Sequential treatment with SN-38 followed by 5-fluorouracil shows synergistic cytotoxic activity in small cell lung cancer cells

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Abstract. Despite the high response rates of small cell lung cancer (SCLC) to first-line cisplatin-based chemotherapies, most patients with SCLC will eventually experience disease progression. Accordingly, novel chemotherapeutic regimens are desired. This *in vitro* study was carried out in order to develop novel chemotherapeutic regimens containing 5-fluorouracil (5-FU) or oral fluoropyrimidine for SCLC. 5-FU was combined with other standard drugs for SCLC (cisplatin, etoposide, an active metabolite of irinotecan and amrubicin) in different schedules. The combination effects were analyzed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and an isobologram method using H69 SCLC cells. Among the examined combinations, synergistic growth inhibition was observed only when H69 cells were treated with 7-ethyl-10-hydroxycamptothecin (SN-38; an active metabolite of irinotecan) followed by 5-FU. The findings of a flow cytometric analysis were consistent with the enhancement of apoptotic cell death by this sequential treatment. This synergism was observed in 4 out of 5 SCLC cell lines tested. The effects of 5-FU and SN-38 on thymidylate synthase (TS) protein expression, an important determinant of 5-FU sensitivity, were assessed by Western blot analysis in H69 cells. Treatment with SN-38 for 24 h suppressed TS protein expression and this low level of TS was maintained for at least 72 h. Pretreatment with SN-38 inhibited the 5-FU-induced increase of TS protein. The synergistic effect induced by the combination of SN-38 and 5-FU may be attributable to the SN-38-induced suppression of TS protein. Furthermore, uracil and 5-chloro-2,4-hydroxypyridine, which are clinically available dihydropyrimidine dehydrogenase inhibitors, enhanced 5-FU-induced growth inhibition. These observations provide evidence supporting the clinical applications of the combination chemotherapy using irinotecan and 5-FU or oral fluoropyrimidines against SCLC.

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Introduction

Small cell lung cancer (SCLC) is a highly aggressive neoplasm characterized by a high growth fraction, short doubling time and a high rate of metastasis (1), although it is sensitive to chemotherapy (2). Systemic chemotherapy prolongs the survival of SCLC patients (2) and cisplatin (CDDP), etoposide (VP-16), topoisomerase I inhibitors [irinotecan (3,4) and topotecan (5)] and amrubicin (AMR) (6) (a totally synthetic 9-amino-anthracycline) are used in regular clinical practice in Japan. Among these chemotherapeutic agents, a combination chemotherapy of CDDP and VP-16 (PE) is thought to be a standard first-line chemotherapeutic regimen worldwide (3,4). The combination of irinotecan and cisplatin (IP) has shown good results, including an 87% in response rate and 12.8-month median survival time (MST) in the treatment of extensive-disease SCLC (3). Despite the high response rates obtained with these first-line CDDP-based chemotherapies, most patients will eventually experience disease progression and thus become candidates for second-line chemotherapy. Accordingly, novel chemotherapeutic regimens, including maintenance chemotherapy, are desired in the treatment of SCLC patients.

The effectiveness of 5-fluorouracil (5-FU) and oral fluoropyrimidine including UFT, a derivative consisting of tegafur and uracil, has been reported in several cancers, especially those arising in the gastrointestinal tract (7). Previously, new generations of oral fluoropyrimidine such as S-1, a derivative consisting of tegafur, potassium oxanate and 5-chloro-2,4-hydroxypyridine (CHDP), which inhibit the degradation of 5-FU and capecitabine have been developed and put into use in the treatment of several solid tumors (8). Several clinical trials have shown the potential of oral fluoropyrimidines for treating advanced non-small cell lung cancer (NSCLC) as a combination chemotherapy (9) or monotherapy (10-12). UFT has also been reported to be effective as adjuvant chemotherapy for lung cancer in certain phase III trials (10,11) and meta-analysis (13). Therefore, oral fluoropyrimidines such as UFT and S-1 are expected to be important drugs in the treatment of NSCLC patients.

On the other hand, information regarding the application of 5-FU or oral fluoropyrimidines in the treatment of SCLC is limited. 5-FU alone or in combination with folic acid did not exert satisfactory antitumor activity in previously treated patients with SCLC (14,15). However, Sbar *et al* reported that a patient with SCLC achieved complete remission with the combination treatment of topotecan and 5-FU in a phase I clinical trial (16). More encouragingly, a combination regimen of 5-FU and cisplatin demonstrated a 77% initial response rate, which is comparable with that of the standard PE therapy (17). These observations suggest that even if 5-FU as a single agent may be less effective in treating SCLC compared with other frequently used anticancer drugs, 5-FU or oral fluoropyrimidine might exert synergistic activity when combined with proper chemotherapeutic agents.

Previously, we reported the schedule-dependent synergism of 5-FU (*in vitro*) or UFT (in an animal model) and vinorelbine against NSCLC (18). We proposed that this synergistic effect is attributable to the vinorelbine-induced suppression of thymidylate synthase (TS) protein, which is an important determinant of 5-FU sensitivity (19). Based on these experiments, we conducted phase I/II clinical trials for NSCLC using vinorelbine and UFT and obtained promising results (unpublished data). If the same mechanism could function in SCLC cells, it may be possible to develop a 5-FU or oral fluoropyrimidine-containing chemotherapeutic regimen that is expected to improve the poor prognosis of SCLC patients. However, the efficacy of this combination of drugs has not been studied experimentally.

In the present *in vitro* studies, we investigated the combined efficacy of 5-FU and other standard drugs for SCLC and the optimal schedule for drug treatment in order to develop 5-FU or oral fluoropyrimidine-containing chemotherapy against SCLC.

Materials and methods

Chemicals and reagents. 5-FU (a gift from Kyowa Hakko Kogyo, Co., Ltd, Tokyo, Japan), CDDP (a gift from Nippon Kayaku, Co., Ltd, Tokyo, Japan), VP-16 (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 7-ethyl-10-hydroxycamptothecin (SN-38; a gift from Daiichi Pharmaceutical, Co., Ltd, Tokyo, Japan) were dissolved in dimethylsulfoxide and stored at -20˚C. AMR (a gift from Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) was dissolved in distilled water and stored at -20˚C after filtration. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chemical Industries) was dissolved in phosphate-buffered saline (PBS) and stored at -20˚C. A dihydropyrimidine dehydrogenase (DPD) inhibitor, uracil (Wako Pure Chemical Industries), was dissolved in 1 M of sodium hydroxide. Another DPD inhibitor CHDP (a gift from Taiho Pharmaceutical Co., Ltd., Saitama, Japan) was dissolved in dimethylsulfoxide. The two DPD inhibitors were stored at -20˚C.

Cell lines and cultures. The H69 and H209 human SCLC cell lines were provided by Drs A.F. Gazdar and H. Oie (NCI-Navy Medical Oncology Branch, NIH, Bethesda, MD). The Lu135 and Lu139 human SCLC cell lines were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The human SCLC cell line 87-5 was provided by Dr S. Kobayashi (Miyagi Prefectural Semine Hospital, Miyagi, Japan) through the Cell Resource Center for Biomedical Research. All of these SCLC cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The cells were grown in a humidified atmosphere of 5% CO_2, 95% air.

MTT assay. The cytotoxic activity of chemotherapeutic agents was determined by an MTT assay. In the simultaneous combinations of drugs, H69 cells were counted with a hematocytometer and $1x10^4$ cells were treated with various concentrations of the indicated drugs simultaneously for 72 h using 96-well flat bottom multiplates (BD Falcon, Franklin, NJ). In the sequential combinations, H69 cells were counted with a hematocytometer and $4x10^5$ cells were treated with 4 ml of various concentrations of 5-FU, CDDP, VP-16, SN-38 or AMR for the indicated time using 6-well flat bottom multiplates (Sumitomo Bakelite Co., Tokyo, Japan). After the treatment, H69 cells were harvested and washed with PBS. The cells were incubated in 100 μl medium containing various concentrations of CDDP, VP-16, SN-38, AMR or 5-FU for the indicated time using 96-well flat bottom multiplates. After the indicated treatment, the MTT solution (10 mg/ml in PBS) was added (10 μl/well). Plates were further incubated for 4 h at 37˚C. Thereafter, the formazan crystals formed were dissolved by adding 100 μl of 0.04 N HCl in 2-propanol. Absorption was measured by a microplate reader (MPR-A4i; Tosoh Corporation, Tokyo, Japan) at 570 nm (reference filter 650 nm). Measurements were performed in triplicate. DMEM (100 μl) with 10 μl MTT-solution and 100 μl 2-propanol was used as a blank solution. Dose-response curves were plotted on the basis of the data derived from the MTT assay.

Sequential exposure to SN-38 followed by 5-FU was evaluated in the H209, Lu139, Lu135 and 87-5 cells by the MTT assay in the same way as described above.

Isobologram method. The combination effects of 5-FU and other agents, CDDP, VP-16, SN-38 or AMR, were analyzed by the isobologram method as described previously (20). We used the concentration producing 50% inhibition of cell growth (IC_{50}) to evaluate dose-response interactions between 5-FU and CDDP, VP-16, SN-38 or AMR.

Cell lysis and Western blot analysis. Cells were lysed in a modified radioimmune precipitation buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 40 mM ß-glycerophosphate and 2 mM Na_3VO_4) and insoluble material was removed by centrifugation. The protein concentration was determined by means of a Bio-Rad Protein assay (Bio-Rad, CA) and lysates containing 30 μg of total cellular protein were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis and visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech) using goat anti-rabbit IgGs coupled to horseradish peroxidase as a

secondary antibody (Amersham Pharmacia Biotech). The rabbit polyclonal primary antibody against recombinant human TS (RTSSA) was provided by Taiho Pharmaceutical Co. An anti-ß-actin antibody (Sigma, Tokyo, Japan) was used as a loading control.

Flow cytometric analysis. After the indicated treatments, H69 cells were collected, washed and re-suspended in 1 ml PBS. The cells were fixed in ice-cold 70% ethanol overnight at 4˚C and re-suspended in 0.5 ml of PBS containing propidium iodide (50 μg/ml) and RNase A (1 μg/ml). Cell fluorescence was analyzed on a Becton FACScan, using cell Quest software (Becton Dickinson, Mountain View, CA). Apoptotic cells (cells with fractional DNA content; Sub-G_1 cells) were defined on a histogram and expressed as percentages.

Results

Isobologram analysis assessing the effects of combinations of 5-FU with CDDP, VP-16, SN-38 or AMR. In order to investigate whether there was synergistic cytotoxic activity in the combination of 5-FU with other cytotoxic drugs in SCLC cell lines, we used H69 cells. The effects of the combined treatment with 5-FU and CDDP, VP-16, SN-38 or AMR were analyzed by the isobologram method using the MTT assay. Instead of irinotecan, SN-38 was used in these *in vitro* experiments since SN-38 is an active metabolite of irinotecan (21). H69 cells were treated according to three schedules: A) simultaneous combination treatment of 5-FU with CDDP, VP-16, SN-38 or AMR for 72 h; B) treatment with CDDP, VP-16, SN-38 or AMR for 24 h followed by 5-FU for 72 h and C) treatment with 5-FU for 72 h followed by CDDP, VP-16, SN-38 or AMR for 24 h. The continuous infusion (CI) of 5-FU has been revealed to be more effective than bolus administration in the treatment of colon cancer (22). In addition, oral fluoropyrimidine is administered for a designated period, which can be long. Thus we set the exposure time of H69 cells to 5-FU to be 72 h considering the clinical use of 5-FU, CI or oral fluoropyrimidines. As shown in Fig. 1, at best, additive interactions were observed when H69 cells were treated with the simultaneous combination of 5-FU and another chemotherapeutic agent (CDDP, VP-16, SN-38 or AMR) or the sequential combination of 5-FU followed by another drug. Similarly, additive interactions were observed in the treatment of CDDP, VP-16 or AMR followed by 5-FU. However, synergistic interaction was observed only when H69 cells were sequentially exposed to SN-38 followed by 5-FU.

Flow cytometry assessing the cytotoxic activities of SN-38, 5-FU and the sequential combination in H69 cells. To examine whether apoptotic cell death is enhanced by the sequential combination of SN-38 followed by 5-FU, we performed flow cytometric analysis to focus on the accumulation of the Sub-G_1 cell population (Fig. 2). H69 cells were treated with either 10 nM of SN-38 for 24 h followed by normal medium for an additional 48 h, 10 μM of 5-FU for 48 h or their sequential combination. As shown, the SN-38 and 5-FU treatments increased the Sub-G_1 cell population by 13 and 16.3%, respectively, compared with the population of

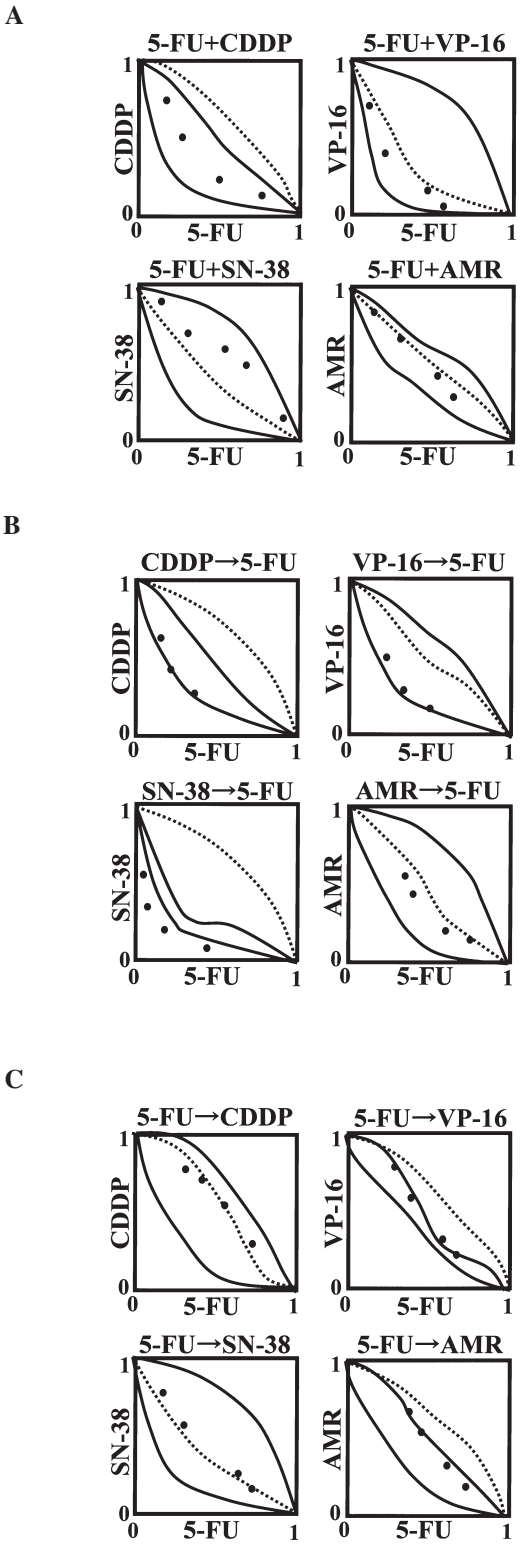

Figure 1. Isobologram analysis assessing the effects of combinations of 5-FU with CDDP, VP-16, SN-38 or AMR. H69 cells were treated according to three different schedules: (A) simultaneous combination treatments of 5-FU with CDDP, VP-16, SN-38 or AMR for 72 h; (B) treatments with CDDP, VP-16, SN-38 or AMR for 24 h followed by 5-FU for 72 h and (C) treatments with 5-FU for 72 h followed by CDDP, VP-16, SN-38 or AMR for 24 h. The envelopes of additivity are defined by three isoeffect lines constructed from the dose-response curves of the single agents in the indicated schedules. The concentration of 5-FU, CDDP, VP-16, SN-38 or AMR alone that produced IC_{50} is expressed as 1 on the ordinate and the abscissa. The plotted data points show the relative values of the concentrations producing IC_{50} when exposed to each treatment schedule.

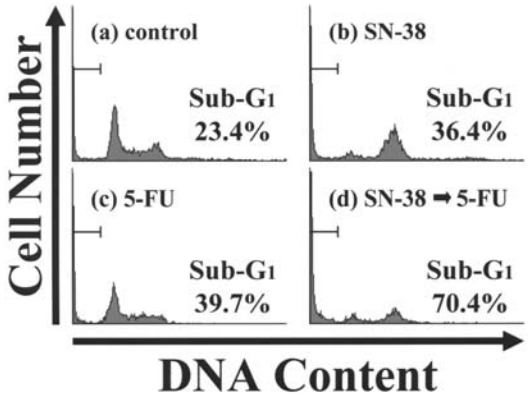

Figure 2. Flow cytometry assessing the cytotoxic activities of SN-38, 5-FU and their sequential combination in H69 cells. H69 cells were (a) untreated for 72 h (control) or treated with (b) 10 nM of SN-38 for 24 h followed by normal medium for an additional 48 h, (c) 10 μM of 5-FU for 48 h or (d) their sequential combination. Cells were fixed, stained and analyzed by flow cytometry as described in Materials and methods. The Sub-G1 cell population is expressed as a percentage of total cell counts.

untreated control cells. On the other hand, the sequential combination increased the Sub-G_1 cell population by 47%, which exceeded the simple sum of the increases achieved by SN-38 and 5-FU alone. These results suggest that apoptotic cell death is enhanced by the sequential combination of SN-38 followed by 5-FU.

Effects of 5-FU and SN-38 on TS protein expression levels in H69 cells. To clarify the mechanisms by which the sequential combination of SN-38 followed by 5-FU exerts the synergistic activity, we assessed the effects of SN-38, 5-FU or the sequential combination on TS protein levels in H69 cells by Western blot analysis.

H69 cells were treated with 10 μM of 5-FU for 6-48 h (Fig. 3A upper panel) or various concentrations of 5-FU for 24 h (Fig. 3A lower panel). The treatment of H69 cells with 5-FU increased the TS protein level in a time- and concentration-dependent manner.

Evaluating the effect of SN-38 on TS. In the SN-38 treatment, TS protein was decreased after 48 h of exposure to SN-38 at a concentration of 10 nM, which was the IC_{50} value for a 72-h treatment with SN-38 alone (data not shown). Since the treatment with SN-38 for 24 h followed by 5-FU exerted synergistic activity, the time course of TS protein levels was assessed after 24 h exposure of H69 cells to SN-38 (Fig. 3B). Even a 24-h treatment with 10 nM SN-38 decreased TS protein expression after 48 h and this low level of TS protein continued, at least, for 72 h. In the concentration assessment, 5 nM of SN-38 was sufficient in reducing TS protein.

TS protein expression in H69 cells was evaluated after sequential treatment with 10 nM SN-38 for 24 h followed by 10 μM 5-FU for 24 h (Fig. 3C). As shown, the pretreatment with SN-38 apparently inhibited the increase of TS protein levels caused by 5-FU treatment in H69 cells.

Isobologram analysis assessing the effects of the sequential treatment with SN-38 and 5-FU in other SCLC cell lines. The

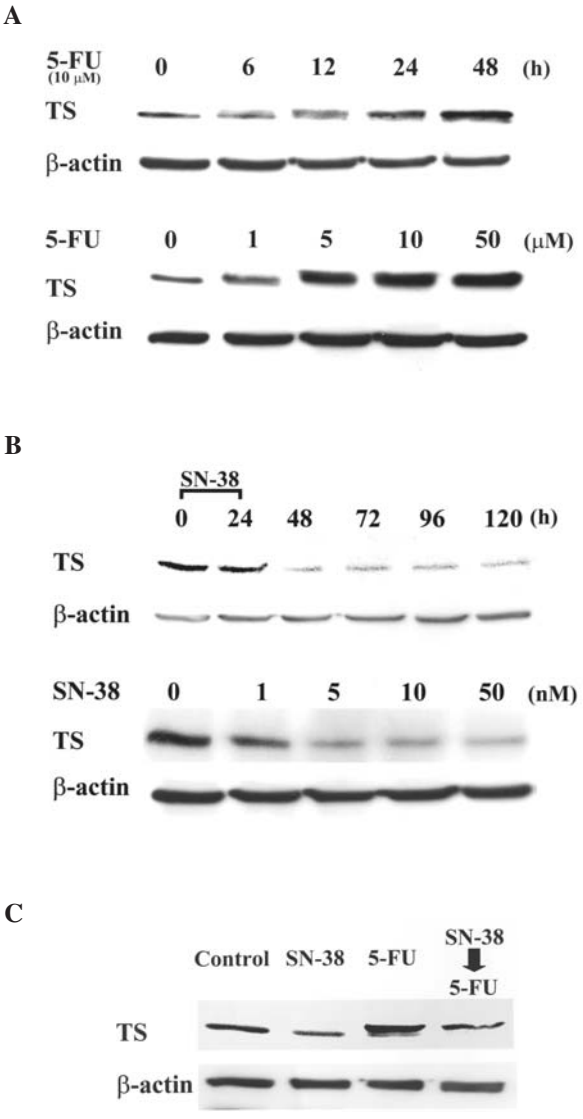

Figure 3. Effects of 5-FU and SN-38 on TS protein expression in H69 cells. (A) H69 cells were treated with 10 μM of 5-FU for the indicated times (upper panel) and with the indicated concentrations of 5-FU for 24 h (lower panel). (B) H69 cells were treated with 10 nM of SN-38 for 24 h, washed with PBS and then incubated with the SN-38-free control medium. The time of the SN-38 treatment is depicted by the bar and the indicated times represent the timing of the cell harvest (upper panel). The H69 cells were treated with the indicated concentrations of SN-38 for 24 h and cultured in a normal medium for an additional 24 h (lower panel). (C) H69 cells were untreated or treated with 10 nM SN-38 alone for 24 h, 10 μM 5-FU alone for 24 h, or 10 nM SN-38 for 24 h followed by 10 μM 5-FU for 24 h. Total cell lysates of each treatment were subjected to Western blot analysis with an anti-TS antibody (RTSSA) as described in Materials and methods. ß-actin was used as a loading control.

sequential treatment with SN-38 and 5-FU showed synergistic cytotoxic activity in H69 cells. To evaluate the generality of this synergism in other SCLC cell lines, we tested the sequential combination effects in H209, 87-5, Lu139 and Lu135 cells. The cells were treated with various concentrations of SN-38 for 24 h, were harvested and washed with PBS and then were exposed to various concentrations of 5-FU for 72 h. Only an additive effect was observed in 87-5 cells. However, the synergistic effects were detected in the other 3 SCLC lines tested in these experiments (Fig. 4).

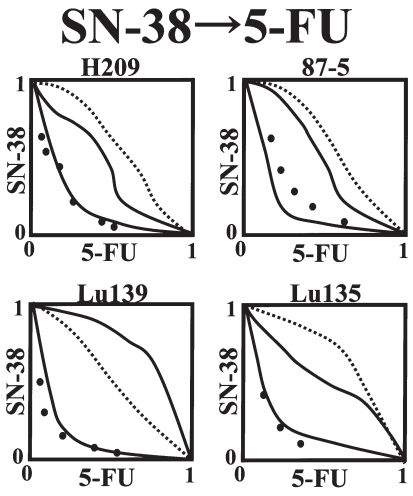

Figure 4. Isobologram analysis assessing the effects of the sequential treatment with SN-38 and 5-FU in other SCLC cell lines. H209, 87-5, Lul39 and Lu135 cells were treated with sequential exposure to SN-38 for 24 h followed by 5-FU for 72 h. The envelopes of additivity are defined by three isoeffect lines constructed from the dose-response curves of the single agents in the indicated schedules. The concentration of 5-FU or SN-38 alone that produced IC_{50} is expressed as 1 on the ordinate and the abscissa. The plotted data points show the relative values of the concentrations producing IC_{50} when cells were treated with the sequential combination of SN-38 and 5-FU.

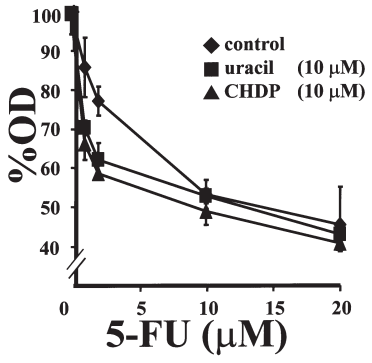

Figure 5. Enhancement of the growth inhibitory effect of 5-FU by DPD inhibitors in H69 cells. H69 cells were treated with various concentrations of 5-FU in the presence or absence of 10 μM uracil or CHDP for 72 h. The cell growth inhibition was assessed by an MTT assay and expressed as the percentage of optical density (% OD) relative to that of the 5-FU-untreated cells. Data are presented as the mean ± standard deviation of three separate experiments.

Including the results from H69 cells, the synergistic effects of sequential treatment with SN-38 followed by 5-FU were observed in 4 of the 5 SCLC cell lines tested in our study.

Enhancement of the growth inhibitory effect of 5-FU by dihydropyrimidine dehydrogenase (DPD) inhibitors in H69 cells. UFT and S-1 contain uracil and CHDP, respectively, as DPD inhibitors. Considering the clinical application of these drug products to SCLC, we evaluated the effects of these DPD inhibitors on 5-FU-induced growth inhibition in H69 cells.

In preliminary experiments, neither 10 μM of uracil nor 10 μM of CHDP exerted growth-inhibitory effects in H69 cells (data not shown). In the presence or absence of these concentrations of uracil or CHDP, the growth inhibition induced by various concentrations of 5-FU was determined by an MTT assay after 72 h of treatment (Fig. 5). As shown, uracil and CHDP enhanced 5-FU-induced growth inhibition at 5-FU concentrations lower than IC_{50}.

Discussion

This *in vitro* study was carried out in order to clarify the potential of 5-FU in combination with other chemotherapeutic agents in the treatment of SCLC. A synergistic effect was observed only when SCLC cells were treated with SN-38 followed by 5-FU in a sequence-dependent manner. Furthermore, our observations in the present study suggest that the inhibition of TS protein expression, which was caused by SN-38 pretreatment and continued for at least 72 h, is an important mechanism of the synergistic effects of sequential treatment with SN-38 and 5-FU in SCLC cell lines.

Using H69 cells as a model system of SCLC, we examined the effects of the combination of CDDP, VP-16, SN-38 or AMR with 5-FU on growth inhibition. Obvious synergistic growth inhibition was observed only when H69 cells were treated with SN-38 followed by 5-FU. Based on the flow-cytometric analysis, this synergism was attributable to the enhancement of apoptotic cell death. This sequential combination exerted synergistic growth inhibition in three out of four SCLC cell lines other than the H69 cells, a finding that suggests that this interaction can be expected in a majority of the SCLC cell lines. On the other hand, only additive interactions were observed in the simultaneous combination of 5-FU with CDDP, VP-16, SN-38 or AMR; treatment with CDDP, VP-16 or AMR followed by 5-FU; or the reverse sequence of 5-FU followed by other agents.

The purpose of the present study was to provide experimental evidence in order to help develop novel chemotherapeutic regimens containing 5-FU or oral fluoropyrimidine for SCLC. Our results indicate the potential of sequential combinations of irinotecan followed by 5-FU or oral fluoropyrimidine in the treatment of SCLC.

There are several reports that support the combination of 5-FU or oral fluoropyrimidine with irinotecan. For example, pretreatment with SN-38 before the 5-FU treatment showed synergistic effects in human colon cancer cells (23). In fact, a chemotherapeutic regimen, FOLFIRI, which consists of 5-FU in conjunction with folic acid and irinotecan, is one of the standard treatments against metastatic colorectal cancer (24). Instead of 5-FU, oral fluoropyrimidine combined with irinotecan exerted promising anti-tumor activity in gastric cancer (25). Thus, given that our *in vitro* experiments can be adapted for clinical efficacy, as in gastrointestinal cancer, the combination of irinotecan followed by 5-FU or oral fluoropyrimidine is expected to be an effective treatment for SCLC.

To clarify the mechanism by which SN-38 and 5-FU interact synergistically, we focused on TS protein, since this enzyme is one of the principal targets of 5-FU-based chemotherapy (26) and is a well-established determinant for sensitivity to 5-FU (27). We have shown that TS protein is induced by 5-FU treatment itself in SCLC cells, in accordance with other reports using other cancer cells (27,28). It has been

postulated that the 5-FU-induced increase of TS is related with the resistance to 5-FU (19). On the other hand, the treatment of H69 cells with SN-38 decreased TS expression levels. Furthermore, the 5-FU-induced increase of TS protein was decreased by the sequential combination of SN-38 followed by 5-FU. Given these results, we propose that the synergistic effect achieved by the combination of SN-38 and 5-FU is attributable to the SN-38-induced suppression of TS protein.

To our knowledge, there have been no reports of TS expression in clinical specimens of SCLC. However, the TS levels in SCLC can be speculated upon based on previous research. Retinoblastoma (Rb) protein is deficient in a majority of SCLC cases (29). When Rb is inactivated by phosphorylation, E2F, a transcription factor, is activated and induces TS protein expression (30). In theory, TS protein levels should be high in SCLC, leading to 5-FU resistance. This speculation may explain the failure of 5-FU monotherapy in the treatment of SCLC patients in previous clinical studies (14,31). The results of the present study suggest a potential combination chemotherapy that could be effective for overcoming 5-FU resistance even though TS protein is increased by Rb deficiency. There is a report by Ichikawa *et al* that is notable in relation to these findings. They found that although intratumor TS gene expression predicts a response to S-1 monotherapy in gastric cancer, this predictive factor did not correlate with the antitumor effects when S-1 was combined with irinotecan (32). If the combination of irinotecan and S-1 is effective regardless of TS expression, it is likely that irinotecan suppresses TS function in a clinical setting as well as an active metabolite of irinotecan, SN-38, decreased TS protein in this *in vitro* study. Further study is necessary in order to confirm this speculation.

Recent clinical studies recommend CI of 5-FU rather than bolus administration (33,34), whereas oral fluoropyrimidines such as UFT and S-1 can be used as an alternative to CI of 5-FU through the maintenance of systemic 5-FU concentrations by means of the DPD inhibition induced by uracil or CHDP (33). In addition, DPD is present in cancer cells and high expression levels of this enzyme confer 5-FU resistance to cancer cells (35). Although the expression level of DPD in SCLC is still unknown, uracil and CHDP enhanced 5-FU-induced growth inhibition in H69 cells at relatively low concentrations of 5-FU. These observations support the clinical use of oral fluoropyrimidines that contain DPD inhibitors in the treatment of SCLC.

The decrease of TS expression by SN-38 became clear after a 48-h exposure to SN-38 in H69 cells. However, the treatment with SN-38 for 24 h was sufficient in reducing the TS expression and the low levels of TS protein lasted for at least 72 h even in SN-38-free medium. In spite of the unclear mechanisms of this phenomenon, the prolonged decrease of TS protein has potentially important implications for the clinical application of 5-FU or oral fluoropyrimidine. If cancer cells are more sensitive to 5-FU during TS suppression, these observations may give theoretical validity to the use of the continuous administration of 5-FU or oral fluoropyrimidine for several days after irinotecan administration in a clinical setting.

In conclusion, our study showed that sequential treatment with SN-38 followed by 5-FU had synergistic cytotoxicity against 4 of the 5 SCLC cell lines. These findings may be at least partially attributed to the suppression of the TS protein caused by SN-38 pretreatment, resulting in increased chemosensitivity to 5-FU. Although 5-FU is not used in the present clinical practice in treatment of SCLC patients, we believe that our study provides evidence supporting the clinical applications of the combination chemotherapy using irinotecan and 5-FU CI or oral fluoropyrimidines, such as UFT, S-1 or capecitabine, as a combined chemotherapy against SCLC.

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