Jpn. J. Cancer Res. 82, 742–746, June 1991

Competitive Binding Radioassay for the Determination of 5-Fluorodeoxyuridine and 5-Fluorodeoxyuridine-5′-monophosphate Levels in Plasma and Tumor Tissue

Seiji Miyata,[1] Hidetada Mikami,[1] Masaru Tai,[1] Takako Hori[1,3] and Hiroshi Fujita[2]

[1]Research Laboratories, Toyama Chemical Co., Ltd., 2-4-1 Shimookui, Toyama 930 and [2]Department of Bacteriology, School of Dental Medicine, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230

A competitive binding radioassay was developed to measure 5-fluoro-2′-deoxyuridine (FUDR) as well as 5-fluoro-2′-deoxyuridine monophosphate (FdUMP). FdUMP has been measured by a competitive binding radioassay with thymidylate synthase as the binding enzyme (TS assay). FUDR was enzymatically converted to FdUMP by thymidine kinase, and then the converted FdUMP was measured by the competitive binding assay to determine the concentration of FUDR in plasma and tumor tissue. As little as 100 pg/ml of FUDR or 50 pg/ml of FdUMP can be detected quantitatively by this method. When TS assay and high-performance liquid chromatography were compared for the measurement of FUDR and FdUMP levels in plasma and tumor tissue of Ehrlich carcinoma (EC)-bearing mice following administration of FUDR, a close agreement was observed for FUDR levels, though low FdUMP levels were detectable only by the TS assay method. The examination of intracellular metabolism of FUDR in EC cells by this method showed that metabolic conversion of FUDR into FdUMP or 5-fluorouracil is rapid. Thus, we have established a highly sensitive method for measuring not only FdUMP but also FUDR with TS assay. This should be very useful for experimental and clinical studies on fluoropyrimidines.

Key words: FUDR — FdUMP — Thymidylate synthase — Thymidine kinase — Binding radioassay

5-Fluorouracil (5-FU) and other fluoropyrimidine derivatives have been widely used for the treatment of certain types of cancers.[1] The mechanisms of their cytotoxic actions include: (1) inhibition of thymidylate synthase by 5-fluoro-2′-deoxyuridine monophosphate (FdUMP), which decreases *de novo* production of thymidine nucleotides for DNA synthesis; (2) inhibition of RNA splicing by incorporation of 5-fluorouridine triphosphate into RNA.[2-5] In tumor cells, thymidine kinase is thought to be responsible for not only the conversion of 5-fluoro-2′-deoxyuridine (FUDR) into FdUMP but also the salvage pathway for FdUMP blockage of thymidylate synthase.[6,7] Thymidine phosphorylase converts 5-FU to FUDR.[8,9] Therefore, the cytotoxicity is thought to depend on enzymatic metabolism of fluoropyrimidine in tumor cells. Determination of FUDR and FdUMP levels in plasma and tumor tissue is necessary to elucidate the antitumor mechanisms of fluorinated pyrimidines.

Competitive binding radioassay with thymidylate synthase for FdUMP (TS assay) is a highly sensitive and specific method.[10] On the other hand, FUDR has been measured by high-performance liquid chromatography (HPLC)[11] or a bioassay method.[12,13] However, their sensitivities for FUDR are inadequate in view of the strong *in vitro* cytotoxicity at quite low concentrations. Therefore the purpose of the present study was to establish a more sensitive assay for measuring the concentration of FUDR as well as FdUMP in plasma and tumor tissue. FUDR was enzymatically converted to FdUMP by partially purified thymidine kinase and the converted FdUMP was measured by competitive binding radioassay to determine the concentration of FUDR. The present method is highly sensitive and should be useful for experimental and clinical studies on fluoropyrimidine anticancer drugs. By the present method, plasma and tumor levels of FUDR and FdUMP were measured in Ehrlich carcinoma (EC)-bearing mice after intravenous administration of FUDR, and intracellular metabolism of FUDR or 5-FU in EC cells was also examined.

MATERIALS AND METHODS

Chemicals and reagents Bovine serum albumin (BSA), FdUMP, streptomycin and *dl*-L-tetrahydrofolate (THF) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Adenosine triphosphate (ATP) was purchased from Wako Pure Chemicals Industries Ltd. (Tokyo). 5-FU was prepared by Kyowa Hakko Co., Tokyo. FUDR were purchased from Daikin Industries Ltd. (Tokyo).

Animal and tumors Male ddY mice (5 weeks old) were purchased from SLC (hamamatsu). Ehrlich ascites carcinoma cells were kindly supplied by Cancer Institute, Kanazawa University, Kanazawa. Cells were maintained

[3] To whom all correspondence should be addressed.

by serial intraperitoneal passage in ddY mice and transplanted subcutaneously into the inguinal region of ddY mice at 5×10^6 cells/head. FUDR was administered intravenously to mice on day 7 after tumor transplantation. The mice were killed at various times, and their plasma and solid tumor tissues were obtained. The tumor was homogenized with 3 volumes of cold phosphate-buffered saline (PBS) with a Physcotron blender (Nichion, Chiba).

Intracellular metabolism in EC cells EC cells were harvested on day 7 after the intraperitoneal inoculation of ddY mice, washed with PBS and adjusted to 1×10^7 cells/ml. Cells were incubated with 100 μg/ml of 5-FU or FUDR in RPMI-HEPES medium (pH 7.4) containing 10% fetal calf serum (FCS). After incubation for various times, cells were immediately washed with cold PBS 3 times. Intracellular FUDR and FdUMP levels were determined by TS assay and 5-FU was measured by the HPLC method.

Preparation of thymidine kinase Thymidine kinase was partially purified from Ehrlich ascites carcinoma cells according to the method of Cheug and Prusoff.[14] Cells harvested from the peritoneal cavity of tumor-bearing mice on day 7 after inoculation were washed 3 times with PBS and were packed by centrifugation (800*g*, 5 min). The washed cells were suspended in 50 m*M* Tris-HCl (pH 7.8) containing 1 m*M* dithiothreitol and disrupted with a VR-200P Ultrasonicator (Tomy, Tokyo). The preparation was centrifuged at 105,000*g* for 60 min and the supernatant was collected. Streptomycin solution (10%) was then added to 8 volumes of supernatant. The suspension was stirred for 30 min and the precipitates were discarded after centrifugation at 13,000*g* for 20 min. Solid ammonium sulfate was added to the supernatant fluid to make 35% saturation, and the suspension was stirred at 4°C for 60 min. The precipitate was collected by centrifugation at 13,000*g* for 40 min, dissolved in stabilizing buffer (20 m*M* Tris-HCl, pH 7.5, 1 m*M* thymidine, 10% glycerol) and dialyzed overnight against the same buffer.

Purification of thymidylate synthase Thymidylate synthase was purified from methotrexate-resistant *Lactobacillus casei* according to the modified method of Dunlap *et al.*[15] and stored under nitrogen at 4°C. The specific activity of purified thymidylate synthase was determined to be 0.954 unit/mg protein.

Preparation of [6-^{3}H]FdUMP [6-^{3}H]FdUMP (16.4 Ci/mmol) was enzymatically synthetized from [6-^{3}H]-FUDR (New England Nuclear, Boston) in 70 m*M* Tris-HCl, (pH 7.8) containing 7.5 m*M* ATP, 7.5 m*M* $MgCl_2$, 30 m*M* KF, 0.05 mg/ml BSA, and thymidine kinase, partially purified from Ehrlich ascites carcinoma. It was then purified by chromatography on DEAE cellulose, and the purity (97.5%) was determined by HPLC.

Separation of FUDR and FdUMP Samples of the plasma or 25% homogenates of tumors were vigorously shaken with 2 volumes of methanol. The mixture was centrifuged at 2,000*g* for 10 min and then extracted with 66% methanol by centrifugation at 2,000*g*. The supernatants were combined and evaporated at 35°C under reduced presure. Then the residue was dissolved in 2 ml of 50 m*M* NH_4HCO_3, and applied to DEAE cellulose minicolumn (1×3 cm) both for separation of FdUMP and FUDR and for removal of dUMP. The column was washed with 12 ml of 100 m*M* NH_4HCO_3 and the first 8 ml of eluate was pooled as FUDR fraction. The column was further washed with 8 ml of 300 m*M* NH_4HCO_3, and the eluate was collected as FdUMP fraction. FUDR and FdUMP fractions were lyophilized and FdUMP was measured by TS assay after the removal of NH_4HCO_3 by methanol extraction.

Conversion of FUDR to FdUMP FUDR was converted to FdUMP by incubation with suitably diluted thymidine kinase in a solution containing 7.5 m*M* ATP, 7.5 m*M* $MgCl_2$, 30 m*M* KF, 0.05 mg/ml BSA and 70 m*M* Tris-HCl, pH 7.8, for 1 h at 37°C. The thymidine kinase activity was determined to be 0.1 unit/mg protein. FdUMP was purified by the above chromatography on DEAE cellulose using stepwise NH_4HCO_3 gradient elution, and lyophilized, and insoluble material was removed by methanol extraction.

Procedure of TS binding assay The assay procedure was carried out as described by Murinson *et al.*[10] The assay was performed in a 12–75 mm plastic tube, which contained the following reactants in a final volume of 0.5 ml: a) 200 μl of serially diluted sample or standard solution, b) 5 mg/ml of BSA, c) 0.1 μmol of methylenetetrahydrofolate, which was freshly prepared by allowing 620 μmol of formaldehyde to react with 10 μmol of *dl*-L-tetrahydrofolate, d) 13 nCi of [^{3}H]FdUMP (150 pg), and 100 μl of suitably diluted thymidylate synthase solution. The mixtures were incubated in the dark for 2 h and 0.5 ml of ice-cold 5% trichloroacetic acid (TCA) solution was added. After centrifugation at 3,000 rpm for 5 min, a 750 μl aliquot of the supernatant was placed in 10 ml of scintillator and the unbound [^{3}H]FdUMP was determined by counting in a liquid scintillation spectrophotometer.

HPLC assay Samples of 0.5 ml of the plasma or 25% homogenate of tumor tissue were precipitated with methanol and the supernatants were applied to a reversed LC-SORB silicagel column (0.5×1 cm). Fractions eluted with 20% methanol were further applied to a DEAE Sepharose column (1×1.5 cm), and separated by elution with 90% methanol (FUDR and 5-FU) or 6 *N* formic acid-methanol (FdUMP). FUDR and 5-FU fractions were further purified on a Q-Sepharose column (1×1.5 cm) eluted with acetic acid-methanol. Samples

were injected into an HPLC apparatus (Shimadzu liquid chromatograph, model LC-3A) with a UV detector set at 254 nm. The normal-phase chromatography of 5-FU and FUDR was performed on Develosil 60-3 using a mobile phase of CH_2Cl_2-AcOH-MeOH (200:1:14, v/v). FdUMP was chromatographed in the reverse-phase mode on Develosil ODS-5 using a mobile phase of MeOH-1 *M* AcOH, AcONa (pH 4)-H_2O-tetrabutyl-*n*-ammonium bromide (TBA) (15 ml:15 ml:170 ml:0.2 g).

RESULTS

Competitive binding assay for FUDR and FdUMP The procedure is fundamentally based on competition between [^{3}H]FdUMP and unlabeled FdUMP for binding to thymidylate synthase. A linear standard curve (Fig. 1) was obtained between FdUMP concentration and LOGIT B/Bo in the concentration range of 25 pg to 1,000 pg/ml. No interference was observed with the plasma and tumor blanks, which was prepared in the same manner as in FUDR procedure.

Recovery studies in plasma and homogenate of tumor tissue gave recoveries of 66% (FdUMP) and 49% (FUDR) in plasma and 57% (FdUMP) and 56% (FUDR) in tumor. Therefore, the detection limit was determined as 50 pg/ml (FdUMP) or 100 pg/ml (FUDR).

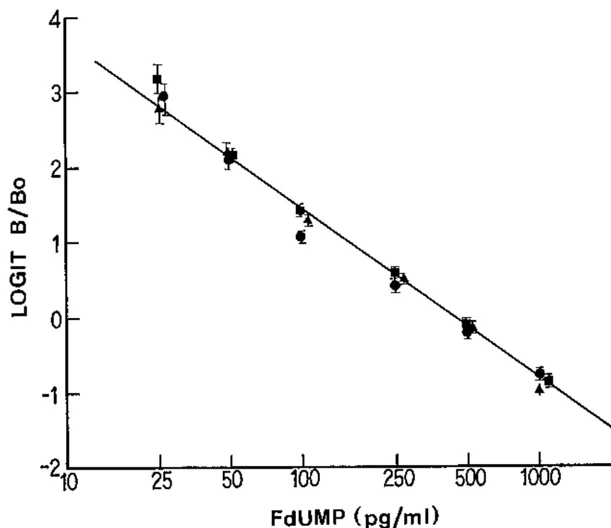

Fig. 1. Standard curve of unlabeled FdUMP against LOGIT B/Bo in the presence or absence of FUDR blank. The assay was carried out as described in "Materials and Methods." The LOGIT B/Bo (y) can be obtained from the following expression: LOGIT B/Bo$=\ln[(B/Bo)/(1-B/Bo)]$, B/Bo$=$Fxs$-$F/Fxs$-$Fo. Fxs is the dpm value of unbound [^{3}H]FdUMP in the presence of an excess (100 ng/ml) of unlabeled FdUMP, F is the dpm value of unbound [^{3}H]FdUMP in the presence of various doses of unlabeled FdUMP (x), and Fo is the dpm value of unbound [^{3}H]FdUMP in the absence of unlabeled FdUMP (●, buffer; ▲, tumor blank; ■, plasma blank). The curve (buffer) is a negative exponential ($y=5.971-2.298x$, $r=0.993$, $P<0.01$) and is not significantly different from that of the plasma blank ($y=6.241-2.336\log x$, $r=0.994$, $P<0.01$) or tumor blank ($y=6.039-2.308\log x$, $r=0.998$, $P<0.01$). Bars represent SE.

FUDR and FdUMP levels in the plasma and tumor tissue of EC-bearing mice after the administration of FUDR ddY mice with a 7-day growth of Ehrlich solid tumor received intravenous injection of FUDR (100 mg/kg). Then at 0.25, 0.5, 1, 4, 7, 24, and 48 h after the administration, the plasma and tumor tissues were removed and FUDR and FdUMP levels were quantitated (Fig. 2). In plasma, the FUDR level was about 10,000 ng/ml at 0.25 h, then rapidly decreased to 0.3 ng/ml at 24 h, when measured by TS assay (Fig. 2A, B). This shows that in plasma FUDR is detectable for as long as 24 h by TS assay, although only for 4 h by HPLC. FdUMP levels in plasma could be measured only by TS assay up to 1 h.

In tumor tissue, FUDR was measurable for as long as 7 h by TS assay, although for only 1 h by HPLC (Fig. 2C, D). FUDR level in the tumor was about 600 ng/g at 0.25 h then rapidly decreased to 1.8 ng/g at 7 h. FdUMP was measurable only by TS assay and the level in tumor tissue was 75.7 ng/g at 0.25 h, and 1.8 ng/g at 48 h. A close agreement was observed between the results obtained by TS assay and HPLC.

Intracellular metabolism in EC cells EC cells obtained from the peritoneal cavity were incubated with 100 μg/ml of 5-FU or FUDR in RPMI-HEPES medium (pH 7.4) containing 10% FCS. The intracellular metabolism of FUDR is shown in Fig. 3A. Intracellular levels of FdUMP and 5-FU were about 100-fold higher than that of FUDR, when compared at any time point during the incubation period. FdUMP concentration in cells rapidly increased and reached at a maximal level within 10 min, while the 5-FU level increased slowly.

The intracellular metabolism of 5-FU is also shown in Fig. 3B. FUDR was below the measurable level, while FdUMP was observed to be about 10 ng/10^7 cells. 5-FU level was 10-fold higher than that of FdUMP.

DISCUSSION

The present competitive binding radioassay was demonstrated to be useful for the measurement of both FUDR and FdUMP. The precision of measurement of TS assay was proved by comparison with the HPLC method; the results by TS assay were in close agreement with those by HPLC. With respect to the detection limit of the TS assay method, it was about 100-fold lower than

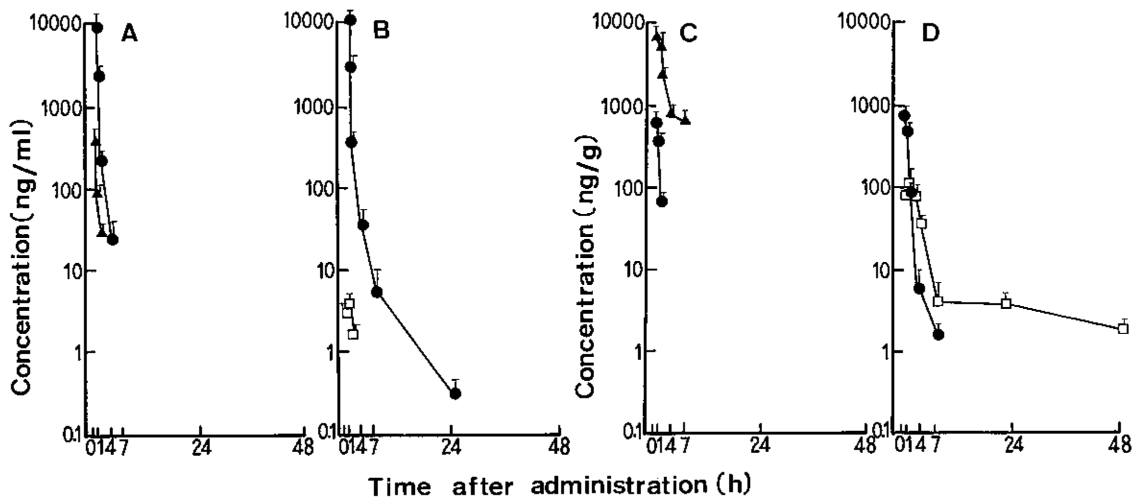

Fig. 2. Concentrations of 5-FU (▲), FUDR (●) and FdUMP (□) in the plasma (A and B) and tumor tissues (C and D) of EC-bearing mice after the administration of FUDR at the dose of 100 mg/kg. FUDR was given intravenously to EC-bearing mice on day 7 after tumor inoculation. At the indicated time, the plasma and tumor were promptly removed, and FUDR and their metabolites were measured as described in "Materials and Methods." Assay was performed by HPLC (A and C) or TS assay (B and D). Values are means for 3 groups of mice. Bars represent SE.

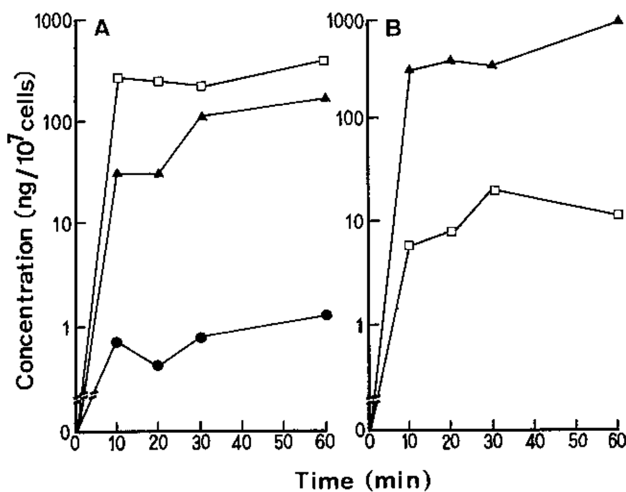

Fig. 3. Intracellular metabolism of FUDR (A) and 5-FU (B) in EC cells. Cells at a concentration of 1×10^7 cells/ml were incubated with 100 μg/ml FUDR or 5-FU in RPMI-HEPES (pH 7.4) containing 10% FCS. After Various times, 5-FU (▲), FUDR (●) and FdUMP (□) concentrations were measured as described in "Materials and Methods."

that of the HPLC method; thus we could follow the pharmacokinetics of FUDR in plasma and tumor for a longer period. The metabolism of fluoropyrimidine in plasma and tissue has previously been studied by using radiolabeled drugs.[2,16] An HPLC method has also been developed to measure FUDR level.[11] However, the detection limit is inadequate taking into account the postulated threshold level of FUDR in tumor tissue. Myers *et al.* reported a spectrophotometric method based on the inhibition of thymidylate synthase activity to measure FdUMP.[17] Furthermore, a competitive binding radioassay for FdUMP has been developed based on competition between unlabeled and labeled FdUMP for thymidylate synthase. In this study, we developed the present TS assay by making the above competitive binding radioassay applicable to FUDR. For this purpose, FUDR was converted to FdUMP by thymidine kinase. This assay has the following advantages: 1) the detection limit of FUDR is reduced to the same level as that of FdUMP; 2) the assay procedure is convenient and rapid.

Little is known about the intracellular metabolism of FdUMP, FUDR and 5-FU because of the low sensitivity of conventional assays for measuring FUDR. However, it has now become possible to examine precisely the intracellular metabolism by using the TS assay. The present *in vitro* study on the intracellular metabolism of FUDR by using the TS assay showed that the concentration of FdUMP or 5-FU and 100-fold higher than that of FUDR in EC cells. However, when cells were treated with 5-FU, the levels of FdUMP and FUDR were respectively below one-tenth or one-thousandth of the 5-FU level. These results indicate that the conversion of FUDR to 5-FU or that of FUDR to FdUMP proceeded rapidly,

but the metabolism of 5-FU to FUDR is slow in EC cells. A number of authors have postulated that thymidine kinase catalyzes the conversion of FUDR to FdUMP, and that thymidine phosphorylase is involved in the conversion of FUDR to 5-FU and *vice versa*.[6-9] Therefore intracellular metabolism of fluoropyrimidine is considered to differ depending on the enzymatic activities in the cells used.

FUDR has strong cytotoxicity *in vitro* but it is not applicable *in vivo* because of its rapid excretion and metabolism.[18] Although the mechanism of FUDR cytotoxicity seems to be time-dependent inhibition of DNA synthesis by FdUMP,[3,4] FdUMP levels decreased rapidly in tumor tissue after FUDR administration in this study. On the other hand, the cytotoxic action of 5-FU seems to involve not only the inhibition of DNA synthesis by FdUMP blockage of TS but also the inhibition of RNA splicing.[3,5] These considerations may account for the lower therapeutic efficacy of FUDR as compared to 5-FU.

By means of the present competitive binding assay, we were able to measure low levels of FUDR as well as FdUMP. This competitive binding radioassay is highly sensitive, and should be useful for pharmacokinetic analysis of FUDR and FdUMP in plasma or tissue samples from animals and patients, and for *in vitro* studies, e.g., on intracellular metabolism or metabolic pathways.

(Received January 10, 1991/Accepted March 20, 1991)

REFERENCES

1) Tokyo Cancer Chemotherapy Cooperative. Group study of fluorinated pyrimidines. *In* "Fluoropyrimidines in Cancer Therapy," ed. K. Kimura, S. Fujii, M. Ogawa, G. P. Bodey and P. Alberto, pp. 242–250 (1983). Elsevier Science Publishers B.V., Amsterdam.
2) Bosch, L., Harbers, H. and Heidelberger, C. Studies on fluorinated pyrimidine. V. Effect on nucleic acid metabolism *in vitro*. *Cancer Res.*, **18**, 335–343 (1958).
3) Hartman, K. Y. and Heidelberger, C. Studies on fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase. *J. Biol. Chem.*, **236**, 3006–3018 (1961).
4) Langerback, R. J., Danenberg, P. V. and Heidelberger, C. Thymidylate synthetase: mechanism of inhibition by 5-fluoro-2′-deoxy-uridylate. *Biochem. Biophys. Res. Commun.*, **48**, 1565–1571 (1972).
5) Wilkinson, D. S., Tlsty, T. D. and Hanas, R. J. The inhibition of ribosomal RNA synthesis and maturation in Novikoff hepatoma cell by 5-fluorouridine. *Cancer Res.*, **35**, 3014–3020 (1975).
6) Kessel, D. and Wodiusky, I. Thymidine kinase as a determinant of the response to 5-fluoro-2′-deoxyuridine in transplantable murine leukemias. *Mol. Pharmacol.*, **6**, 251–254 (1969).
7) Bresnick, E. and Thompson, U. B. Properties of deoxythymidine kinase partially purified from animal tumor. *J. Biol. Chem.*, **240**, 3967–3973 (1965).
8) Woodman, P. W., Sarrif, A. M. and Heidelberger, C. Specificity of pyrimidine nucleoside phosphorylases and phosphorolysis of 5-fluoro-2′-deoxyuridine. *Cancer Res.*, **40**, 507–511 (1980).
9) Chu, M. Y., Naguib, F. N. M., Iltasch, M. H., el Kouni, M. H., Chu, S. H., Cha, S. and Calabresi, P. Potentiation of 5-fluoro-2′-deoxyuridine antineoplastic activity by the uridine phosphorylase inhibitors benzylacyclouridine and benzyloxybenzylacyclouridine. *Cancer Res.*, **44**, 1852–1856 (1984).
10) Murinson, D. S., Anderson, T., Schwartz, H. S., Myers, C. E. and Chabner, B. A. Competitive binding radioassay for 5-fluorodeoxyuridine 5′-monophosphate in tissue. *Cancer Res.*, **39**, 2471–2475 (1979).
11) Benvenuto, J. A., Lu, K. and Loo, T. L. High-pressure liquid chromatographic analysis of ftorafur and its metabolites in biological fluids. *J. Chromatogr.*, **134**, 219–222 (1977).
12) McAllister, R. M., Grunmeier, P. W., Corieli, L. L., and Blakemore, W. S. A quantitative technique for the measurement of *in vitro* cytotoxicity of 5-fluoro-2′-deoxyuridine, nitrogen mustard (NH_2), and other antitumor agents. *Cancer*, **12**, 938–943 (1959).
13) Kanazawa, F., Hoshi, A., Kuretani, K., Saneyoshi, M. and Kawaguchi, T. Antitumor activity of 3′,5′-diesters of 5-fluoro-2′-deoxyuridine against murine leukemia L1210 cells. *Cancer Chemother. Pharmacol.*, **6**, 19–23 (1981).
14) Cheng, Y-C. and Prusoff, W. H. Mouse ascites sarcoma 180 deoxythymidine kinase. General properties and inhibition studies. *Biochemistry*, **13**, 1179–1185 (1974).
15) Dunlap, R. B., Harding, N. G. L. and Heuneckens, F. M. Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*. *Biochemistry*, **10**, 88–97 (1971).
16) Diasio, R. B. and Bowen, D. Rapid simultaneous analysis of fluoropyrimidine metabolite pools by a high performance liquid chromatographic-isotopic method. *Proc. Am. Assoc. Cancer Res.*, **19**, 132 (1978).
17) Myers, C. E., Young, R. C., Johns, D. G. and Chabner, B. A. Assay of 5′-fluorodeoxyuridine 5′-monophosphate and deoxyuridine 5′-monophosphate pools following 5-fluorouracil. *Cancer Res.*, **34**, 2682–2688 (1974).
18) Birnie, G. D., Kroeger, H. and Heidelberger, C. Studies of fluorinated pyrimidines. XIII. The degradation of 5-fluoro-2′-deoxyuridine and related compounds by nucleoside phosphorylase. *Biochemistry*, **2**, 566–572 (1962).