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 fluoropyrimidines 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.

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 fluoropyrimidines 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 fluoropyrimidines 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.

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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 Daichi Pharmaceutical, Co., Ltd, Tokyo, Japan) were dissolved in dimethylsulfoxide and stored at -20°C. AMR (a gift from Takeda Chemical Industries, Ltd, Osaka, Japan) was dissolved in distilled water and stored at -20°C after filtration. Dimethylglycerophosphate and 2 mM Na3VO4) 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 was determined by an MTT assay as described previously (20). We used the concentration producing 50% inhibition of cell growth (IC50) 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 radiolabeled precipitation buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxocholate, 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 β-glycerophosphate and 2 mM Na3VO4) 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-β-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-G1 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-G1 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-G1 cell population by 13 and 16.3%, respectively, compared with the population of

![Figure 1](https://via.placeholder.com/150)

**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 IC50 is expressed as 1 on the ordinate and the abscissa. The plotted data points show the relative values of the concentrations producing IC50 when exposed to each treatment schedule.
untreated control cells. On the other hand, the sequential combination increased the Sub-G1 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 IC50 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 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).
In the presence or absence of these 10 μM of CHDP exerted growth-inhibitory effects in H69 cells. Considering the clinical application of these DPD inhibitors in H69 cells, we evaluated the effects of these DPD inhibitors on 5-FU-induced growth inhibition 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 envelopes of additivity were defined by the iso-effect 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 IC50 is expressed as 1 on the ordinate and the abscissa. The IC50 when cells were treated with the sequential combination of SN-38 and 5-FU or uracil or CHDP enhanced 5-FU-induced growth inhibition at 5-FU concentrations lower than IC50.

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 chemotheraphy 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 even though 5-FU 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.

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