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In vitro enhancement of fluoropyrimidine-induced cytotoxicity by leucovorin in colorectal and gastric carcinoma cell lines but not in non-small-cell lung carcinoma cell lines*

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Summary. Leucovorin (LV) increases the cytotoxic effect of fluorouracil (FUra) and 5-fluoro-2′-deoxyuridine (FdUrd) by enhancing the formation of the fluorodeoxyuridine monophosphate (FdUMP) thymidylate synthase (TS) 5,10-methylenetetrahydrofolate (mTHF) ternary complex. To study the difference in the efficacy of this combination against different tumors, we compared the effect of LV (20 μM) on the cytotoxicity of FUra, FdUrd, and 5-fluorouridine (FUrd) in vitro against cell lines of five colorectal carcinomas (CC), five gastric carcinomas (GC), and four non-small-cell lung carcinomas (NSCLC) using the colony-forming assay. At the concentration used in the experiments, LV alone failed to inhibit colony formation in any of the cell lines tested. The NSCLC cell lines were more resistant to FdUrd than were the CC and GC lines. LV modulated the cytotoxicity of FdUrd in all five CC lines and in three of the five GC lines but failed to do so in any of the NSCLC lines. In addition, following 20 h treatment with 1 μM [^{3}H]-FdUrd, formation of the FdUMP/TS/mTHF ternary complex was enhanced by LV in the LV-sensitized CC and GC cell lines but not in the LV-refractory NSCLC lines. These in vitro data corresponded well to the results of clinical trials. Therefore, the colony-forming assay may be useful for the identification of the sensitivity of tumors according to phenotype.

Introduction

For several decades, 5-fluorouracil (FUra) has been widely used to treat various types of cancer. However, its efficacy has been relatively low [22]. To improve the clinical results, several biochemical modulators of FUra have been investigated. Using such approaches, recent randomized clinical trials have demonstrated a higher response rate for FUra-LV as compared with FUra alone in patients with metastatic colorectal carcinoma [13, 14, 33, 34]. Phase II trials against gastric [1], breast [12, 39], and head and neck carcinomas [42, 44] also suggest a higher response rate for this combination chemotherapy.

In contrast to LV-sensitized cancers such as colorectal carcinoma, patients with non-small-cell lung carcinoma did not respond to FUra-LV therapy in a phase II trial conducted in the National Cancer Center Hospital, Japan. No complete response or partial response was seen in 14 patients who had not received prior chemotherapy [30].

In recent investigations, inhibition of the growth of a number of human colorectal, gastric, and non-small-cell lung carcinoma (NSCLC) cell lines by fluorinated pyrimidines and leucovorin was studied using a tetrazolium dye-based growth-inhibition assay for rapid screening [31, 32, 40]. The results indicated little, if any, enhancement of the effects of FUra by leucovorin in any of the colorectal carcinoma cell lines tested, although the conclusions drawn by the authors indicated otherwise [31, 32]. Moreover, the degree of enhancement of fluorinated pyrimidine-induced cytotoxicity by LV was higher in NSCLC cell lines than in colorectal carcinoma cell lines [40]. These data do not seem to agree with the above-mentioned clinical results.

Using a soft-agar plating assay as an index of cytotoxicity Keyomarsi and Moran [19] studied a combination of fluoropyrimidines and LV and found that the activity of both FUra and FdUrd was substantially enhanced by LV in a murine leukemia cell line [19]. It can also be argued that tumor-cell kill is more relevant than growth inhibition to the therapeutic efficacy of a drug or a combination regimen. Therefore, in an effort to determine whether the in

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Abbreviations: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FUrd, 5-fluorouridine; FdUMP, 5-fluoro-2′-deoxyuridine monophosphate; FUTP, fluorouridine triphosphate; FBS, fetal bovine serum; LV, leucovorin; mTHF, 5,10-methylenetetrahydrofolate; NSCLC, non-small-cell lung carcinoma; SDS, sodium dodecyl sulfate; TS, thymidylate synthase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; IC_{50}, 50% inhibitory concentration; TCA, trichloroacetic acid

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Table 1. Characteristics of the cell lines used in the present study

Cancer	Cell line	Histology	Plating efficiency in soft agar (%)
Colorectal carcinoma	WiDr	Adenocarcinoma	13
	DLD-1	Adenocarcinoma	23
	LoVo	Adenocarcinoma	8
	COLO201	Adenocarcinoma	17
	COLO320DM	Adenocarcinoma	9
Gastric carcinoma	TMK-1	Adenocarcinoma	19
	KATO III	Signet-ring-cell carcinoma	10
	MKN-28	Adenocarcinoma	17
	MKN-45	Adenocarcinoma	21
	MKN-74	Adenocarcinoma	13
NSCLC	PC-7	Adenocarcinoma	9
	PC-9	Adenocarcinoma	20
	PC-13	Large-cell carcinoma	7
	PC-14	Adenocarcinoma	19

vitro cellular response of various types of cancer would correspond to the clinical results, in the present study we reexamined the effects of FUra, FdUrd, and fluorouridine (FUrd) in the presence of LV on the ability of cultured cells to proliferate.

FUra and its deoxyribonucleoside, FdUrd, can be metabolized to 5-fluoro-2′-deoxyribonucleoside-5′-monophosphate (FdUMP) [9]. FdUMP inhibits thymidylate synthase (TS) by forming a covalent complex with an enzyme that usually occurs in the presence of the folate cofactor for the TS reaction, 5,10-methylenetetrahydrofolate (mTHF) [5, 11, 37]. This ternary complex is kinetically stabilized by increased concentrations of mTHF and its polyglutamate derivatives [20, 23, 35], and this stabilization is currently thought to explain the enhancement of FUra- and FdUrd-induced tumor-cell growth inhibition obtained in the presence of reduced folates such as LV [folinic acid, (6-R,S)5-formyltetrahydrofolate] [6, 15, 16, 19, 20, 41].

We also examined the formation of the TS/FdUMP/mTHF ternary complex in the LV-sensitized and LV-refractory cell lines relative to the enhancement of the drug effects by the reduced folate. Our results suggest that the cytotoxic synergism achievable with this combination is substantially greater in colorectal and gastric carcinoma cell lines than in NSCLC cell lines.

Materials and methods

Drugs and chemicals. RPMI 1640 medium and phosphate buffered saline (PBS) were purchased from Nissui Pharmaceutical Co. (Tokyo). The active agents were obtained from the following sources: FUra, from Kyowa Hakko Co. Ltd. (Tokyo); FUrd, from Mitsui Pharmaceutical Inc.; l-leucovorin [folinic acid, the calcium salt of (6-S)5-formyltetrahydrofolate] from Lederle Co. (Tokyo); and [6-^{3}H]-FdUrd (sp. act., 17.9 Ci/mmol), from New England Nuclear. Unless otherwise stated, all other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cell lines and culture. WiDr, DLD-1, LoVo, COLO 210, and COLO 320DM colorectal carcinoma cell lines were obtained from the American Type Culture Collection (Rockville, Md.). TMK-1, KATO III, MKN-28, MKN-45, and MKN-74 gastric carcinoma cell lines were kindly donated by Dr. Y. Shimada, National Cancer Research Center (Hospital; Japan), and PC-7, PC-9, PC-13, and PC-14 NSCLC cell lines were kindly donated by Prof. Y. Hayata of Tokyo Medical College. The characteristics of the cell lines used are summarized in Table 1. The cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated dialyzed fetal bovine serum (FBS; Cell Culture Laboratories, Cleveland, Ohio), penicillin (100 IU/ml), and streptomycin (100 μg/ml) and were passaged twice a week. The cultures were not allowed to remain at confluence prior to passage.

All experiments were done using cells in the exponential phase of growth. New cultures were established from mycoplasma-free frozen stocks every 2 months, and cultures were tested for mycoplasma contamination (Hoechst stain kit for detection of mycoplasma in cell culture; Flow Laboratories, Inc., McLean, Va.) prior to their replacement by newly thawed stocks.

Colony-forming assay. The colony-forming assay was carried out in a double-layer soft-agar system as previously described [29], except that the same culture medium was used to maintain the culture in the lower layer. First, fluorinated pyrimidines and LV were added to the medium, followed by the cells at the desired densities and, finally, the agar solution. The mixture consisting of cells, drugs, and soft-agar solution in culture medium was plated on the lower layer in a well of a six-well multidish (Libro). After plating of the upper layer, the plates were incubated at 37° C in humidified air containing 5% CO_2 for 9–20 days until control colonies had formed. All assays were performed in triplicate and were repeated more than two times. Colonies exceeding 50 μm in diameter were counted using an automatic particle counter (CP-2000, Shiraimatsu Co., Tokyo). The biologically active l-isomer of leucovorin [8] was used for the combination experiments. All drugs were dissolved in distilled water and stored at –20° C; they were diluted with culture medium just prior to each experiment.

Detection of the FdUMP/TS/mTHF ternary complex by SDS-PAGE. The ternary complex FdUMP/TS/mTFH was detected according to the electrophoretic method described by Ullman et al. [41]. Cells were treated for 20 h with 1 μM [^{3}H]-FdUrd in culture medium and then washed with ice-cold PBS. The cells were lyzed by freezing and thawing using dry ice/ethanol and a temperature of 37° C, respectively. The cellular lysate was centrifuged at 12,500 *g* for 15 min and the supernatant was removed. The supernatant was precipitated with 5% ice-cold trichloroacetic acid (TCA) and then centrifuged at 12,500 *g* for 5 min. The TCA remaining in the precipitate was removed by washing with ethanol/ethyl ether (1 : 1, v/v) and dissolved with a small volume of sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After measurement of protein content by Bradford's method [10], an equivalent amount of proteins were separated by SDS-PAGE according to Laemmli's method [21]. Following electrophoresis, gels were fixed with 30% acetic acid in methanol for 30 min and treated with E^3 HANCE (New England Nuclear) and distilled water. After drying, the gels were placed in contact with X-ray film and were exposed at –70° C for 3 weeks.

Results

Dose response for the LV-sensitizing effect on fluorinated-pyrimidine-induced cytotoxicity

Figure 1 shows the dose-response curve generated for the effect of LV on the cytotoxicity of FdUrd in the LoVo colon-adenocarcinoma cell line. A sensitizing effect of LV on FdUrd cytotoxicity was observed at an LV concentration of approximately 0.003 μM, and this effect reached a plateau at about 0.3 μM. At the concentration used in this experiment, LV alone failed to inhibit colony formation in

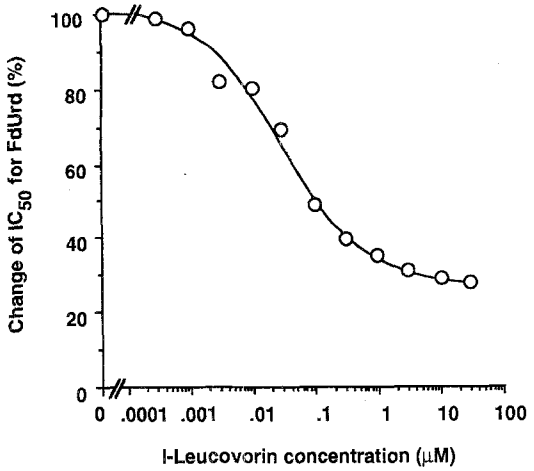

Fig. 1. Cytotoxicity of FdUrd in LoVo colorectal-carcinoma cell line exposed to LV. LoVo cells were exposed to the indicated concentrations of LV and to varying concentrations of FdUrd for 10 days in soft agar. Results represent the IC_{50} values obtained using FdUrd with in the presence of LV against those obtained in the absence of LV as determined from dose-response curves based on the mean values of triplicate determinations

Table 2. Effect of LV on the cytotoxicity of FUra in human carcinoma cell lines

Cancer	Cell line	IC_{50} of FUra (μM)		Ratio[a]
		-LV	+LV	
Colorectal carcinoma	WiDr	1.68±0.29	1.13±0.70	1.46
	DLD-1	4.77±1.30	5.75±1.62	0.83
	LoVo	3.30±0.42	2.14±0.03	1.54
	COLO201	3.72±1.28	1.20±0.48*	3.09
	COLO320DM	0.94±0.23	0.68±0.01	1.38
Gastric carcinoma	TMK-1	0.49±0.16	0.21±0.03*	2.40
	KATO III	2.24±0.04	0.50±0.17***	4.52
	MKN 28	2.04±0.02	1.49±0.04	1.37
	MKN 45	0.24±0.02	0.22±0.01	1.06
	MKN 74	3.42±1.43	3.23±1.35	1.06
NSCLC	PC-7	6.25±2.78	6.23±3.56	1.00
	PC-9	1.01±0.42	0.67±0.35	1.51
	PC-13	2.78±1.78	2.50±1.96	1.11
	PC-14	3.50±0.89	2.40±1.25	1.46

Data represent mean values ± SD for triplicate assays
[a] Ratio = IC_{50} of FUra in the absence of LV/IC_{50} of FUra in the presence of LV
* P <0.05, ** P <0.0001 vs the IC_{50} value obtained using FUra in the absence of LV (Student's unpaired t-test)

Table 3. Effect of LV on the cytotoxicity of FdUrd in human carcinoma cell lines

Cancer	Cell line	IC_{50} of FdUrd (μM)		Ratio[a]
		-LV	+LV	
Colorectal carcinoma	WiDr	0.0125±0.005	0.0040±0.0015*	3.09
	DLD-1	0.1295±0.052	0.0370±0.0113*	3.50
	LoVo	0.0344±0.014	0.0089±0.0033**	3.85
	COLO201	0.1160±0.016	0.0215±0.0092**	5.40
	COLO320DM	0.0805±0.009	0.0175±0.0064**	4.60
Gastric carcinoma	TMK-1	0.0860±0.0320	0.0230±0.0106*	2.40
	KATO III	0.5000±0.2100	0.0580±0.0198*	4.52
	MKN 28	0.0880±0.0260	0.0300±0.0156*	2.93
	MKN 45	0.0285±0.0097	0.0138±0.0069	2.07
	MKN 74	0.3200±0.1267	0.4200±0.0980	0.76
NSCLC	PC-7	4.30±2.62	4.60±1.58	0.93
	PC-9	0.73±0.21	0.40±0.26	1.83
	PC-13	1.30±0.09	0.93±0.31	1.40
	PC-14	2.02±1.36	1.70±0.87	1.19

Data represent mean values ± SD for triplicate assays
[a] Ratio = IC_{50} of FdUrd in the absence of LV/IC_{50} of FdUrd in the presence of LV
* P <0.05, ** P <0.0001 vs the IC_{50} value obtained using FdUrd in the absence of LV (Student's unpaired t-test)

Table 4. Effect of LV on the cytotoxicity of FUrd in human carcinoma cell lines

Cancer	Cell line	IC_{50} of FUrd (μM)		Ratio[a]
		-LV	+LV	
Colorectal carcinoma	WiDr	0.750	0.640	1.17
	DLD-1	1.510	1.290	1.17
	LoVo	0.065	0.062	1.05
	COLO201	1.380	0.810	1.70
	COLO320DM	0.780	0.620	1.26
Gastric carcinoma	TMK-1	0.024	0.024	0.98
	KATO III	0.054	0.022	2.45
	MKN 28	0.021	0.020	1.03
	MKN 45	0.006	0.005	1.13
	MKN 74	0.061	0.053	1.15
NSCLC	PC-7	0.029	0.016	1.81
	PC-9	0.052	0.030	1.73
	PC-13	0.118	0.105	1.12
	PC-14	2.400	2.390	1.00

Data represent mean values for duplicate assays
[a] Ratio = IC_{50} of FUrd in the absence of LV/IC_{50} of FUrd in the presence of LV

any of the cell lines tested. On the basis of these data and previously reported findings [16, 31], 20 μM LV was selected as the concentration at which the effects of LV modulation were maximal.

Cytotoxicity of fluorinated pyrimidines in human cell lines from different organs

In the colony-forming assay, cellular sensitivity to FUra (Table 2) was slightly higher in gastric carcinoma cell lines than in colorectal or NSCLC cell lines (mean IC_{50} values: 1.68±1.15, 2.88±1.41, and 3.39±2.18 μM, respectively). Cellular sensitivity to FdUrd (Table 3) was significantly higher in colorectal (mean IC_{50}, 0.075±0.038 μM) and gastric carcinoma cell lines (mean IC_{50}, 0.205±0.189 μM) cell lines than in NSCLC cell lines (mean IC_{50}, 2.09±1.57 μM; P <0.05). Cellular sensitivity to FUrd (Table 4) was highest in gastric carcinoma cell lines (mean IC_{50}, 0.13±0.23 μM), followed by NSCLC (mean IC_{50}, 0.6±1.2 μM) and colorectal carcinoma (mean IC_{50}, 0.91±0.58 μM) cell lines. The IC_{50} values for FUrd differed significantly between colorectal carcinoma and gastric carcinoma (P <0.01). Thus, responses to the three fluorinated pyrimidines differed in the different tumor cell

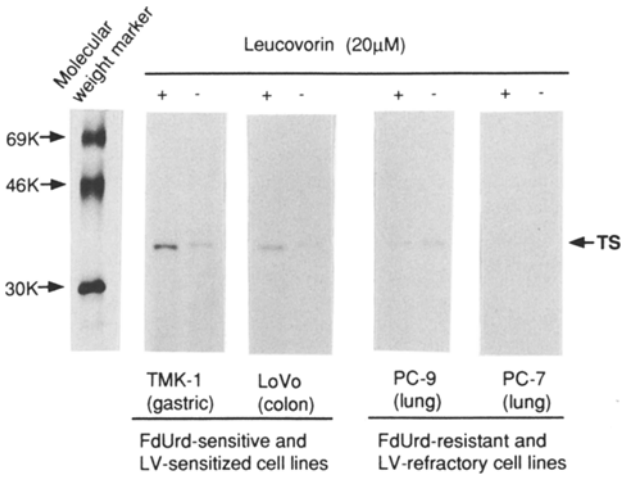

Fig. 2. [^{3}H]-FdUMP/TS/mTHF ternary complex formation in LV-sensitized and LV-refractory cell lines. After 20 h treatment with 1 μM [^{3}H]-FdUrd in the presence (+) and absence (–) of LV, cells were lyzed by freezing and thawing. The soluble cellular proteins were analyzed by SDS-PAGE and fluorography. The mobility of the molecular weight markers is indicated by *arrows*

lines. Colorectal carcinoma cell lines were more sensitive to FdUrd than to FUrd or FUra. However, FUrd was the most potent agent in gastric and NSCLC carcinoma cell lines, although the gastric carcinoma cell lines were also sensitive to FdUrd. The NSCLC cell lines were more resistant to FdUrd than were the colorectal or gastric carcinoma cell lines.

Effect of LV on the cytotoxicity of fluorinated pyrimidines in cultured cell lines from different organs

LV sensitized cells to FUra in only a few colorectal (COLO201) and gastric (TMK-1, KATO III) carcinoma cell lines but failed to sensitize any of the NSCLC cell lines (Table 2). On the other hand, LV enhanced FdUrd cytotoxicity in all of the colorectal carcinoma cell lines and in some of the gastric carcinoma cell lines failed to do so in the NSCLC cell lines (Table 3). It is possible that higher concentrations of LV are required for the modulation of FdUrd cytotoxicity in NSCLC cell lines. Therefore, we assayed the modulatory effect of 200 μM LV on FdUrd cytotoxicity in the LV-refractory NSCLC cell lines. However, the use of a 10-fold higher concentration of LV did not significantly change the effect of an IC_{50} dose of FdUrd on the NSCLC cell lines (data not shown). In experiments using FUrd (Table 4), which is thought to be mainly metabolized to FUTP, resulting in RNA damage [3], LV modulated the cytotoxicity of FUrd in only one gastric carcinoma cell line (KATO III). These data demonstrate that the cellular response to LV modulation differs in different types of tumors.

Covalent ternary-complex formation involving TS binding

We attempted to determine the formation of the ternary complex FdUMP/TS/mTHF in the LV-sensitized cell lines TMK-1 and LoVo and in the LV-refractory cell lines PC-7 and PC-9. Figure 2 illustrates the binding of [^{3}H]-FdUMP to TS, which yields the ternary complex FdUMP/TS/mTHF. The results demonstrate LV-enhanced ternary-complex formation in LV-sensitized cell lines but not in LV-refractory lines. When cells were treated with FdUrd alone, the amount of ternary-complex formation noted in FdUrd-sensitive colorectal and gastric carcinoma cell lines was also greater than that observed in FdUrd-resistant NSCLC cell lines. These data correspond to the results of the colony-forming assay.

Discussion

Numerous studies on the effects of LV on fluorinated-pyrimidine-induced cytotoxicity [19, 20, 24, 25, 41] have demonstrated an augmentation of the growth-inhibitory activity of FdUrd against mouse or human leukemic cells in culture. Evans et al. [15, 16] also reported that a 2- to 3-fold increase in the growth-inhibitory potency of FUra and FdUrd was induced by LV in murine sarcoma 180 and human Hep-2 carcinoma cells in vitro. Enhancement of the therapeutic effects of FUra by LV has been clinically documented in gastrointestinal [1, 13, 14, 33, 34], head and neck [42, 44], and breast cancer [12, 39]. However, in phase II trials, patients with NSCLC [30] and hepatocellular carcinoma [45] have not responded to this combination. Therefore, identification of the responding and nonresponding tumor phenotypes would contribute substantially to our understanding of the limitations of FUra/LV chemotherapy.

In the present study, LV enhanced FUra-induced cytotoxicity in some gastrointestinal carcinoma cell lines but failed to do so in any of the NSCLC cell lines tested. Following continuous exposure of cells to drugs, marked cytotoxic synergism between LV and FdUrd was also observed in five human colorectal carcinoma cell lines and three gastric carcinoma cell lines but not in any of the NSCLC cell lines. We also demonstrated enhancement of the formation of covalent ternary complex in the LV-sensitized colorectal and gastric cell lines but not in the LV-refractory NSCLC cell lines. This synergistic interaction was markedly more similar to the clinical results than was that previously found in gastrointestinal carcinoma cell lines by a rapid assay of cell growth using tetrazolium dye staining [31, 32]. In those studies, a 2- to 3-fold shift in the growth-inhibitory activity of FdUrd was observed in only 4 of the 11 colorectal carcinoma cell lines investigated and in only 1 of the 4 gastric carcinoma cell lines tested. Moreover, the growth inhibition by FdUrd in the remaining cell lines either was affected to a smaller extent or remained unaffected by the addition of LV. In addition, these authors reported that the modulation by LV of FUra and FdUrd cytotoxicity in lung-carcinoma cell lines was greater than that previously found in colorectal and gastric cell lines by the same group [40]. The reason for the discrepancy in these results remains unclear.

However, Keyomarsi and Moran [19] have reported that experiments based on the growth inhibition caused by combinations of fluoropyrimidines and LV clearly under-

estimate this synergism in murine L1210 leukemia cells as compared with the results of colony-forming assays. Therefore, the evaluation index for this synergistic effect might yield differing results. Therefore, we think that the colony-forming assay may be useful for the identification of the in vitro sensitivity of tumors according to phenotype.

FUra inhibits cell growth by at least two mechanisms, including the incorporation of FUra as FUTP into RNA and the inhibition of TS [2]. LV increases the cytotoxicity of the fluorinated pyrimidines by stabilizing the FdUMP/TS complex, thereby inhibiting dTMP synthesis in the DNA synthetic pathway, but it has no effect on the nuclear processing of RNA [11, 15, 37]. In the present study and previous investigations [19, 31], the enhancement by LV of the cytotoxicity of FdUrd was greater than its enhancement of FUra cytotoxicity. FdUrd is rapidly metabolized to FdUMP and acts principally to inhibit TS [18]. Therefore, it would be reasonable to expect that LV would have a greater modulatory effect on the growth-inhibitory activity of FdUrd than on that of FUra. Indeed, the cytotoxicity of FUrd, which manifests mainly as RNA damage, was enhanced in only one of the cell lines tested. Our data demonstrate that LV-sensitized colorectal and gastric carcinomas show higher sensitivity to FdUrd alone as compared with NSCLC cell lines. In addition, colorectal carcinoma cell lines are more resistant to FUrd than are gastric carcinoma cell lines. These data suggest that TS inhibition by fluoropyrimidines may be more effective in colorectal carcinoma cell lines and may have only a minor effect on NSCLC cell lines.

Numerous factors are undoubtedly important determinants of FdUrd cytotoxicity in mammalian cells, including differences in pools of mTHF and its polyglutamates [23, 26, 36], in the net accumulation of FdUrd [38], in the activation of FdUrd by thymidine kinase [27], in FdUMP hydrolysis [17], and in either the levels of TS [43] or the kinetics of enzyme binding with FdUMP [4, 6] and dUMP pools [28]. Any of these factors may differ between FdUrd/LV (FdUrd)-sensitive colorectal carcinoma cell lines and FdUrd/LV (FdUrd)-refractory NSCLC cell lines. We are currently investigating further the mechanism underlying the response of different tumor types to fluorinated pyrimidine-LV combinations.

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