Cancer Chemother Pharmacol (2004) 53: 296–304
DOI 10.1007/s00280-003-0725-6

ORIGINAL ARTICLE

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The efficacy of the combination therapy of 5-fluorouracil, cisplatin and leucovorin for hepatocellular carcinoma and its predictable factors

Received: 24 April 2003 / Accepted: 4 September 2003 / Published online: 20 December 2003
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Abstract *Purpose*: Several clinical trials in human neoplasms have demonstrated the effectiveness of combination therapy with 5-fluorouracil (FUra), cisplatin (CDDP), and leucovorin (LV). Thymidylate synthase (TS), the target enzyme of FUra, and dihydropyrimidine dehydrogenase (DPD), the rate-limiting catabolic enzyme of pyrimidines, have both been reported to be predictors of the response to FUra-based chemotherapies. Therefore, we aimed to clarify the effects of a combination of the three drugs against hepatoma cells and to determine the role of these two enzymes using in vitro models. *Methods*: Five human hepatoma cell lines (Hep3B, HepG2, HuH7, PLC/PRF/5 and Chang) were used. Cytotoxicity was determined after exposure to various concentrations and combinations of antitumor agents. The combination effects of FUra and CDDP in terms of synergy, additivity or antagonism were evaluated by median effect analysis. The mRNA levels of TS and DPD were measured by quantitative real-time PCR. Expression of TS and DPD proteins was also investigated. *Results*: LV alone did not show any cytotoxicity, although it enhanced the cytotoxicity of FUra, but not that of CDDP. Synergistic enhancement was observed with the combination of FUra and CDDP against all cells. The median combination index at fraction 0.5 was 0.554 (range 0.273–0.616). All cells expressed TS and DPD with median relative quantities of mRNA normalized to that of HuH7 cells of 1.04 (range 1.00–1.32) and 1.18 (range 0.88–1.55), respectively. A strong correlation was found between the IC_{50} of FUra and the mRNA level of DPD ($r = 0.912$, $P = 0.0295$). *Conclusions*: LV and CDDP enhanced the cytotoxicity of FUra, which provided a rationale for the regimen combining the three drugs for the treatment of hepatocellular carcinoma. DPD plays an important role in the sensitivity to FUra, and the DPD mRNA expression level may be used to predict the response to FUra-based chemotherapy for HCC.

Keywords Hepatocellular carcinoma · Chemotherapy · Thymidylate synthase · Dihydropyrimidine dehydrogenase

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in Southeast Asia and Japan [12, 16]. Screening of patients with liver cirrhosis as a population at high risk of developing HCC and the development of imaging modalities such as ultrasonography and computed tomography have improved the early detection of HCC [19]. Treatment modalities for HCC at an early stage with good hepatic function are nearly established. These include hepatic resection, percutaneous ethanol injection [9], transcatheter arterial embolization [24], microwave coagulation [38], and radiofrequency ablation [23]. Unfortunately, many patients cannot be treated with these established therapies and still suffer with advanced HCC. For these patients, chemotherapy is the only remaining treatment choice. Many chemotherapy regimens have been tried in the effort to control advanced HCC, although their effectiveness is unsatisfactory and the prognosis in these patients is extremely poor [11, 22, 25, 27, 28, 40]. Recently, intra-arterial infusion chemotherapy has been tried in advanced HCC, and the usefulness of chemotherapeutic regimens based on 5-fluorouracil (FUra) has been reported [1, 29, 35, 44, 49]. FUra has been used in the treatment of human neoplasms for many years. Attempts to increase its antitumor activity by adding other agents such as cisplatin (CDDP) [1, 29,

44], methotrexate [8, 46, 48], mitomycin C [39], leucovorin (LV) [14, 31, 43, 47], and interferon-α [35, 46] have been reported.

In in vitro studies, both CDDP and LV have been found to enhance the cytotoxicity of FUra against murine and human neoplasms by increasing the intracellular reduced folate pool [20, 26, 37, 41, 42, 50]. Reduced folate is considered to enhance the cytotoxicity of FUra by both forming and maintaining a stable ternary complex with the FUra metabolite 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) and the target enzyme thymidylate synthase (TS) [10, 37, 50]. Stabilization of the ternary complex increases the inhibition of DNA synthesis and therefore enhancing cytotoxicity. This mechanism of this antitumor effect is termed biochemical modulation, a process that alters the metabolism of tumor cells. Given this concept, several clinical trials have demonstrated the effectiveness of this combination therapy for the treatment of patients with a variety of cancers [1, 14, 29, 31, 43, 44, 49]. However, interaction of the three drugs in combination has not yet been proven in human hepatoma cells.

Recently, several studies have demonstrated that sensitivity to FUra is correlated with the activities of essential enzymes which determine the intratumoral FUra concentration [17, 20, 26, 32, 33, 48]. Dihydropyrimidine dehydrogenase (DPD), the initial and rate-limiting enzyme of pyrimidine catabolism, and TS, the target enzyme of FUra, have been investigated in patients with colorectal cancer and other neoplasms. Intratumoral expression of TS and DPD is correlated with the response and prognosis in patients treated with FUra-based chemotherapy [2, 3, 21, 36].

Thus, in this study, we aimed to clarify the mechanism involved in the interaction of CDDP and LV with FUra using in vitro models of the three-drug combination therapy for HCC. We also investigated the correlation between the cytotoxicity of this combination therapy and the expression levels of TS and DPD.

Materials and methods

Chemicals and drugs

FUra was purchased from Wako Pure Chemical Industries (Osaka, Japan). CDDP was purchased from Sigma (Saint Louis, Mo.). LV was purchased from Schircks Laboratories (Jona, Switzerland).

Cell lines and cell cultures

The human hepatoma cell lines Hep3B, HepG2, Huh7 and PLC/PRF/5 were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Chang was purchased from the American Type Culture Collection (Manassas, Va.). Cells were cultured in Earle's minimal essential medium (Gibco Invitrogen, Carlsbad, Calif.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma) in 75-cm^2 tissue culture flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, N.J.) at 37°C in an atmosphere containing 5% CO_2.

Table 1 Study design for the MTT cytotoxicity assay (*F* FUra, *P* CDDP, *L* LV). See Materials and methods for details

Plate no.	Regimen	FUra (μg/ml)	CDDP (μg/ml)	LV (μg/ml)
1	F	0.005–500	–	–
2–7	FP	0.005–500	0.0025–25	–
8	FL	0.005–500	–	10
9–14	FPL	0.005–500	0.0025–25	10
15	P	–	0.0025–25	–
16	PL	–	0.0025–25	10
17	L	–	–	0.001–100

Cytotoxic assays

Cells were seeded into 96-well microplates (Falcon) at 1.5×10^4 cells/ml (200 μl/well) and incubated for 24 h to allow cell attachment before measurement of cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All cell lines were in logarithmic growth at the time of assay. For each cell line, 17 plates were used per test (Table 1). Following a previous report that the maximal stabilization of the ternary complex of reduced folate with FdUMP and TS is obtained at a total LV concentration of about 10 μg/ml [34], we used LV at 10 μg/ml in combination assays with FUra and CDDP.

Fresh medium containing each drug alone or the drugs in combination was added to each well 24 h after seeding cells into the microplates. After addition of drugs, the microplates were incubated for 72 h and then cell viability was evaluated by the MTT colorimetric assay using a Cell Proliferation Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Spectrophotometric absorbance was measured using a microtiter plate reader (ImmunoMini NJ-2300; Nalge Nunc International KK, Tokyo, Japan). The absorbance of the formazan product was measured at a wavelength of 570 nm, and the reference wavelength was 660 nm. The surviving fraction of cells was expressed as a percentage in relation to the mean absorbance in control wells. Dose-response curves were generated from each plate, and the IC_{50} (the drug concentration causing a 50% reduction in the survival of treated cells), IC_{30} and IC_{70} values were obtained from each curve.

Data analysis

The combination effects of FUra and CDDP in terms of synergy, additivity or antagonism were evaluated by median effect analysis [6]. The experimental data were compared with a simulated curve corresponding to an additive effect using CalcuSyn software (Biosoft, Cambridge, UK). This comparison enabled calculation of the combination index (CI) which was plotted as a function of the fraction affected [6]. $CI = 1$ indicates additivity, $CI < 1$ indicates synergism, and $CI > 1$ indicates antagonism. The synergy between FUra and CDDP was analyzed using a fixed ratio (FUra:CDDP 20:1) [4, 5, 7], similar to the ratio used clinically [1, 29]. Dose combinations (FUra/CDDP) for synergy testing were (μg/ml): 0.05/0.0025, 0.5/0.025, 5/0.25, 50/2.5, 158.1/7.905, and 500/25.

Immunofluorescence staining

Rabbit polyclonal antibodies to recombinant human TS and DPD were gifts from Postmarketing Research Laboratory, Taiho Pharmaceutical Company (Tokushima, Japan). Monoclonal anti-β-actin (mouse IgG) was purchased from Sigma. Cells cultured in collagen-coated culture slides (Falcon) were washed twice in phosphate-buffered saline (PBS) for 5 min at room temperature and were fixed with pure ethanol. Cells on the slides were incubated with normal goat serum (Nichirei, Tokyo, Japan) for 30 min at room temperature. Cells were incubated with primary antibodies (diluted 1:100 with PBS) for 2 h at 37°C and stained with Alexa

Fluor 488 goat anti-rabbit antibody or Alexa Fluor 488 goat anti-mouse antibody (Molecular Probes, Eugene, Ore.). Finally, cells were mounted with a SlowFade Light Antifade Kit with DAPI (Molecular Probes), examined under a fluorescent microscope and analyzed using Qfluoro fluorescence imaging software version 3.0 (Leica Microsystems, Wetzlar, Germany).

SDS-PAGE and immunoblotting analysis

Cells were cultured in a six-well plate (Falcon) at 70% confluence. Following rinsing in PBS, lysis buffer (CelLytic M, Sigma) was added and incubation continued 15 min at room temperature. After centrifugation of the lysed cells, supernatants were collected. A volume of 30 μl lysate from each extract was separated by 5–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, Calif.) using the Bio-Rad electrotransfer system. Nonspecific binding was blocked by incubating with 5% non-fat dried milk (ECL blocking agent; Amersham Pharmacia Biotech, Little Chalfont, UK) in PBS/Tween 20 (0.05% v/v) overnight at 4°C. The membranes were incubated with the primary antibody diluted 1:1000 with PBS/Tween 20 (0.05% v/v) for 1 h at 37°C, and then probed with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish-peroxidase conjugated anti-mouse IgG diluted 1:1000 with PBS/Tween 20 (0.05% v/v) (Amersham). After the final wash, chemiluminescence was detected with an ECL-Plus kit (Amersham) and analyzed using an LAS1000 imaging analyzer (Fuji Photo Film, Tokyo, Japan). The bands were quantified densitometrically using NIH Image version 1.61 (National Institutes of Health, Bethesda, Md.). The quantities of TS and DPD protein were standardized to β-actin as an internal control and were normalized to HuH7 cells.

Quantitation of mRNA

Total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. During the protocol, contaminating small DNA was removed by DNaseI digestion using an RNase-free DNase set (Qiagen). The RNA was immediately reverse-transcribed using an Omniscript reverse transcriptase kit (Qiagen) with random hexamers (Applied Biosystems, Foster City, Calif.) and RNase inhibitor (Applied Biosystems). Quantitative real-time PCR amplification was performed using specific target double-labeled fluorogenic probes with an ABI Prism 7700 sequence detection system (Perkin Elmer/Applied Biosystems). Primers and probes were selected with Primer Express software version 1.0 (Perkin Elmer/Applied Biosystems). The sequences were as follows:

- TS (accession no. NM001071): forward primer (p763) GGCCTCGGTGTGCCTTT; reverse primer (p825) GATGTGCGCAATCATGTACGT; TaqMan probe (p781) AACATCGCCAGCTACGCCCTGC
- DPD (accession no. NM000110): forward primer (p2501) AAGTGGTCTTCAGTTTCTCCATAGTG; reverse primer (p2586) CTTCGATCACAGTGAAATCCTGAT; TaqMan probe (p2533) CCGTCCTCCAGGTATGCAGTGCCAT

The expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal standard. The primers and probe used to amplify and detect GAPDH were from Applied Biosystems (Pre-developed TaqMan Assay Reagents, endogenous control GAPDH). The reporter dye (6-carboxy-fluorescein for TS and DPD, VIC for GAPDH) was covalently attached to the 5′ end, and the quencher dye 6-carboxy-tetramethyl-rhodamine was incorporated into the 3′ end of the probe sequence.

Each PCR plate contained control cDNA samples isolated from HuH7 cells and serially diluted to allow correction for plate-to-plate variability. During the PCR, the reporter signal was normalized to an internal passive reference dye (ROX) to correct for any non-PCR-related fluctuations in fluorescence signal. The cycle number at which the normalized fluorescent signal exceeded the threshold (C_T) was inversely proportional to the log of the quantity of input cDNA (set at ten times the background fluorescence). By assigning relative mRNA values to wells containing the calibrator, the C_T for each well was converted to the quantity of mRNA. A graph was constructed with input cDNA on the *x*-axis and mRNA quantity on the *y*-axis. Relative mRNA expression was determined by dividing the slope of the regression line for the target of interest by the slope of the regression line for GAPDH. DPD mRNA and TS mRNA were both normalized to HuH7. The experiment was performed in triplicate on each mRNA.

Statistical analysis

Statistical analyses were performed with the standard statistical software StatView-J version 5.0 (SAS Institute, Cary, N.C.). One-way ANOVA and the Post-hoc test were applied to compare the absorbance of the control wells with that of wells with 10 μg/ml LV alone. A paired *t*-test was applied to compare the IC values without and with LV. Graphical analysis was performed with CalcuSyn (Biosoft). The strengths of the linear associations between pairs of variables were determined by the Pearson product moment correlation coefficient: $r \geq 0.7$, strong correlation; $r = \geq 0.4$ to < 0.7, weak correlation. Differences were regarded as significant for $P < 0.05$.

Results

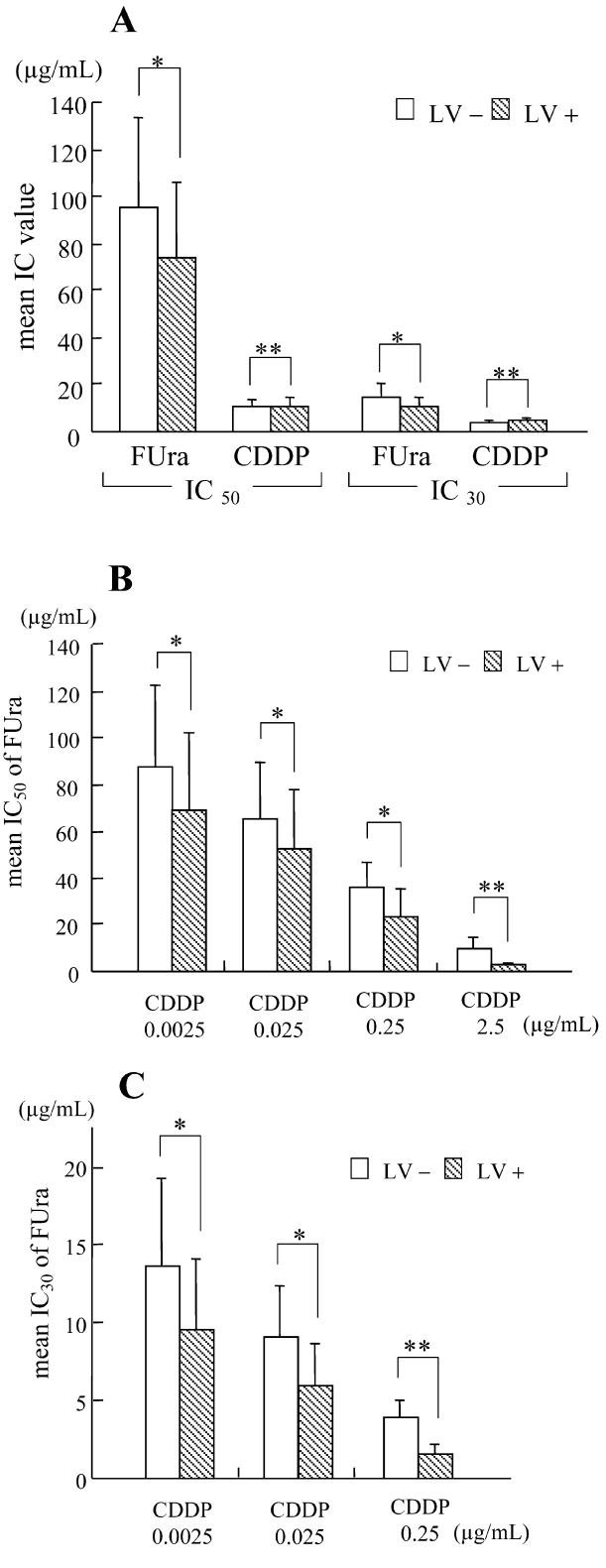
Cytotoxicity of LV alone and its effects on the cytotoxicity of FUra and CDDP

LV had no cytotoxicity at a concentration of 10 μg/ml. Survival in the presence of LV is shown in the second column of Table 2. One-way ANOVA and the Post-hoc test indicated no differences between the absorbances of the control wells and wells with 10 μg/ml LV alone in all cell lines. LV at 100 μg/ml had minimal cytotoxicity with median survival of 92.4% (range 91.6–95.0%, data not shown). In this study, LV did not affect cell growth in the concentration range 0.0001–10 μg/ml.

The cytotoxicities of FUra and CDDP without or with LV are shown in Table 2 and Fig. 1A. The IC_{50} and IC_{30} values of FUra in combination with LV at 10 μg/ml were significantly less than those of FUra alone (mean of five

Table 2 Effects of LV (10 μg/ml) alone and on the effects of FUra and CDDP against human hepatoma cell lines at 50% and 30% effect levels

Cell line	LV alone (cell survival, % control)	Inhibitory concentration (μg/ml)				
		Effect level (%)	FUra		CDDP	
			Alone	With LV	Alone	With LV
Hep3B	101.3 ± 2.12	50	227	192	3.75	3.84
		30	37.2	24.9	2.25	2.32
HepG2	97.5 ± 2.28	50	17.2	12.5	19.4	22.3
		30	1.96	1.26	7.57	8.70
HuH7	102.4 ± 2.48	50	65.4	36.1	7.00	7.37
		30	5.80	3.48	2.06	2.44
PLC/PRF/5	100.8 ± 3.11	50	95.6	85.6	6.27	6.18
		30	16.4	14.9	1.88	1.89
Chang	102.8 ± 1.86	50	62.8	43.1	14.8	14.8
		30	11.8	7.79	6.48	6.61



cell lines, $P < 0.05$, paired *t*-test; Fig. 1A), i.e. FUra with LV was significantly more cytotoxic than FUra alone. On the other hand, no differences were found between the IC_{50} and IC_{30} values of CDDP with LV at 10 μg/ml and those of CDDP alone (Fig. 1A). LV at 10 μg/ml decreased the IC_{50} of FUra in combination with CDDP at

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Fig. 1 A Mean IC_{50} and IC_{30} values of FUra and CDDP without or with LV (mean of five cell lines). **B, C** Mean IC_{50} and IC_{30} values of FUra together with CDDP at various concentrations without or with LV (*bars* SEM; $*P < 0.05$, **not significant)

0.0025, 0.025 and 0.25 μg/ml and the IC_{30} of FUra in combination with CDDP at 0.0025 and 0.025 μg/ml, i.e. LV enhanced the cytotoxicity of FUra in combination with CDDP (Fig. 1B, C). Thus, LV enhanced the cytotoxicity of FUra alone and in the presence of CDDP but did not affect the cytotoxicity of CDDP.

Combination effect of FUra and CDDP

The combination effects of FUra and CDDP in the five cell lines investigated are shown in Fig. 2. Each experimental point and the corresponding curves indicating the CI values were generated by median effect analysis using the CalcuSyn software. The lower the CI value, the greater the synergy, and conversely the higher the CI value, the greater the antagonism. Synergy was identified in all five cell lines for the medium to high fraction levels indicating high doses of the drugs. The median CI at fraction 0.5 was 0.554 (range 0.273–0.616).

Protein expression of TS and DPD

All cell lines expressed TS and DPD in the cytosol as shown by fluorescence staining (Fig. 3A–D). The levels of expression of TS and DPD were determined by Western blotting (Fig. 3E) and the median relative quantities of TS and DPD protein normalized to those of HuH7 cells were 1.19 (range 0.84–6.74) and 1.00 (range 0.52–1.04), respectively. No correlation was observed between TS and DPD protein expression levels and the IC of FUra (data not shown).

Quantitation of TS and DPD mRNA expression

The relative quantities of TS and DPD mRNA were calculated using GAPDH mRNA as internal standard and were normalized to HuH7 cells. The quantities of TS and DPD mRNA are shown in Table 3. The median relative quantities of TS and DPD mRNA were 1.04 (range 1.00–1.31) and 1.19 (range 0.88–1.52). The relationships between the quantities of TS and DPD mRNA and the IC_{50} values of FUra are shown in Fig. 4A, B. Cell lines with lower expression of TS and DPD tended to be more sensitive to FUra than those with higher expression. A strong and statistically significant correlation was observed between DPD mRNA expression level and the IC_{50} of FUra ($r = 0.912$, $P = 0.0295$).

The ratio of the IC value of FUra without LV to that with LV was determined. The ratio indicates the degree to which the cytotoxicity of FUra was enhanced by LV. The degree of correlation between the ratio and the

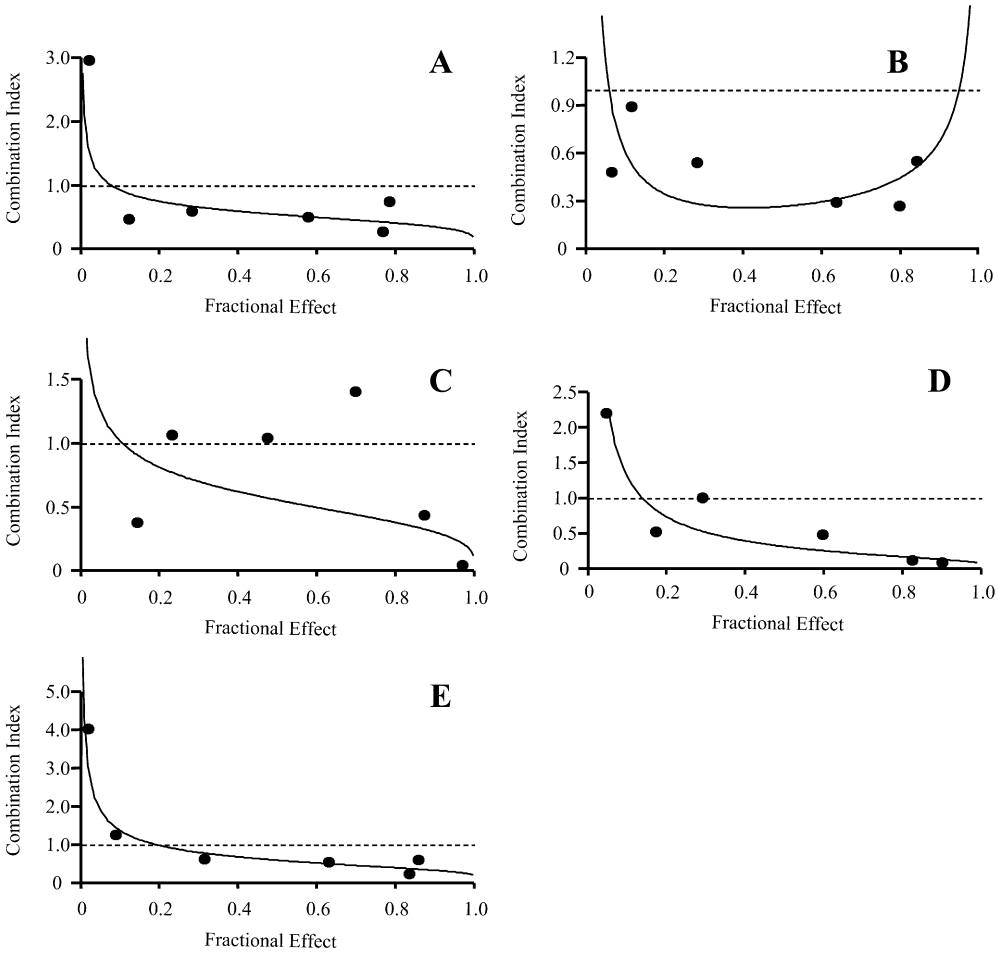

Fig. 2A–E Data resulting from the combination of FUra and CDDP in Chang (**A**), Hep3B (**B**), HepG2 (**C**), HuH7 (**D**), and PLC/PRF/5 (**E**) cells analyzed by the median effect analysis program (CalcuSyn, Biosoft, Cambridge, UK)

expression levels of TS and DPD mRNA was also determined (Fig. 4C, D). A negative correlation was found between the ratio and the mRNA expression levels of TS ($r = -0.832$, $P = 0.0913$).

Discussion

The cytotoxic mechanism of FUra is considered to involve the formation of a ternary complex with TS and with reduced folate. Therefore, the higher the expression of intratumoral TS, the lower the sensitivity to FUra [17, 32, 33, 48]. A previous study has shown that CDDP enhances the cytotoxicity of FUra by inhibiting methionine transport and increasing the ternary complex [41], in addition to its original effect (inhibition of DNA synthesis). Based on these rationales for combining FUra and CDDP, clinical efficacy of low-dose FUra and CDDP in patients with advanced HCC has been reported [1, 29, 44]. On the other hand, LV is successfully used as the exogenous folate in combination with FUra for the treatment of the patients with colorectal cancers and HCC [30, 31, 43, 47]. Thus, the formation of a ternary complex is important for FUra-based chemotherapy, and TS is one of the most crucial factors determining the sensitivity to FUra.

DPD is an initial and late-limiting catabolic enzyme of FUra. DPD catabolizes FUra to 5-fluoro-dehydrouracil in normal liver tissue. Both in vitro and in vivo experiments have shown that the intratumoral activity of DPD is related to the antitumoral effect of FUra [15, 17, 45]. Several clinical trials have demonstrated that tumoral DPD activity is correlated with the response and the prognosis of patients treated with FUra-based chemotherapy [18, 36]. DPD, as well as TS, is considered a key enzyme determining the sensitivity to FUra.

Chemotherapy is the only remaining treatment choice for patients with advanced HCC. Therefore prediction of chemosensitivity and identification of nonresponsive patients are extremely important. If resistance to FUra could be predicted, harmful and useless therapy could be avoided. Furthermore, these patients might be able to receive alternative chemotherapeutic regimens as primary therapy. These therapeutic tactics will contribute to the improvement of the response rate to chemotherapy of advanced HCC.

In the current study, all five hepatoma cell lines expressed TS and DPD. TS and DPD protein expression was observed in the cytosol by immunofluorescent staining. Both TS and DPD mRNA levels in cell lines were quantifiable using real-time PCR amplification. A strong and statistically significant correlation was found

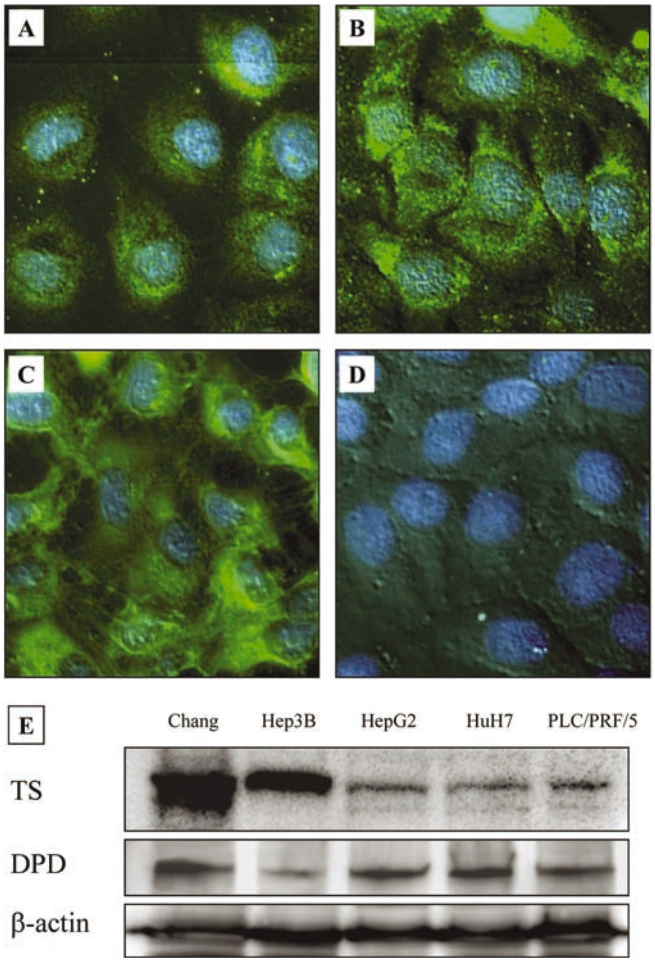

Fig. 3 A–D Typical immunofluorescence staining of TS and DPD (**A** TS in HuH7 cells, **B** DPD in Hep3B cells, **C** β-actin in HuH7 cells, **D** no primary antibody control in PLC/PRF/5 cells). **E** Western blot analysis of TS and DPD in Chang, Hep3B, HepG2, HuH7, PLC/PRF/5 cells. The bands were quantified densitometrically using NIH Image version 1.61

Table 3 Relative quantity of mRNA normalized to that of HuH7 cells. The data are presented as means ± SEM ($n = 3$)

	Cell line				
	Hep3B	HepG2	PLC/PRF/5	Chang	HuH7
TS	1.25 ± 0.094	1.01 ± 0.037	1.31 ± 0.075	1.04 ± 0.080	1
DPD	1.52 ± 0.122	0.88 ± 0.088	1.19 ± 0.068	1.24 ± 0.189	1

between DPD mRNA expression and the IC_{50} of FUra ($r = 0.912$, $P = 0.0295$). This result indicates that DPD, the rate-limiting enzyme in the catabolic pathway of pyrimidines, is one of the most important factors in the treatment of FUra-based chemotherapy for HCC. Conversely, TS mRNA expression levels showed a weak tendency to correlate with the IC_{50} of FUra. Previous studies have shown that low TS expression is associated with sensitivity to FUra [2, 3, 21, 36]. Thus we expected to observe a correlation between TS mRNA expression and the IC_{50}. This result may be attributable to the dose of FUra used. In in vitro models high doses of chemotherapeutic drugs tend to be used since the tumor cells are generally resistant to death induced by anticancer drugs. In this study, the IC_{50} values of FUra were in the range 17.2–237 μg/ml. In contrast, following clinical administration by continuous infusion the plasma level of FUra is about 0.5 μg/ml [13]. Under circumstances where there is excessive FUra in the tumor cells, the intracellular FUra concentration may be much more affected by its catalyzing enzyme than by the target enzyme.

No correlation was found between the protein expression levels of TS or DPD and the IC values of FUra (data not shown), although the protein expression levels might be more appropriate to reflect the enzymatic activity than mRNA expression. The results may be attributable to the technical problem in measuring the density of immunoblots and the insufficiency of repeated investigations since adequate volumes of specific antibodies were difficult to obtain. To examine the intratumoral expression levels of TS and DPD using clinical samples (such as needle biopsy specimens), mRNA quantitation may be more suitable, since the TaqMan method is able to quantify the mRNA with comparatively small amounts of samples.

The current study showed that LV had no cytotoxicity in the concentration range used. In contrast, LV at 10 μg/ml enhanced the cytotoxicity of FUra and FUra with CDDP. The degree of enhancement was influenced by the level of expression of TS mRNA. Cell lines expressing lower levels of TS mRNA showed increasing ratios of IC_{50} values without LV to those with LV ($r = -0.832$, $P = 0.0913$). This finding agrees with the role of LV as a biochemical modulator of FUra.

CDDP inhibited the growth of all five cell lines in a dose-dependent manner. Furthermore, CDDP enhanced the cytotoxicity of FUra, and this combination was synergistic at medium to high fraction levels. However, no correlation was observed between the mRNA expression level of TS/DPD and the extent of the synergy (data not shown). In addition, the cytotoxicity of the two-drug combination (FUra with CDDP) was increased with LV at 10 μg/ml. These results provide a rationale for the use of three-drug combination therapy in the treatment of HCC. Although previous studies have shown that CDDP enhances the cytotoxicity of FUra by inhibiting methionine transport and increasing the ternary complex, our results indicate other possible mechanisms for the synergistic effect of this combination. Kim et al. have reported that apoptosis is involved in the cell death induced by the combination of FUra and CDDP in gastric carcinoma [20]. Furthermore, both Fas and its receptor-independent mitochondrial pathway contribute to the cytotoxicity of this combination. Anticancer drugs are considered to induce apoptosis through a DNA damage pathway, although the enhanced cytotoxicity of the combination of FUra and CDDP may involve the death receptor pathway or the mitochondrial pathway.

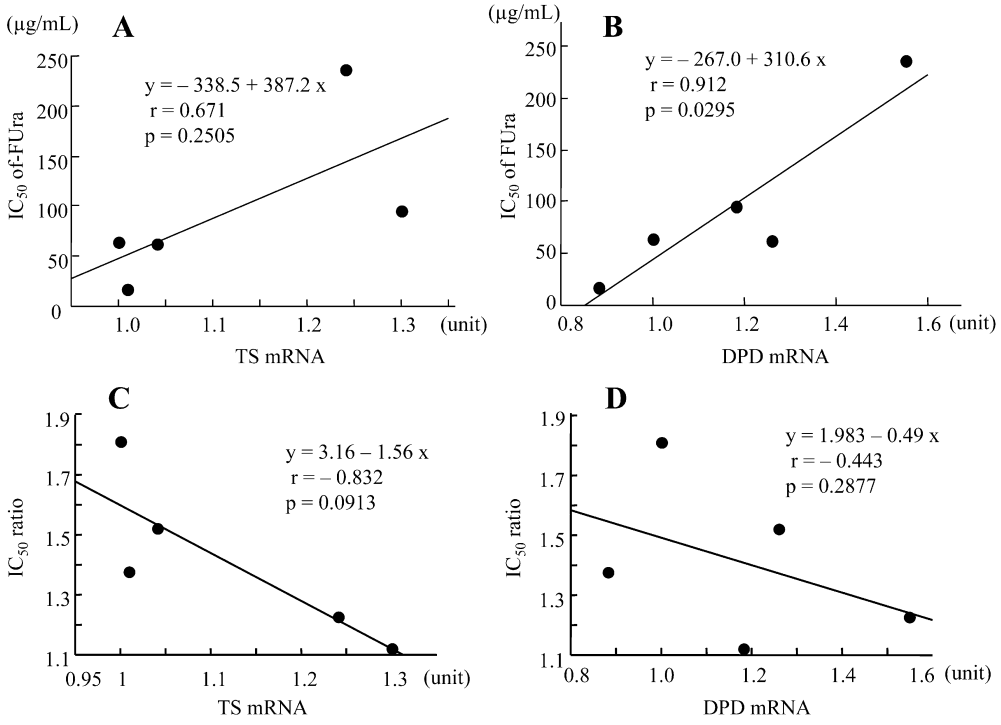

Fig. 4 A, B Relationship between relative mRNA expression of TS (**A**) and DPD (**B**) and the IC_{50} values of FUra in five cell lines. **C, D** Relationship between relative mRNA expression of TS (**C**) and DPD (**D**) and the ratio of the IC_{50} of FUra without LV to that with LV in five cell lines

In conclusion, the use of this in vitro model has clarified the roles of CDDP and LV in three-drug combination therapy for HCC as follows: (1) LV enhances the cytotoxicity of both FUra and the combination of FUra with CDDP; and (2) CDDP enhances the cytotoxicity of FUra in a synergistic manner not only by increasing the ternary complex but also by other possible mechanisms. In human hepatoma cell lines, TS and DPD play important roles in determining sensitivity to FUra. Furthermore, the mRNA expression levels of these key enzymes may be used to predict the response to FUra-based chemotherapy of patients with advanced HCC.

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