single agent, even though the combined data do not reach the supra-additive area.

Cisplatin is commonly used in lung cancer chemotherapy and is considered to be one of the most active agents. It has been shown to induce intra-strand DNA cross-links.[23] The addition of cisplatin to Topo 1 inhibitors has been reported to result in additive or synergistic cytotoxicity *in vitro* or *in vivo*.[3,7,24] A phase II study of CPT-11 combined with cisplatin in patients with advanced NSCLC[25] also demonstrated a synergistic effect. These results may reflect an effect of Topo 1-directed drugs on the repair of DNA damage caused by alkylating agents. Alternatively, the alkylating agents might promote unscheduled DNA synthesis and thereby provide replication forks that can interact with Topo 1–DNA. Our data and previous reports demonstrated that simultaneous administration of CPT-11 and cisplatin is suitable for clinical application and is worthy of clinical trial.

Taxol, a new antitubular agent, has been called the best new anticancer agent developed from natural products, showing remarkable clinical efficacy in advanced ovarian cancer, breast cancer as well as lung cancer.[1] It enhances the polymerization of tubulin to stable microtubles and also interacts stoichiometrically with microtubles.[26,27] It blocks cells in the mitotic phase of their circle and such cells are unable to replicate normally. In the present study we found a synergistic cytotoxic effect on NCI N417 cells and an additive effect on PC-9 cells when SN-38 and Taxol were administered simultaneously. This is consistent with a previous report in which Taxol synergistically combined with topotecan, another water-soluble derivative of CPT, against human teratocarcinoma cells.[28] Although the mechanism of the synergism between SN-38 and Taxol is not clear, our data indicate that the study of the combination of SN-38 with Taxol in clinical trials for human lung cancer is highly warranted.

BLEO had an additive effect with SN-38 on NCI N417 cells and a marginal supra-additive effect on PC-9 cells. A similar effect in combination on leukemia cells was also reported.[7] It is considered to be suitable for combination with CPT-11 or SN-38.

Our findings show the different combination effects of 5-FU and SN-38 on two lung cancer cell lines. A supra-additive effect was observed on PC-9 cells; however, a protective effect on NCI N417 cells was observed.

In previous studies, a combination of SN-38 with S-phase-specific agents (methotrexate and 5-FU) re-

sulted in different effects. Simultaneous administration of SN-38 and methotrexate caused a protective effect, while SN-38 and 5-FU are found to be additive on human leukemia cells.[7] The mechanism is unclear. 5-FU markedly slowed down cell progression through the S phase, but did not completely stop it. Therefore, the cells replicating DNA in the presence of 5-FU, even at slow rates, can still be sensitive to CPT-11. When they were combined, a synergistic effect occurred. However, if S-phase-specific agents completely stop DNA replication, when they were combined with CPT-11, an antagonistic effect occurred.

In summary, we have found the cytotoxic effect of SN-38 in combination with other anticancer agents depends on the cell lines used and the anticancer agents combined. CPT-11 or SN-38 had a supra-additive effect with VP-16 on both NSCLC and SCLC cells; when combined with cisplatin, a supra-additive effect was seen SCLC cells and additive effect in NSCLC. Clinical report on patients with these combinations confirmed the data we obtained on these combinations. BLEO is suitable for combination with SN-38 and is worthy of clinical trial. Although ADR and 5-FU can be synergistically combined with SN-38 on NSCLC, these combinations give rise to the opposite effect (antagonism) on SCLC. They should be avoided in combination with SN-38 or CPT-11 for the chemotherapy of SCLC. For NSCLC, SN-38 is suitable for combining with the drugs checked in this study on chemotherapy. Furthermore, we demonstrated that SN-38 and Taxol are two of the most effective anticancer agents. When they were combined, a supra-additive and an additive effect were observed in SCLC and NSCLC, respectively. We strongly suggest this combination is well studied *in vitro* and *in vivo*. It may have an unexpected effect on the chemotherapy of lung cancer.

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