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Increased Sensitivity to Long-term 5-Fluorouracil Exposure of Human Colon Cancer HT-29 Cells Resistant to Short-term Exposure

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A 5-fluorouracil (5-FU)-resistant subline of human colon cancer HT-29 cells was developed by repeated 1-h exposure *in vitro* to 5-FU. This subline (HT-29/5-FU/S) had 8-fold resistance to 5-FU in a 1-h exposure assay. However, it had rather increased sensitivity to 5-FU when assayed after a continuous 96-h exposure to it. Significantly less 5-fluorouridine-5′-triphosphate was produced in the resistant cells, leading to a lower level of 5-FU incorporation into the cellular RNA. The reduced activity of orotate phosphoribosyltransferase might explain these results. In contrast, the HT-29/5-FU/S cells were more sensitive to the inhibition of *in situ* thymidylate synthase (TS) by 5-FU than were the parent cells. The lower *in situ* TS activity may have made HT-29/5-FU/S cells more sensitive to TS inhibition by 5-FU as compared with the parent cells. The fact that HT-29/5-FU/S was more resistant to short-term 5-FU exposure but more sensitive to long-term exposure than the parent line confirmed the existence of different modes of action of 5-FU, depending on the exposure time.

Key words: HT-29 human colon cancer — 5-Fluorouracil-resistance — Dual actions of 5-FU — Exposure time

5-FU is a unique anticancer agent with two major pharmaco-biochemical actions: of its active metabolites, FUTP is fraudulently incorporated into RNA and impairs the multiple functions of RNA, while FdUMP blocks the catalytic activity of TS by forming a ternary covalent complex with its co-substrate, 5, 10-CH_2-FH_4, which inhibits DNA synthesis. Thus 5-FU potentially exerts both RNA- and DNA-directed actions against target cells.

One of the factors determining which action 5-FU preferentially exerts on the target cells seems to be their innate biochemical characteristics of 5-FU metabolism. If the resultant intracellular concentration of FUTP or FdUMP is lower than the effective level, 5-FU may exert only one of its dual actions. However, it seems to exert dual actions against the majority of cancer cells, although the efficacy of such actions varies. Another determinant is the length of the exposure time to 5-FU. We have previously developed a method for the kinetic analysis of the cell-killing action of anticancer agents which can classify them into cell cycle phase-nonspecific (type I) drugs and cell cycle phase-specific (type II) drugs.[1, 2] According to this analysis, FUrd and FdUrd showed typical kinetic profiles for type I and II drugs, respectively. 5-FU had a FUrd-like kinetic profile for cell killing action when cells were exposed for a short time and a FdUrd-like profile when they were continuously exposed for a long time.[3, 4] These results suggested that 5-FU exerts RNA-directed action under short exposure conditions and DNA-directed action under long, continuous exposure conditions. The former requires a high 5-FU concentration, while for the latter, a low concentration is sufficient.

In view of the dual actions of 5-FU, which depend on the exposure time, it might be possible to develop a subline which acquires resistance to short/long exposure to 5-FU, but does not show cross-resistance to long/short exposure. Based on this idea, we have established some 5-FU-resistant sublines of human gastro-intestinal cancer cell lines by repeated long-term exposure to 5-FU. However, we failed to obtain a subline of the type we had expected. In the present study, we tried repeated 1-h exposure of human colon cancer HT-29 cells to 5-FU, and established a subline named HT-29/5-FU/S. This subline is resistant to short-term exposure to 5-FU, exhibited no cross-resistance to long-term exposure to 5-FU, and had rather higher sensitivity than its parent cells.

MATERIALS AND METHODS

Chemicals [^{3}H]5-FU (655 GBq/mmol) was purchased from NEN Life Science Products (Wilmington, DE); [^{3}H]FdUMP (481 GBq/mmol), [^{3}H]FUrd (740 GBq/mmol) and [^{3}H]dUrd (740 GBq/mmol) were from Moravek Biochemicals (Brea, CA); Sulforhodamine B was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

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[2] The abbreviations used are: 5-FU, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluoro-2′-deoxyuridine; FUTP, 5-fluorouridine-5′-triphosphate; FdUMP, 5-fluoro-2′-deoxyuridylate; 5,10-CH_2-FH_4, 5,10-methylenetetrahydrofolate; dUrd, 5′-deoxyuridine; OPRT, orotate phosphoribosyltransferase; PRPP, 5-phosphoribosyl-1-pyrophosphate; TS, thymidylate synthase.

Cell lines HT-29, a human colon cancer cell line, was obtained from American Type Culture Collection (Rockville, MD) through Dainippon Pharmaceutical Co., Tokyo. A 5-FU-resistant subline, HT-29/5-FU/S, was established by repeated 1-h exposure of HT-29 cells to increasing concentrations of 5-FU (0.5 to 10 m*M*).

Drug sensitivity assays The sensitivity of cells *in vitro* was determined by means of the sulforhodamine B assay[5] with a minor modification. Briefly, cells were cultured with the drug for 96 h (continuous exposure), or for 1 h with the drug followed by 96 h without the drug (1-h exposure). After culture, the adherent cells were fixed in 10% trichloroacetic acid, washed and dried. Sulforhodamine B (0.4% wt/vol. in 1% acetic acid) was added to stain the cells, and unbound sulforhodamine B was removed by washing with 1% acetic acid. The cells were air-dried, and the bound sulforhodamine B was solubilized in 10 m*M* unbuffered Tris base (pH 10.5). The optimal density was read on an automated spectrophotometric plate reader at a wavelength of 525 nm.

5-FU incorporation into RNA Cells at the logarithmic growth phase were incubated with 300 μM [^{3}H]5-FU for 1 h at 37°C. Four million cells harvested with trypsin were washed twice with phosphate-buffered saline, then treated twice with 2.5 ml of 10% (wt/vol.) perchloric acid, and RNA was extracted by shaking the precipitated residue in 5 ml of 1 *N* perchloric acid for 18 h at 4°C. After centrifugation, the supernatant was transferred to a tube, to which another 5 ml of 1 *N* perchloric acid washing the residue was added. The radioactivity levels in aliquots of the RNA extract were measured using a Beckman model LS 7500 scintillation counter (Fullerton, CA) and the RNA content was estimated by measuring the UV absorbance at 260 nm.

Assay for intracellular active metabolites of 5-FU Approximately 10^6 cells were incubated at 37°C with 300 μM [^{3}H]5-FU for 1 h to measure FUTP level or with 10 μM [^{3}H]5-FU for 5 h to measure FdUMP level. The cells were washed twice with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, disrupted in 1 ml of 1% NP-40 in a lysis buffer (10 m*M* Tris-HCl, 5 m*M* EDTA, 50 m*M* NaF and 50 m*M* NaCl; pH 7.5), and centrifuged at 75,000*g* for 20 min at 4°C in a Beckman model TL-100 ultracentrifuge (Palo Alto, CA). Cold 6 *M* trichloroacetic acid was added to 500 μl of the supernatant to produce a final concentration of 0.6 *M* and the mixture was centrifuged. The supernatant was added to 2 vol. of cold Freon containing 17.7% tri-*n*-octylamine, and the mixture was vortexed and centrifuged. The last procedure was repeated until the pH of the aqueous phase became 7, when this phase was separated and filtered through Ultrafree C3HV. The filtrate was analyzed using a Shimadzu model LC-10A HPLC (Tokyo) equipped with an Inertsil ODS column (250×6.0 mm, 4-mm particle size; GL Sciences, Tokyo). The elution proceeded as described previously.[6] The radioactivity of fractions containing FUTP or FdUMP was measured.

Assay of 5-FU-anabolizing enzymes The activities of uridine/thymidine phosphorylase and kinase and OPRT were measured as described by Peters *et al*.[7] Briefly, phosphorylase was assayed using 0.5 m*M* [^{3}H]FUrd or [^{3}H]FdUrd, 2.5 m*M* β-mercaptoethanol and 100 m*M* sodium phosphate buffer (pH 6.4); kinase was assayed using 0.5 m*M* [^{3}H]FUrd or [^{3}H]FdUrd, 5 m*M* ATP, 5 m*M* $MgCl_2$, 15 m*M* 2-glycerophosphate and 50 m*M* Tris-HCl (pH 8.0); OPRT was assayed using 0.5 m*M* [^{3}H]5-FU, 2 m*M* PRPP, 5 m*M* $MgCl_2$, 15 m*M* 2-glycerophosphate, 0.6 m*M* α,β-methyleneadenosine diphosphate and 50 m*M* Tris-HCl (pH 8.0). These mixtures were incubated at 37°C with 40–50 μl of the cytosol in a total volume of 100 μl for 20 min, and the reaction was stopped by heating at 90–100°C. After centrifugation at 14,000 rpm for 2 min in a Tomy model MR-150 centrifuge (Tokyo), 20 μl of the supernatant was charged with the cold carrier on a PEI-cellulose thin-layer chromatography sheet and developed with water. The spots of substrate and product were distinguished under UV and excised, and the level of radioactivity in each was measured.

***In situ* TS assay** The *in situ* TS activity was measured as described previously.[8] Cells (8×10^6 cells/ml) were incubated with or without 5-FU for 5 h at 37°C. [5-^{3}H]dUrd (final 37 kBq/ml) was added 2 h after the start of the incubation. The reaction was terminated by transferring 100-μl aliquots into centrifuge tubes containing 200 μl of a 10% activated charcoal suspension in 4% aqueous trichloroacetic acid. The tubes were vigorously stirred by vortexing, then centrifuged at 14,000 rpm for 2 min in a Kubota model KM-15200 microfuge (Tokyo). The radioactivity in 150 μl of the supernatant was determined in a scintillation counter.

RESULTS

Sensitivity to 5-FU and FdUrd When cells were exposed to various concentrations of 5-FU for 1 h and cultured without the drug for 96 h, the IC_{50} values of 5-FU for HT-29 and HT-29/5-FU/S were 0.1 and 0.77 m*M*, respectively (Fig. 1A), which indicated 8-fold resistance. However, when cells were continuously exposed to 5-FU for 96 h, the IC_{50} values of 5-FU for HT-29 and HT-29/5-FU/S were 0.14 and 0.033 μM, respectively. These results sdemonstrate that HT-29/5-FU/S was resistant to short-term exposure, but became more sensitive to long-term exposure to 5-FU (Fig. 1B). HT-29/5-FU/S also showed significant collateral sensitivity to FdUrd (Fig. 1C).

5-FU incorporation into RNA The amount of 5-FU incorporated into the cellular RNA was measured after incubation of the cells with 300 μM [^{3}H]5-FU for 1 h and

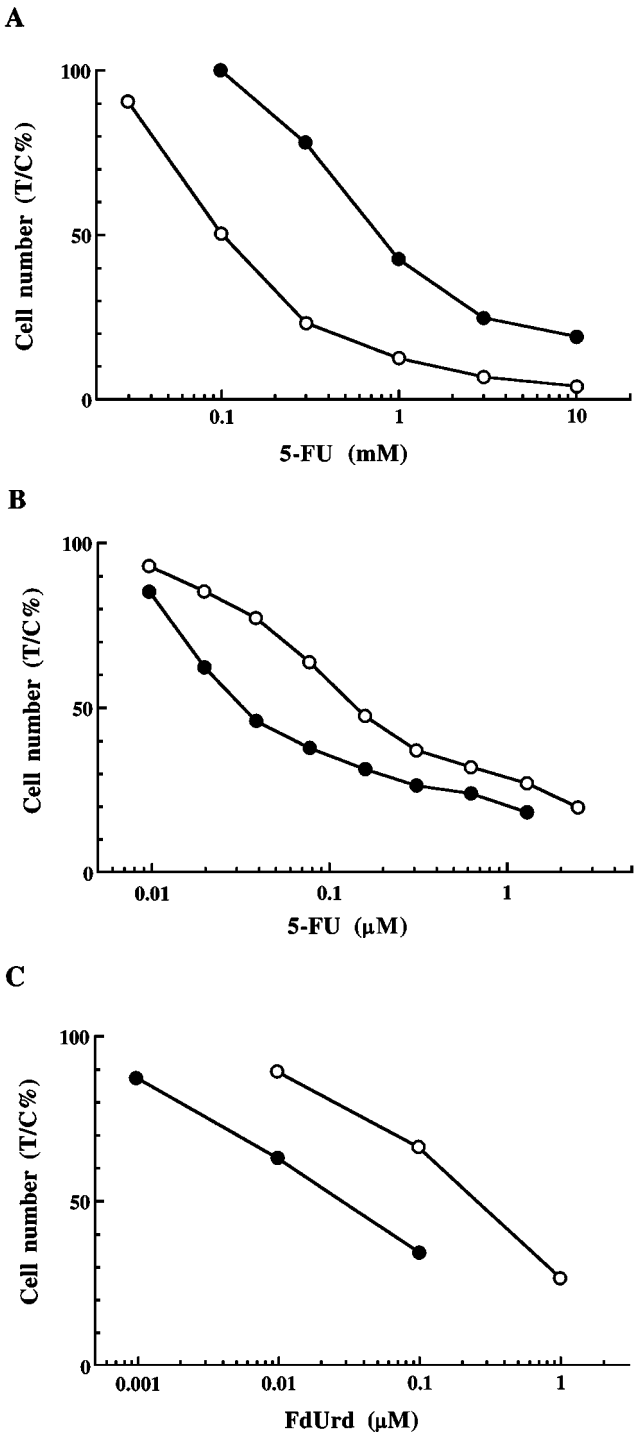

Fig. 1. Sensitivity of HT-29 and HT-29/5-FU/S cells to 5-FU and FdUrd. Cells were exposed to various concentrations of 5-FU for 1 h then cultured without the drug for 96 h (A) or cultured with 5-FU (B) or FdUrd (C) continuously for 96 h. The relative cell number was measured by a sulforhodamine B assay. Each point is the mean of 4 determinations with a coefficient of variation of less than 10%. ○ HT-29; ● HT-29/5-FU/S.

after post-incubation for 24 h in drug-free medium. Under both conditions, the amount of 5-FU incorporated into RNA in HT-29/5-FU/S was 60% less than in HT-29 cells (Fig. 2). The *P* values (Student's *t* test) were 0.0003 for A and 0.0002 for B.

Levels of active metabolites of 5-FU Intracellular FUTP level was assessed after 1-h incubation with 300 *μM* [^{3}H]5-FU (Table I). The level in HT-29/5-FU/S cells was one-fourth of that in HT-29 cells. FdUMP level was measured after 5-h incubation with 10 *μM* [^{3}H]5-FU. It was also lower in the resistant cells, but the difference was not as marked as in the case of FUTP.

Activities of 5-FU-anabolizing enzymes The activities of five enzymes involved in 5-FU anabolism were measured (Table II). Only OPRT showed lower activity in HT-29/5-FU/S cells. Uridine phosphorylase/kinase and thymidine phosphorylase exhibited rather higher activity in the resistant cells. There was no significant difference in thymidine kinase activity between the two cell lines.

***In situ* TS activity and its sensitivity to 5-FU** Cells were incubated with or without 5-FU for 5 h and the *in situ* TS activity was determined for the last 3 h (Fig. 3). The basal levels of TS activity measured in the absence of 5-FU were 70.4±7.1 and 35.3±7.1 (pmol/mg protein/3 h) for HT-29 and HT-29/5-FU/S cells, respectively; the TS activity in the 5-FU resistant cells was about 50% lower than that in the parent cells (*P*=0.0004). The values of percentage inhibition of *in situ* TS in HT-29 and HT-29/5-FU/S cells were 0 and 21% at 1 *μM* 5-FU, and 40 and 85% at 10 *μM* 5-FU, respectively. This demonstrates that HT-29/5-FU/S cells were more sensitive to TS inhibition by 5-FU than HT-29 cells.

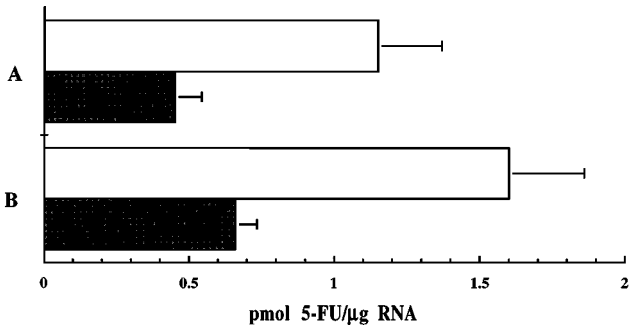

Fig. 2. The incorporation of 5-FU into RNA in HT-29 and HT-29/5-FU/S cells. Cells were exposed to 300 *μM* 5-FU for 1 h (A) or incubated without the drug for 24 h after 1 h exposure to 300 *μM* 5-FU (B) and then RNA was extracted as described in "Materials and Methods." Each column is the mean of 3 determinations with the bar indicating the standard deviation. The open and shaded columns denote HT-29 and HT-29/5-FU/S, respectively.

Table I. FdUMP/FUTP Levels in HT-29 and HT-29/5-FU/S Cells

Substrate	Incubation time (h)	Products (pmol/ mg protein)			*P* value
		Metabolite	HT-29	HT-29/5-FU/S	
300 μM 5-FU	1	FUTP	679±48	160±14.6	0.0036
10 μM 5-FU	5	FdUMP	4.14±0.13	2.28±0.57	0.0209

Table II. Enzyme Activity of 5-FU Anabolism in HT-29 and HT-29/5-FU/S Cells

Enzyme	Activity (nmol/mg protein/20 min)		*P* value
	HT-29	HT-29/5-FU/S	
Uridine phosphorylase	2.60±0.72	7.15±1.05	0.0285
Uridine kinase	4.62±0.48	8.16±0.36	0.0105
Orotate phosphoribosyltransferase	7.47±0.34	4.64±0.24	0.0041
Thymidine phosphorylase	11.5±1.7	21.7±4.7	0.0774
Thymidine kinase	17.2±1.8	14.5±1.2	0.261

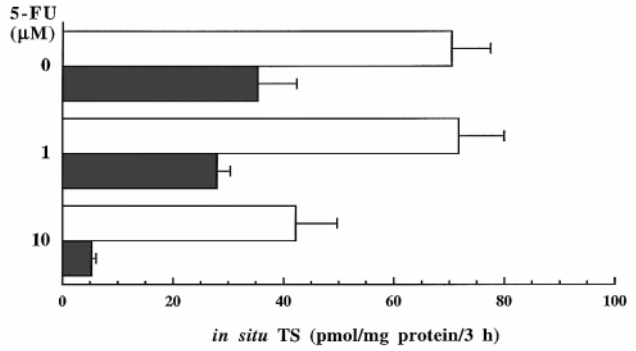

Fig. 3. *In situ* thymidylate synthase inhibition by 5-FU in HT-29 and HT-29/5-FU/S cells. Cells were incubated with or without 5-FU for 5 h, and [^{3}H]dUrd was added 2 h later after the start of incubation. *In situ* TS activity was measured as described in "Materials and Methods." Each column is the mean of 3 determinations with the bar indicating the standard deviation. The open column denotes HT-29 and the shaded one denotes HT-29/5-FU/S.

DISCUSSION

We have established two sublines of human stomach[9] and colon (Inaba *et al*., unpublished data) cancer cell lines with acquired resistance to long-term exposure to 5-FU. Both of them also showed resistance to short-term exposure to 5-FU. The major mechanism of resistance in these sublines was the decreased production of two active metabolites, FUTP and FdUMP. In the present study, we established a subline of human colon cancer HT-29 cells by repeated 1-h exposure to stepwise-increasing concentrations of 5-FU. This subline manifested 8-fold resistance to 5-FU in a 1-h exposure assay. In contrast, it showed rather higher sensitivity to 5-FU and FdUrd under a 96-h exposure condition (Fig. 1).

Aschele *et al*. also established 5-FU-resistant sublines of human colon cancer (HCT-8) using two different exposure schedules.[10] One subline developed by repeated 4-h exposure to 5-FU showed 3-fold resistance to short-term exposure to 5-FU but retained full sensitivity to long-term exposure. Decreased incorporation of 5-FU into cellular RNA was found in this cell line. Another subline developed after repeated 7-day exposure to 5-FU exhibited 10-fold increased resistance to long-term exposure and marked cross-resistance to short-term exposure to 5-FU.[11] Decreased expression of folypolyglutamate synthetase was reported as a novel mechanism of resistance to 5-FU in this cell line.[12]

We studied the mechanism of resistance to short-term exposure and the increased sensitivity to long-term exposure to 5-FU in HT-29/5-FU/S cells. A significant reduction of 5-FU incorporation into cellular RNA was observed in HT-29/5-FU/S cells, and this may account for the resistance to 1-h exposure to 5-FU, in view of our previous kinetic analysis of the cell-killing action of 5-FU. In accordance with this, significantly reduced production of FUTP was observed (Table I). However, a comparative study of pyrimidine enzymes involved in 5-FU activation for the two cell lines did not reveal the cause of the decrease of FUTP. OPRT activity was significantly lower, but the activity of uridine phosphorylase/kinase was rather higher in HT-29/5-FU/S than in HT-29 cells. If the latter pathway is not effective because of a deficiency in the

ribose-1-phosphate pool, the decreased activity of OPRT may contribute to the decreased production of FUTP.

An increased level of TS as a target enzyme of 5-FU can be a mechanism of resistance to 5-FU. Copur *et al*. reported elevated levels of TS protein and m-RNA and amplification of the TS gene in human colon H630 cells which acquired resistance to continuous exposure to 5-FU.[13] Therefore, we determined the basal level of *in situ* TS activity and the inhibitory effect of 5-FU. The basal activity of the *in situ* TS in HT-29/5-FU/S cells was significantly lower than that in HT-29 cells. Probably as a result of the lower TS activity, the inhibitory effect of 5-FU on *in situ* TS was more potent in HT-29/5-FU/S than in HT-29 cells (Fig. 3). This could explain the increased sensitivity of HT-29/5-FU/S cells to long-term exposure to 5-FU, in which DNA-directed action through TS inhibition seems to be predominant. The difference in the effective concentrations of 5-FU (see Figs. 1 and 3) might be caused by the difference in exposure time between these two experiments.

In conclusion, an HT-29 subline that had acquired 8-fold increased resistance to short-term exposure to 5-FU showed collateral sensitivity to the same drug under a long-term exposure condition. This result provides additional evidence for the hypothesis that 5-FU exerts different modes of cytotoxic action depending on the exposure time and treatment schedule.

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REFERENCES

1) Ozawa, S., Sugiyama, Y., Mitsuhashi, Y., Kobayashi, T. and Inaba, M. Cell killing action of cell cycle phase-non-specific antitumor agents is dependent on concentration-time product. *Cancer Chemother. Pharmacol*., **21**, 185–190 (1988).
2) Ozawa, S., Sugiyama, Y., Mitsuhashi, J. and Inaba, M. Kinetic analysis of cell killing effect induced by cytosine arabinoside and cisplatin in relation to cell cycle phase specificity in human colon cancer and Chinese hamster cells. *Cancer Res*., **49**, 3823–3828 (1989).
3) Inaba, M., Mitsuhashi, J. and Ozawa, S. Kinetic analysis of 5-fluorouracil action against various cancer cells. *Jpn. J. Cancer Re*s., **81**, 1039–1044 (1990).
4) Inaba, M. and Mitsuhashi, J. Flow cytometric analysis of cell-killing actions of 5-fluorouracil in human colorectal cancer cells. *Oncol. Res*., **6**, 303–309 (1994).
5) Monks, A., Scudiero, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J. and Boyd, M. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst*., **83**, 757–766 (1991).
6) Grem, J. L. and Fischer, P. H. Alteration of fluorouracil metabolism in human colon cancer cells by dipyridamole with a selective increase in fluorodeoxyuridine monophosphate levels. *Cancer Res*., **46**, 6191–6199 (1986).
7) Peters, G. J., Laurensse, E., Leyva, A., Lankelma, J. and Pinedo, H. M. Sensitivity of human, murine, and rat cells to 5-fluorouracil and 5′-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res*., **46**, 20–28 (1986).
8) Yalowich, J. C. and Kalman, T. I. Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 leukemia cells *in vitro*. *Biochem. Pharmacol*., **34**, 2319–2324 (1985).
9) Inaba, M., Mitsuhashi, J., Sawada, H., Miike, N., Naoe, Y., Daimon, A., Koizumi, K., Tsujimoto, H. and Fukushima, M. Reduced activity of anabolizing enzymes in 5-fluorouracil-resistant human stomach cancer cells. *Jpn. J. Cancer Res*., **87**, 212–220 (1996).
10) Aschele, C., Sobrero, A., Faderan, M. A. and Bertino, J. R. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules. *Cancer Res*., **52**, 1855–1864 (1992).
11) Sobrero, A. F., Aschele, C., Guglielmi, A. P., Mori, A. M., Melioli, G. G., Rosso, R. and Bertino, J. R. Synergism and lack of cross-resistance between short-term and continuous exposure to fluorouracil in human colon adenocarcinoma cells. *J. Natl. Cancer Inst*., **85**, 1937–1944 (1993).
12) Wang, F.-S., Aschele, C., Sobrero, A., Chang, Y.-M. and Bertino, J. R. Decreased folypolyglutamate synthetase expression: a novel mechanism of fluorouracil resistance. *Cancer Res*., **53**, 3677–3680 (1993).
13) Copur, S., Aiba, K., Drake, J. C., Allegra, C. J. and Chu, E. Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil. *Biochem. Pharmacol*., **49**, 1419–1426 (1995).