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Enhancement of the antitumour activity of 5-fluorouracil (5-FU) by inhibiting dihydropyrimidine dehydrogenase activity (DPD) using 5-chloro-2,4-dihydroxypyridine (CDHP) in human tumour cells

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Abstract

The purpose of this study was to evaluate the use of 5-chloro-2,4-dihydroxypyridine (CDHP), a potent inhibitor of dihydropyrimidine dehydrogenase (DPD), to enhance the antitumour activity of the fluoropyrimidines. In an *in vitro* study, CDHP did not influence cell proliferation by itself. However, CDHP did inhibit 5-fluorouracil (5-FU) degradation and enhanced 5-FU cytotoxicity in a concentration-dependent manner in two human tumour cell lines (MIAPaCa-2 and HuTu80) with relatively high basal DPD activity. CDHP exhibited a maximum effect at a molar ratio (CDHP:5-FU) of more than 0.2. However, CDHP did not have any effect on 5-FU cytotoxicity in the CAL27 tumour cell line, which has a relatively low basal DPD activity, even at concentrations where the DPD activity is almost completely inhibited. In an *in vivo* study, the maximal tolerable doses (MTD) of tegafur (FT) and a combination of FT and CDHP at a molar ratio of 1:0.4 (FT/CDHP) for nude mice were determined by oral administration for 14 consecutive days. After a single oral administration of either FT or FT/CDHP at the MTD, the 5-FU serum concentration–time profiles were almost the same for both treatment strategies. When nude mice bearing subcutaneous (s.c.) MIAPaCa-2 cells were treated with either FT or FT/CDHP at the MTD, the FT/CDHP treatment showed a significantly higher antitumour effect than the FT treatment (tumour growth inhibition: FT/CDHP, 51±12%; FT, 21±25%; $P<0.05$). However, the host-body weight suppression induced by FT/CDHP and FT was equivalent. These findings suggest that the combination of fluoropyrimidine and CDHP for the treatment of tumours with a high basal DPD elicits a greater antitumour effect than treatment with fluoropyrimidines alone and we suggest that CDHP inhibits the degradation of 5-FU in the tumour.

Keywords: Dihydropyrimidine dehydrogenase; Enzyme inhibitors for therapeutic use; *In vitro* and *in vivo* antitumour efficacy; Fluorouracil pharmacokinetics; Drug synergism; Human tumours

1. Introduction

In mammalian systems, 5-fluorouracil (5-FU) is catabolised to α-fluoro-β-alanine (FBAL), losing its cytotoxicity in the process. Catabolism occurs mainly in the liver via three enzymes: dihydropyrimidine dehydrogenase (DPD, EC1.3.1.2), dihydropyrimidinase (EC3.5.2.2), and ß-ureidopropionase (EC3.5.1.6). DPD is the rate-limiting enzyme in the catabolic process. Recently, encouraging clinical results have led to the development of a new generation of oral fluoropyrimidines, commonly referred to as DPD inhibitory fluoropyrimidines (DIF), that specifically target DPD [1,2].

Recently, we have developed S-1, a DIF consisting of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (Oxo) in a molar ratio of 1:0.4:1 [3]. FT, which is a prodrug of 5-FU, functions as an effector. Both CDHP and Oxo, which do not exhibit antitumour activities by themselves, act as modulators. CDHP competitively inhibits DPD approximately 180 times more effectively than uracil *in vitro* [4], leading to the retention of 5-FU in the blood for a prolonged period [3,5–7]. Oxo, which competitively inhibits the conversion of 5-FU to 5-fluorouridine 5′-monophosphate by orotate phosphoribosyltransferase (EC2.4.2.10), is mainly distributed in the gastrointestinal (GI) tract after oral administration in rats, leading to the relief of the GI toxicity induced by 5-FU [3,8]. In Japan, S-1 is used clinically for the treatment of gastric and head and neck tumours.

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Etienne and colleagues were the first to discuss the clinical relationship between intratumoral DPD levels and the antitumour effect of 5-FU [9]. They treated 62 head and neck cancer patients with biomodulated 5-FU. In each case, a tumoral and non-tumoral biopsy sample was obtained before treatment, and cytosolic DPD activities were measured. Patients with a complete response to the treatment exhibited a significantly lower tumoral/non-tumoral DPD activity ratio than partial or non-responding patients. Moreover, several clinical studies have indicated that the tumoral expression level of DPD is associated with the tumour's response to 5-FU in colorectal [10], gastric [11,12], and non-small cell lung [13] cancer patients.

Some *in vitro* studies have discussed the regulation of tumoral DPD activity as a way to biochemically modulate 5-FU. Eniluracil (EU), which is a potent irreversible DPD inhibitor [14,15], enhanced 5-FU cytotoxicity in five human cancer cell lines with a high basal DPD activity [16]. We previously demonstrated that uracil inhibited 5-FU degradation and enhanced 5-FU cytotoxicity in a concentration-dependent manner in two human tumour cell lines with high basal DPD activities [17]. However, whether the regulation of intratumoral DPD activity can enhance 5-FU antitumour activity *in vivo* remains uncertain. Thus, we firstly measured basal DPD activities in 31 human cell lines in culture to select a suitable cell line with high basal DPD activity, because downregulation of DPD appears to occur in culture [18]. Only three cell lines had DPD activities greater than 100 pmol/min/mg protein, including MIAPaCa-2 and HuTu80. We used these cell lines to examine the effects of CDHP on cytosolic 5-FU catabolism and the enhancement of 5-FU cytotoxicity in human cancer cell lines and the relationship between these two effects. Furthermore, we investigated the effect of CDHP on the antitumour activity of fluoropyrimidines in a nude mice model by comparing the maximum antitumour effects of FT and FT/CDHP treatments.

2. Materials and methods

2.1. Drugs

5-FU was purchased from Wako Pure Chemical Industries (Osaka, Japan); FT and CDHP were synthesised by Taiho Pharmaceutical Co. (Tokyo, Japan); all other chemicals were of the highest standard grade commercially available.

2.2. Tumour cell lines

The human pancreas carcinoma line MIAPaCa-2 [19] was purchased from Dainippon Pharmaceutical Co. (Osaka, Japan); the human duodenum adenocarcinoma line HuTu80 (ref. HTB-40) and the human tongue squamous cell carcinoma line CAL27 (ref. CRL-2095) [20] were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.3. Animals

Female nude mice (BALB/c-nu/nu) were purchased from Clea Japan, Inc. (Tokyo, Japan). They were maintained under specific-pathogen-free conditions, and provided with sterile food and water *ad libitum*. All experiments were performed in compliance with the regulations of the Animal Experimentation Committee of Taiho Pharmaceutical Co., Ltd.

2.4. Measurement of DPD activity

The enzyme assay, a modification of the method introduced by Naguib and colleagues [21], has been previously described in detail in Ref. [17]. Briefly, tumour cells were harvested by trypsinisation before reaching confluence (approximately 70% confluence), freeze-thawed in two volumes of homogenisation buffer, and centrifuged at 105 000×g for 1 h at 4°C. The supernatant fluid (cytosol) was collected as the enzyme source. The enzyme reaction mixture, which contained 10 mM potassium phosphate (pH 8.0), 0.5 mM ethyldiamine tetra acetic acid (EDTA), 0.5 mM 2-mercaptoethanol, 2 mM dithiothreitol, 5 mM $MgCl_2$, 20 μM [6–^{14}C]5-FU (56 mCi/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO, USA), 100 μM NADPH (Sigma Chemical Co., St. Louis, MO, USA), and 25 μl of cytosol in a final volume of 50 μl, was incubated at 37 °C for 30 min. DPD activity was determined by measuring the sum of the products formed from 5-FU, i.e., dihydrofluorouracil, α-fluoro-β-ureidopropionate and FBAL. After chemical hydrolysation and neutralisation using KOH and $HClO_4$, a 5 μl aliquot of the supernatant was applied to a thin-layer chromatography plate (silica gel 60 F254, Merck, Darmstadt, Germany), and developed with a mixture of ethanol and 1 M ammonium acetate (5:1, v/v), according to the method of Ikenaka and colleagues [22]. Each product was visualised and quantified using an imaging analyser BAS-2000 (Fujix, Tokyo, Japan).

2.5. Effects of CDHP on 5-FU cytotoxicity

Tumour cells (2000 cells/well) in the exponential growth phase were grown in 96-well plates. Twenty-four hours after plating, the cells were exposed to drugs (5-FU alone, CDHP alone, or various 5-FU/CDHP combinations) for 4 days. The cytotoxic effect of 5-FU was measured by dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay [23]. Results were expressed as follows: growth inhibition (%) = (1−(the mean absorbance detected in treated cells)/(the mean absorbance

detected in untreated control cells))×100. Based on the median effect equation according to Chou and Talalay [24], IC_{50} values were estimated from the regression line of log–logit plots of concentration versus growth inhibition rate. The 5-FU-cytotoxicity enhancement factor (F) was defined as 5-FU IC_{50} without CDHP divided by 5-FU IC_{50} in the presence of CDHP [16].

2.6. Determination of the maximal tolerable doses (MTD) of drugs

FT and FT/CDHP (at a molar ratio of 1:0.4) were dissolved in 0.5% (w/v) hydroxypropylmethylcellulose solution. Nine-week-old normal nude mice were divided into groups (7 mice/group) in order to equalise the mean body weight of mice in each group on day 0. The MTD of each treatment was determined by administering various doses in a 1.2-fold dilution series (FT, 150, 180 and 216 mg/kg/day; FT/CDHP, 6.9/2.0, 8.3/2.4 and 10.0/2.9 mg/kg/day) to mice once daily for 14 consecutive days. The mice were observed daily and weighed twice a week for 5 weeks.

2.7. Determination of 5-FU and CDHP serum levels

Ten-week-old nude mice were divided into two groups, and given a single oral administration of either FT (180 mg/kg) or FT/CDHP (8.3/2.4 mg/kg). Blood samples were drawn at 0.5, 1, 2, 4, 8, and 12 h after administration. The serum was immediately separated and stored at −20°C until analysis.

The analysis of 5-FU and CDHP concentrations has been previously described in detail in Ref. [25]. Briefly, 5-FU and CDHP were extracted with ethyl acetate from the residue obtained after dichloromethane extraction. Pentafluorobenzyl derivatives were then prepared. 5-FU and CDHP were analysed using a negative ion chemical ionisation-gas chromatograph/mass spectrometry. A stable isotope of each compound was used as an internal reference. The area under the curve (AUC) values were determined using the linear trapezoidal method for measured values.

2.8. Effects of CDHP on 5-FU antitumour activity

MIAPaCa-2 cells (2.1×10^6 cells) suspended in 0.1 ml of Hank's solution were transplanted subcutaneously (s.c.) into the right axilla of 7-week-old nude mice. Twenty days later (on day 0), the mice were randomly divided into treatment and control groups (7 mice/group) based on tumour volume (initial volume, 50–200 mm^3). The drugs for the treatment groups and the vehicle solution for the control group were orally administered once daily from day 1 to day 14. In this experiment, tumour volume was not measured after day 0 because MIAPaCa-2 tumours grew gradually in flat and irregular shapes. On day 15, the mice were weighed before being sacrificed with diethylether, and the tumours were removed and weighed. The criteria for assessing antitumour activity and toxicity were calculated as follows: tumour growth inhibition (%) = [1−(mean tumour weight of treated group)/(mean tumour weight of control group))×100; body weight change (g) = (body weight on day 15)−(body weight on day 0)−(tumour weight).

2.9. Statistics

The significance of differences between means was assessed using the Welch's *t*-test.

3. Results

3.1. Inhibitory effect of CDHP on 5-FU catabolism in human tumour cells

We determined the DPD activity of human tumour cells *in vitro*. As shown in Table 1, the basal DPD activities in both the MIAPaCa-2 and HuTu80 cells were comparatively high, while those in CAL27 cells were comparatively low. The inhibitory effect of CDHP on 5-FU degradation *in vitro* was investigated using cytosol samples obtained from these cells. Fig. 1 shows the ratio of 5-FU degradation in a DPD reaction mixture

Table 1
Effect of CDHP on 5-FU cytotoxicity in human cancer cells

Cell line	DPD activity (pmol/min/mg protein)	IC_{50} of 5-FU (μM)		Enhancement factor (F)[a]
		−CDHP	+CDHP (69 μM)	
MIAPaCa-2	101±58	7.3±0.2	3.1±0.2	2.4±0.1
HuTu80	153±11	4.1±0.4	2.6±0.5	1.6±0.1
CAL27	33±7	1.1±0.2	0.9±0.2	1.1±0.1

The growth inhibition rate was assessed by MTT test. The 5-FU concentration causing a 50% growth inhibition compared with the control (IC_{50}) was calculated from the regression line, respectively. The growth inhibition of CDHP (69 μM) alone was less than 6%. Each value is the mean ±Standard Deviation (S.D.) for three separate experiments.

[a] (IC_{50} of 5-FU alone)/(IC_{50} of 5-FU with CDHP).

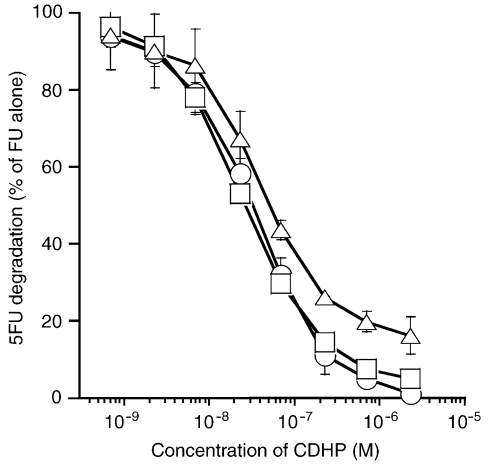

Fig. 1. Effect of CDHP on 5-FU degradation in cytosol extracted from human tumour cells *in vitro*. MIAPaCa-2 (circle), HuTu80 (square), and CAL27 (triangle) were used as the enzyme sources. The concentration of 5-FU was kept at 20 μM in each reaction. Each value is the mean±S.D. (bars) of three separate experiments.

treated with various concentrations of CDHP and compared with an untreated control mixture. In all three cell lines, 5-FU degradation was inhibited by CDHP in a concentration-dependent manner, with a maximum level of inhibition observed at 2.3 μM of CDHP (molar ratio to 5-FU was approximately 0.1).

3.2. CDHP enhancement of 5-FU cytotoxicity in human tumour cells

The MTT assay revealed that 69 μM (equal to 10 μg/ml) of CDHP increased the growth inhibitory effect of 5-FU by 2.4 times in the MIAPaCa-2 cells and 1.6 times in the HuTu80 cells (Table 1). CDHP alone had no effect on cell growth (growth inhibition, less than 6%). When both cells were treated with 5-FU alone (3.8 μM: equal to 0.5 μg/ml), the growth inhibition rate was moderate (25% in MIAPaCa-2 cells and 48% in HuTu80 cells; Fig. 2a and b). When both cells were treated with a combination of the fixed 5-FU concentration (3.8 μM) and various CDHP concentrations (0.0021–6.9 μM), the growth inhibition rate increased in a concentration-dependent manner and reached a plateau (an increase in growth inhibition of approximately 30% compared with 5-FU alone) at 0.69 μM of CDHP (molar ratio to 5-FU was approximately 0.2) (Fig. 2a and b). However, CDHP did not have any effect on 5-FU cytotoxicity in the CAL27 cells (Table 1 and Fig. 2c).

3.3. MTD of the drugs

Various doses of FT and FT/CDHP in a 1.2-fold dilution series were orally administered to normal mice once daily for 14 consecutive days. On day 35, five among the seven mice in the treatment group of FT, 216

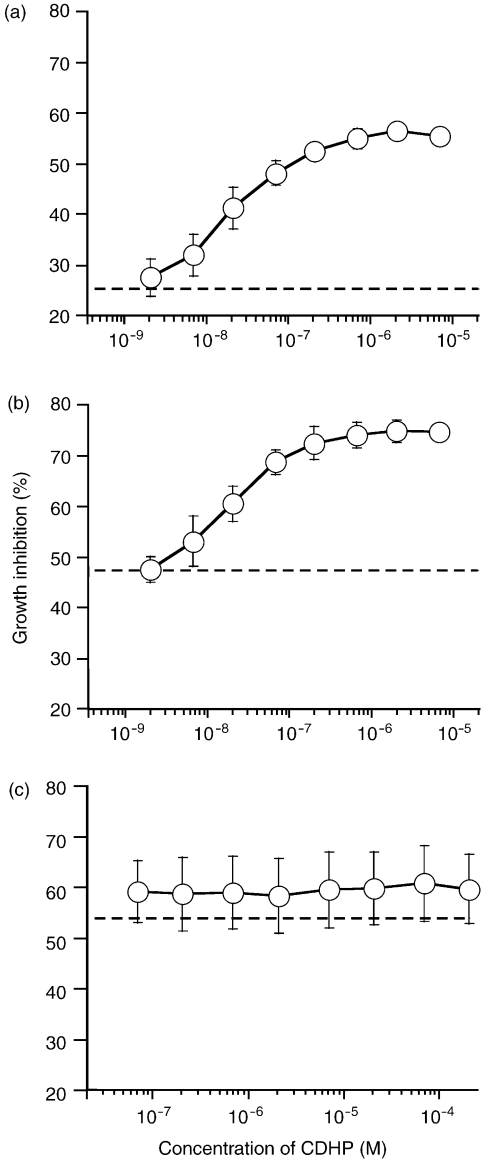

Fig. 2. Effect of CDHP on 5-FU cytotoxicity in human tumour cells. MIAPaCa-2 (a), HuTu80 (b), and CAL27 (c) were examined by the MTT assay. The concentration of 5-FU was kept at 3.8 μM in a and b, and at 0.77 μM in c. Each value is the mean±S.D. (bars) of three separate experiments. Dotted lines represent the growth inhibitory effect of 5-FU alone.

mg/kg/day and FT/CDHP, 10.0/2.9 mg/kg/day were dead, while the rest survived. Therefore, the MTD of each drug was determined as follows: FT, 180 mg/kg/day and FT/CDHP, 8.3/2.4 mg/kg/day. In this treatment, the maximal weight loss was recorded at day 15.

3.4. 5-FU and CDHP serum levels after oral administration

Fig. 3 shows the 5-FU and/or CDHP serum concentration time profiles after a single oral administration of FT and FT/CDHP at the MTD in nude mice. Serum 5-FU decreased in an almost parallel manner in

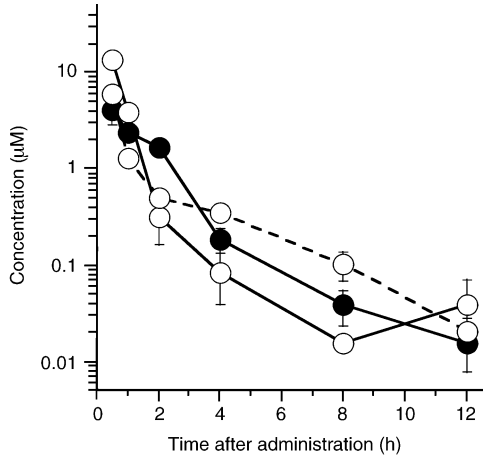

Fig. 3. Serum concentration versus time profiles of 5-FU (solid line) and CDHP (dotted line) in nude mice receiving oral administrations at the MTD of FT alone (180 mg/kg, closed circle) and FT/CDHP (8.3/2.4 mg/kg, open circle). Values are the means and S.D. (bars) of four or five mice.

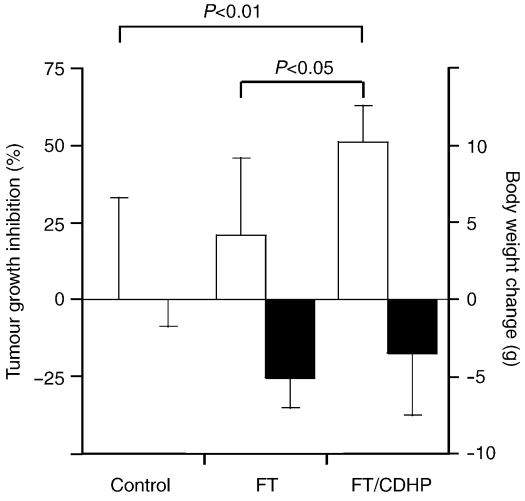

Fig. 4. Effect of CDHP on the antitumour activity of FT against MIAPaCa-2 after oral administration in tumour-bearing nude mice. Treatment groups were dosed at the MTD for 14 consecutive days (days 1–14): FT, 180 mg/kg/day; FT/CDHP, 8.3/2.4 mg/kg/day. The control group received only the administration of vehicle. Tumour and body weight was measured on day 15. The open bars represent tumour growth inhibition and the closed bars represent the change in body weight. Values are the means and S.D. (bars) of seven mice. Control tumour weight, 191±64 mg.

both groups until 12 h after administration. The AUC_{0-12} of 5-FU generated from both drug treatments were as follows: FT, 6.9±0.9 μM h; FT/CDHP, 10.6±0.5 μM h ($P<0.01$). The CDHP profile was almost parallel to the 5-FU profile after the FT/CDHP administration. The $AUC_{0-12\ h}$ of CDHP was 7.0±0.7 μM h, and the molar ratio of CDHP to 5-FU in the serum ranged from 0.3 to 6.7 for each time-point until 12 h after the administration.

3.5. CDHP enhancement of 5-FU antitumour activity in human tumour xenografts

To investigate the effect of CDHP on the antitumour activity of fluoropyrimidines in an animal model, FT and FT/CDHP were orally administered at the MTD for 14 consecutive days to nude mice bearing s.c. MIA PaCa-2 cells. Fig. 4 shows that each drug at the MTD induced an approximately equivalent body weight suppression, as expected. However, the mean tumour growth inhibition in the FT/CDHP group was approximately 30% higher than that in the FT group, and this difference was statistically significant ($P<0.05$). Tumour growth and host body weight were hardly affected by the oral administration of CDHP alone at a dose of 2.4 mg/kg/day for 14 consecutive days (data not shown).

4. Discussion

We previously evaluated uracil as a modulator that enhances the cytotoxicity of 5-FU using MIAPaCa-2 and HuTu80 cells [17]. Uracil enhanced 5-FU cytotoxicity in a concentration-dependent manner similar to that of CDHP. The maximum effect of uracil was almost the same as that of CDHP, although the uracil treatment required a 100-fold higher concentration than that of CDHP. Fischel and colleagues reported that 1 μM of EU enhanced 5-FU cytotoxicity 1.6–3.0-fold in five human cancer cell lines with high basal DPD activities (68–320 pmol/min/mg protein) [16]. These findings agree with our results for CDHP (Table 1) and uracil (1.5–2.0-fold in the two cell lines), suggesting that the potential of DPD inhibitors to enhance 5-FU cytotoxicity does not depend on the kind of inhibitor, but on the concentration of the inhibitor. In the case of CDHP, the growth inhibition rate reached a plateau at 0.69 μM of CDHP when the concentration of 5-FU was at 3.8 μM (Fig. 2a and b). In other words, the minimum effective concentration of CDHP was a molar ratio to 5-FU of 0.2. Generally, tumoral DPD activities in patients seem to be highly variable. McLeod and colleagues investigated DPD activities in 63 consecutive colorectal cancer patients [18]. The median tumoral DPD activity was 54.7 pmol/min/mg protein and ranged from 27.9 to 206.9 pmol/min/mg protein. They also measured DPD activities in five colorectal cancer cell lines, which ranged from 6.8 to 26.8 pmol/min/mg. Based on these results, CAL27 cells seemed to have similar results to the colorectal tumours having the lowest DPD activities, while MIAPaCa-2 and HuTu80 cells had relatively high DPD activities.

According to Cao and colleagues [26], treatment of rats with s.c. Ward colon carcinomas with a combination of FT and EU (FT/EU) at the MTD produced a higher antitumour activity than that of FT alone. In a pharmacokinetic study, plasma 5-FU concentrations ±EU after the administration of both drugs decreased

in parallel, and the AUC of 5-FU generated from FT/EU (27 μM h) was even less than that of FT alone (50 μM h). They hypothesised that the lack of 5-FU catabolites and/or the elevation of endogenous uracil and/or thymine throughout the entire body as a result of the EU treatment improved the antitumour efficacy and/or decreased the host toxicity. They also demonstrated that the 5-FU catabolites, 5-fluoro-5,6-dihydrouracil and FBAL, impaired the antitumour activity of 5-FU with EU treatment in the Ward carcinoma model [27,28]. However, these 5-FU catabolites did not have any affect on 5-FU cytotoxicity in Ward carcinoma cell cultures [27]. In this series of studies, the DPD activity in Ward carcinoma was not mentioned [26–29].

We suggest that the 5-FU antitumour activity was enhanced by CDHP through the inhibition of 5-FU degradation in the tumour, rather than due to a lack of 5-FU catabolites. Supporting evidence for this is provided by the fact that: (1) CDHP is a competitive and reversible inhibitor of DPD [4] and is unlikely to prevent the formation of 5-FU catabolites to the same degree as EU. In rats, the AUC and the half-life of FBAL in plasma were similar to those of 5-FU after the oral administration of S-1 [30]; (2) MIAPaCa-2 cell lines have a high basal DPD activity, and the results of *in vitro* and *in vivo* assays using these cells seem to be consistent and straight-forward.

Unexpectedly, the AUC of 5-FU generated from FT/CDHP (10.6±0.5 μM h) was significantly higher than that of FT alone (6.9±0.9 μM h). The increased AUC may explain a part of the increased activity of the FT/CDHP, however, we still consider the inhibition of tumoral DPD activity to be mainly responsible for this increased activity for the following reasons: It was only within the first hour that the 5-FU concentrations in the FT/CDHP group were higher than those in the FT group (at 12 h, 5-FU almost disappeared). Considering that 5-FU is a time-dependent drug, the retention of a prolonged concentration of 5-FU in blood may be more important for a tumour response to 5-FU than the AUC. We determined the MTD of both drugs in a series of 1.2-fold dose escalation and this is likely to provide more important information. Therefore it was thought that the systemic effect of both drugs were almost equal, even if the AUCs were different.

As mentioned above, the efficacy of CDHP is thought to be determined by its concentration ratio to 5-FU. In the MTT assay, the minimum effective concentration of CDHP was a molar ratio to 5-FU of 0.2. In the *in vivo* study, treatment with FT/CDHP at the MTD was more effective than treatment with FT alone, and the serum concentration of CDHP after the oral administration of FT/CDHP at the same dose in nude mice was above 0.2 (ranging from 0.3 to 6.7, until 12 h after administration). The DPD activity of xenografts collected after the oral administration of 2.9 mg/kg of CDHP to tumour-bearing nude mice decreased to 28% compared with that of untreated xenografts (data not shown). Clinically, a pharmacokinetic study after a single oral administration of S-1 in 12 patients resulted in a plasma AUC_{0-14} of 5-FU of 0.72±0.27 μg h/ml (5.6±2.1 μM h), while that of CDHP was 1.37±0.57 μg h/ml (9.4±3.9 μM h). The plasma half-life of CDHP (3.0±0.5 h) was also longer than that of 5-FU (1.9±0.4 h) [7]. Obviously, the concentration ratio of CDHP to 5-FU in the blood was significantly more than the minimum effective concentration.

In conclusion, we demonstrated that CDHP inhibits 5-FU degradation and enhances 5-FU cytotoxicity in human tumour cells *in vitro*, and that the oral administration of CDHP has the potential to enhance the antitumour activity of 5-FU against s.c. tumours in nude mice, using human tumour cells with a high basal DPD activity. S-1, an oral fluoropyrimidine that contains CDHP, is expected to be more effective in cancer patients with tumours that exhibit only a minimal response to 5-FU alone because of their high basal DPD activity.

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