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QUARTERLY

Synergistic effect of 5-fluorodeoxyuridine and quinazoline antifolates on murine leukemia self-cultured *in vitro**

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The effect of thymidylate synthase inhibitors, fluorodeoxyuridine (FdUrd) and its two sulphonamide derivatives was examined in the culture of murine leukemia cells — 5178Y (parental subline) and its fluorodeoxyuridine resistant subline 5178Y/F. A synergistic effect of the antimetabolites on cell survival was observed on exposure of the culture of either line to a slightly inhibitory concentration of FdUrd (1 nM) in combination with 2-desamino-2-methyl-10-propargyl-5,8-dideaza-pteroylsulphoglutamate or 2-desamino-2-methyl-10-propargyl-5,8-dideaza-pteroylsulphoglycine. This effect was accompanied by a marked reduction, in both cell lines of intracellular concentration of 5,10-methylenetetrahydro-pteroyl-polyglutamate, although its concentration in the resistant subline was 3 times as high as in the parental line. The inhibitory effect of combined drugs on the cellular pool of folates in 5178Y line depended also on the sequence of drug addition, whereas in the FdUrd resistant line this sequence was without any effect. The results obtained strongly suggest that under certain conditions inhibition of thymidylate synthesis by antifolates is intensified by a prior use of FdUrd.

Fluoropyrimidine antimetabolites, particularly fluorouracil and FdUrd, are cytotoxic in diverse biological systems and have been extensively used in clinical treatment of carcinomas of ovary, breast and gastrointestinal tracts [1]. Primarily the action of these drugs depends on their conversion to FdUMP, which binds tightly to thymidylate synthase (EC 2.1.1.45) in the presence of methylenetetrahydrofolate ($CH_2H_4PteGlu_n$) a substrate of the enzyme [2, 3]. Since thymidylate synthase (TS) is the only enzyme providing *de novo* thymidylate, this enzyme is crucial for DNA biosynthesis and is a target for cancer therapy.

Unfortunately FdUMP is only one of several products derived from FdUrd. The other products can also be incorporated into DNA,

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Abbreviations: TS, thymidylate synthase; $5,10CH_2H_4PteGlu_n$, 5,10-methylenetetrahydropteroyl-polyglutamate; FdUrd, fluorodeoxyuridine; FdUMP, fluorouridinemononucleotide; DMPDDSF(Glu or Gly), 2-desamino-2-methyl-10-propargyl-5,8-dideazapteroyl- sulphoglycine or γ-glutamate; PDDF, 10-propargyl-5,8-dideazafolic acid; pABAGlu, *p*-aminobenzoylglutamate; MTX, methotrexate; TMTX, trimethotrexate; $H_2PteGlu_n$, dihydro-pteroyl-polyglutamate; $H_4PteGlu_n$, tetrahydro-pteroyl-polyglutamate.

RNA and sugars [4–6]. Multiciplity of FdUrd metabolites inspired studies directed toward development of folate analogs of TS inhibitors. 10-*N*-propargyl-5,8-dideazafolate developed by Jones and coworkers in 1981 [7] appeared to be an extremely potent inhibitor of TS. Since that time a whole family of dideazafolates inhibiting TS has been synthesized in different laboratories including ours [8, 9].

It seemed also of interest to examine the effect of two TS inhibitors on cell survival. Studies on the effect of continuous exposure of murine leukemia cells to either FdUrd or the newly synthesized antifolates — sulphonamide derivatives of 10-propargyl-5,8-dideazafolates, a poly-γ-glutamable (DMPDDSFGlu), and a nonpolyglutamable (DMPDDSFGly) (Fig. 1) could provide some information on the mechanism of TS inhibition *via* interference with intracellular pools of folates and DNA synthesis.

MATERIALS AND METHODS

Materials

Fisher's medium, Dulbecco medium, bovine fetal serum, bovine new born serum and trypsin were purchased from Grand Island Biochemical Company, Life Technologies Ltd., Paisley, Scotland.

10-Propargyl-5,8-dideazafolic acid (PDDF) and its *p*-aminosulphonyl derivative (DMPDDSF) were purchased or synthesized as described previously [8, 9].

Trimethotrexate (TMTX) was a gift from Glaxo-Wellcome Company. Methotrexate (MTX), folic acid (both purified, prior to use by ion-exchange — DEAE cellulose chromatography), tetrahydrofolate, ATP, thymidylate synthase (from bovine liver) and dihydrofolate reductase (from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $pABAGlu_n$ was a gift from Prof. Rzeszotarska and Dr. Krzyżanowski from the Opole University.

All radioactive compounds were purchased from Moravek Biochemicals, Inc., Brea, CA, U.S.A. or Amersham, International plc, Amersham, U.K.

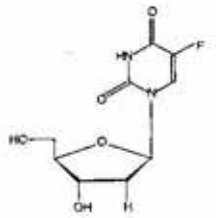
All other chemicals were reagent grade.

Methods

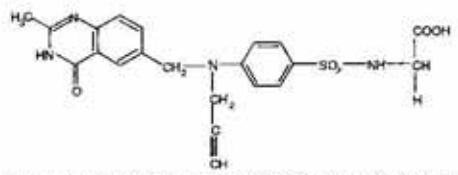
Cell culture. Murine leukemia 5178Y cells free of Mycoplasma were grown in suspension in 60 mm Falcon dishes in Fisher's medium supplemented with 8% of bovine new born serum under a 5% CO_2 atmosphere as described previously [8–10]. Murine leukemia 5178Y/F cells containing 6 times more of TS protein than the parental line [10] were obtained in our laboratory by passaging 5178Y cells on the media containing the increasing concentration of FdUrd, starting from 0.1 nM to reach 80 μM [10] after 18 months.

Cell survival. The 5178Y cells were plated at a density of 1×10^4 cells/well (Bibby Sterilin, Ltd., England). After 2 h the drugs were added for 48 h at the concentration indicated and the number of viable cells was counted in Neubauer camera using the trypan-blue staining.

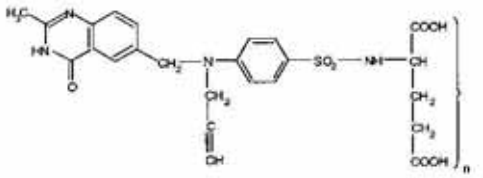
Enzyme assays. For the enzyme assay cells were collected (by centrifugation), washed twice in ice cold phosphate buffer-saline, sonicated in Branson Sonifier 250



5-Fluoro 2'-deoxyuridine (FdUrd)



2-Desamino-2-methyl-10-propargyl-5,8-dideazapteroyl-glycine (DMPDDSFGly)



2-Desamino-2-methyl-10-propargyl-5,8-dideazapteroyl-glutamate (DMPDDSFGlu)

Figure 1. Chemical structures of thymidylate synthase inhibitors.

sonicator in 100 mM phosphate buffer (pH 7.4) containing 10 mM 2-mercaptoethanol, and centrifuged for 20 min at 20 000 × ***g***. The supernatant was used for the enzyme assay.

Folylpolyglutamate synthetase (EC 6.3.2.-17) was assayed according to McGuire *et al.* [11] using tetrahydrofolate (250 μM) as a substrate.

γ-Glutamyl hydrolase (EC 3.4.19.9) was assayed in cell extracts according to Sikora *et al.* [12], or determined by high performance liquid chromatography (Beckman Ultrasil AX 10 μ 0.4 cm × 25 cm column) using pABA-Glu_5 as a substrate.

Cell protein was estimated according to Bradford [13].

Thymidylate synthesis. Deoxyuridylate incorporation was a measure of *de novo* thymidylate biosynthesis by the intact cultured cells. The cells exposed to the drugs were exposed in turn to 1 μCi of [5-^{3}H]dUrd for 40 min and the reaction was terminated by addition of activated charcoal suspended in 0.15 M trichloroacetic acid. Recovery of ^3H_2O radioactivity was a measure of the rate of thymidylate synthesis *in situ* [9]. The results were expressed in pmoles/well.

[6-^{3}H]dUrd incorporation to DNA. Following cell treatment by the drug cultures were exposed to 10 nM (1 μCi) of [6-^{3}H]dUrd for 40 min. Incorporation of the radiolabel into DNA in intact cells was measured following alkaline hydrolysis of RNA in 0.5 M KOH at 37°C according to Hryniuk *et al.* [14].

Determinations of 5,10CH_2H_4PteGlu_n and H_2PteGlu_n. Cells exposed to the drugs were extracted by boiling in a solution containing 50 mM Tris, 1 mM EDTA and 50 mM ascorbate (pH 7.4) and 5,10CH_2H_4PteGlu_n was measured in the supernatant according to Bunni *et al.* [15]. Then the samples were incubated for 30 min with 10 pmol of [^{3}H]FdUMP and 10 pmol of bovine TS in a total volume of 100 μl at 37°C. The reaction was stopped by adding 1% SDS and boiling for 10 min. The [^{3}H]FdUMP-thymidylate synthase-5,10CH_2H_4PteGlu_n complex was quantitated following separation of unreacted [^{3}H]FdUMP by G-25 Sephadex filtration. H_2PteGlu_n was measured after reduction to H_4PteGlu_n by dihydrofolate reductase in the presence of NADPH and assayed as 5,10CH_2H_4PteGlu_n in the presence of 6 mM formaldehyde in the thymidylate synthase reaction. The amount of dihydrofolate was calculated by substracting the amount of 5,10CH_2H_4PteGlu_n present in the initial sample from the amount formed by dihydrofolate reductase.

Standard curves were generated using 5,10CH_2H_4PteGlu_7 and H_2PteGlu_7. The results were expressed as nmol/g cell protein.

Functional interactions between drugs. The combined drug effect was evaluated using the Chou & Talalay [16] analysis based on the median effect principle. The data were analyzed using the concentration-effect statistical program.

RESULTS AND DISCUSSION

Effect on cell survival

The activities of three antimetabolites — FdUrd and DMPDDSFGlu or DMPDDSFGly — inhibitors of thymidylate synthase are shown in Table 1. The decrease in survival of 5178Y cells caused by FdUrd was the most pronounced with I_{50} approximately at 0.002 μM on 48 h exposure to the antimetabolite. In the case of the two antifolates: DMPDDSFGlu and DMPDDSFGly I_{50} values were 3 μM and 20 μM, respectively.

Continuous exposure of 5178Y/F cells to either antifolate caused only a slight increase in I_{50} value in comparison with parental 5178Y cells (Table 1). DMPDDSFGlu appears to be a much potent (about 7 times) inhibitor than DMPDDSFGly in both cell lines due to its ability to undergo polyglutamylation [17, 18].

A possible effect on interaction of the antimetabolites at low inhibitory concentration on cell survival was also tested (Table 2). A 48 h exposure of 5178Y cells successively to DMPDDSFGlu and FdUrd at concentrations corresponding to I_{10} resulted in a more significant lowering of cell survival than expected from the sum of their separate effects. The addition of DMPDDSFGlu for 48 h at the same concentration after 2 h exposure of the cells to FdUrd resulted in even higher syner-

Table 1. Effect of FdUrd, DMPDDSFGlu and DMPDDSFGly on survival of 5178Y and 5178Y/F cells.

The results are the means ± S.D. from 4–6 independent experiments.

Antimetabolite	I_{50} (μM)		I_{10} (μM)	
	5178Y cells	5178Y/F cells	5178Y cells	5178Y/F cells
FdUrd	0.002	0.010	0.001	0.05 ± 0.008
DMPDDSFGly	20.00 ± 1.00	30.00 ± 7.00	1.00 ± 0.10	3.00 ± 0.30
DMPDDSFGlu	3.00 ± 0.70	5.00 ± 1.00	0.20 ± 0.03	0.50 ± 0.03

gistic effect than when the drugs were added at the same time. A slightly lower synergistic inhibitory effect was observed with 5178Y/F cells irrespective of the succession of drug addition (Table 2). We assumed that these results might be related to the differences in the intracellular folate pool between 5178Y and 5178Y/F cells, since the intracellular concentration of folates i.e. $5,10CH_2H_4$-$PteGlu_n$ and $H_2PteGlu_n$ in 5178Y/F cells is about 3 times higher than that in the parental cell line 5178Y [18] (Table 3). This supposition proved to be correct as we have observed that leucovorin (5-CHO-H_4PteGlu), a reduced folate, added at 5 μM concentration together with FdUrd and one of the antimetabolites decreased the number of survived cells by 30% in both cell lines (Table 4) [19]; being without any effect on synergistic action of antifolates (data not shown). One has to take into account that the enzyme, a folate substrate ($5,10CH_2H_4PteGlu_n$) and the antimetabolite (FdUMP) form tertiary complex [20]. Stability of this complex depends on folate concentration [21–23]. Moreover the complex formed in the presence of dideazafolates was found to be more stable than the one formed without an inhibitor [21, 24] which is consistent with our observation (Table 4).

De novo thymidylate biosynthesis

DMPDDSFGlu and DMPDDSFGly exert a moderate inhibition of thymidylate synthesis *de novo* (below 35%) (Table 5). FdUrd showed

Table 2. Effect of low concentration (I_{10}) of FdUrd, DMPDDSFGly and DMPDDSFGlu on survival of 5178Y and 5178Y/F cells.

The drug concentration was the same as in Table 1. Results are the means ± S.D. from 4–6 independent experiments.

Addition			Cell survival (% of control)			
FdUrd	DMPDDSFGlu	DMPDDSFGly	5178Y cells		5178Y/F cells	
			observed	expected[c]	observed	expected[c]
–	–	–	100 ± 5		100 ± 6	
+	–	–	92 ± 1		98 ± 7	
–	+	–	90 ± 1		90 ± 6	
–	–	+	91 ± 3		90 ± 1	
+	+	–	21 ± 3	68.2	25 ± 2	66.5
+	–	+	29 ± 4	69.8	32.4 ± 2	66.7
+[a]	+[a]	–	7 ± 1	68.3	15 ± 7	61.3
+[b]	+[b]	–	15 ± 6	64.2	15 ± 7	67.4

[a]FdUrd was added 2 h after the cells were plated and DMPDDSFGlu was added after another 4 h for 48 h; [b]DMPDDSFGlu was added 2 h after the cells were plated and FdUrd was added after another 4 h for 48 h; [c]The expected values were calculated after [16].

Table 3. Effect of FdUrd combined with DMPDDSFGlu or DMPDDSFGly on cellular concentration of $5,10CH_2H_4PteGlu_n$ and $H_2PteGlu_n$.

The cells were cultured in the presence of drugs at I_{10} concentrations. For details see Materials and Methods. The results are means ± S.D. from 3–4 independent experiments.

Addition			Intracellular concentration (pmoles/mg protein)			
			$5,10CH_2H_4PteGlu_n$		$H_2PteGlu_n$	
FdUrd	DMPD-DSFGlu	DMPD-DSFGly	5178Y cells	5178Y/F cells	5178Y cells	5178Y/F cells
–	–	–	3.6 ± 0.47	10.1 ± 0.2	1.6 ± 0.20	3.1 ± 0.5
+	–	–	1.2 ± 0.20	5.8 ± 0.3	1.3 ± 0.30	2.5 ± 0.4
–	+	–	4.3 ± 0.20	10.3 ± 0.1	1.2 ± 0.10	2.4 ± 0.3
–	–	+	4.6 ± 0.17	9.8 ± 0.1	1.4 ± 0.20	2.2 ± 0.1
+[a]	–	+[a]	0.9 ± 0.20	3.2 ± 0.3	0.8 ± 0.10	1.8 ± 0.1
+[b]	+[b]	–	0.2 ± 0.07	10.0 ± 0.1	0.4 ± 0.07	0.8 ± 0.1

[a]DMPDDSFGly was added 2 h after the cells were plated and FdUrd was added after another 4 h; [b]FdUrd was added 2 h after the cells were plated and DMPDDSFGlu was added after another 4 h.

a much lower inhibition (below 20%) than could be expected from the cell survival. This lack of relation between the effects on cell survival and inhibition of *de novo* thymidylate synthesis suggested that a simple blocking of thymidylate biosynthesis can not be responsible for cytostatic action of FdUrd but it might indicate dissociation of thymidylate from DNA [25].

The combined effect of FdUrd with DMPDDSFGlu or DMPDDSFGly on thymidylate synthesis showed slight synergy in intact cells of both lines (Table 5). However, the assay gives no information on the mechanism of this effect.

Effect of FdUrd combined with DMPDDSFGlu or DMPDDSFGly on [6-^{3}H]dUrd incorporation into DNA. Incorporation of [6-^{3}H]dUrd into DNA was measured to compare FdUrd interference into DNA and thymidylate syntheses. More intensive incorporation of [6-^{3}H]dUMP into DNA in the presence of FdUrd than in the presence of either antifolate indicates than the inhibitory effect of FdUrd was mainly directed to DNA synthesis than towards inhibition of thymidylate synthesis (Table 5). Although antifolates inhibit DNA synthesis, this effect is not as great as their inhibition of thymidylate synthesis and it is probably indirect, due changes in concentration of deoxyribonucleoside-5'-phosphates [24, 25].

The effect of FdUrd combined with DMPDDSFGlu and DMPDDSFGly on the activity of γ-folylpolyglutamate synthase and γ-glutamyl hydrolase. The activities of these two enzymes crucial for cellular polyglutamylation were measured in the extracts of the cells treated with the drugs (Table 6). Only a moderate inhibitory

Table 4. Effect of 5 μM leucovorin (5-CHO-H_4PteGlu) on survival of 5178Y and 5178Y/F cells treated with FdUrd combined with DMPDDSFGlu or DMPDDSFGly at low concentrations (I_{10}).

The compounds are added together 2 h after cells were plated. Results are means ± S.D. from 3–4 independent experiments.

Addition			Cell survival (% of control)	
FdUrd	DMPDDSFGlu	DMPDDSFGly	5178Y cells	5178Y/F cells
+	–	+	60 ± 8	60 ± 10
+	+	–	41 ± 3	45 ± 2

Table 5. Effect of FdUrd combined with DMPDDSFGlu or DMPDDSFGly at low concentration (I_{10}) on *de novo* thymidylate biosynthesis in 5178Y and 5178Y/F cells.

The cells were cultured in the presence of the drug as described in Table 2. *De novo* thymidylate synthesis was measured by incorporation of [5-^{3}H]dUrd or [6-^{3}H]dUrd into DNA as described in Materials and Methods. The results are the means ± S.D. from 3 independent experiments.

Addition			Incorporation (% of control)			
FdUrd	DMPD-DSFGlu	DMPD-DSFGly	[5-^{3}H]dUrd		[6-^{3}H]dUrd	
			5178Y cells	5178Y/F cells	5178Y cells	5178Y/F cells
–	–	–	100 ± 1	100 ± 4	100 ± 5	100 ± 3
+	–	–	72 ± 2	81 ± 5	78 ± 3	81 ± 4
–	+	–	56 ± 4	51 ± 1	42 ± 4	46 ± 5
–	–	+	69 ± 3	65 ± 4	41 ± 1	40 ± 6
+	+	–	41 ± 1	38 ± 3	62 ± 8	63 ± 2
+	–	+	44 ± 1	44 ± 3	64 ± 6	60 ± 1
+[a]	+[a]	–	19 ± 1	18 ± 1	75 ± 2	77 ± 3
+[b]	+[b]	–	30 ± 1	26 ± 3	64 ± 1	52 ± 3

effect on folyl-γ-polyglutamate synthetase activity was observed in the cells exposed to DMPDDSFGlu, probably because of drug polyglutamylation (Table 6).

Effect of FdUrd combined with DMPDDSFGlu and DMPDDSFGly on cellular concentration of $H_2PteGlu_n$ and $5,10CH_2H_4PteGlu_n$. During continuous exposure of 5178Y and 5178Y/F cells to 1 nM and 50 nM FdUrd, respectively, the cellular pool of $5,10CH_2H_4PteGlu_n$ was reduced approximately by 50% with only 20% of reduction in thymidylate synthesis as measured by [5-^{3}H]deoxyuridylate incorporation (Tables 3, 5). The cellular pool of $H_2PteGlu_n$ under the same conditions was slightly decreased. When the cells of either line were exposed to DMPDDSFGly or DMPDDSFGlu at I_{10} con-

Table 6. Effect of FdUrd, DMPDDSFGlu and DMPDDSFGly at I_{10} concentration on the activity of folylpolyglutamate synthetase (FPGS) and γ-glutamyl hydrolase in 5178Y and 5178Y/F cells.

The drugs were added to the cell culture at the same time. The results are means ± S.D. from 3–6 independent experiments.

Addition			Enzyme activity (nmoles/h per mg protein)			
FdUrd	DMPD-DSFGlu	DMPD-DSFGly	FPGS		γ-Glutamyl hydrolase	
			5178Y cells	5178Y/F cells	5178Y cells	5178Y/F cells
–	–	–	0.32 ± 0.01	0.24 ± 0.07	14.0 ± 3.0	18.0 ± 2.0
+	–	–	0.31 ± 0.01	0.20 ± 0.04	13.0 ± 3.0	19.0 ± 3.0
–	+	–	0.18 ± 0.04	0.08 ± 0.01	16.3 ± 4.0	19.0 ± 2.0
–	–	+	0.28 ± 0.04	0.18 ± 0.02	19.0 ± 2.0	22.0 ± 3.0
+	–	+	0.28 ± 0.04	0.19 ± 0.02	16.0 ± 1.0	20.0 ± 1.0
+	+	–	0.17 ± 0.01	0.09 ± 0.02	19.0 ± 3.0	22.0 ± 3.0

centrations the $5,10CH_2H_4PteGlu_n$ pool increased by about 25%, similarly as deoxyuridylate incorporation. The inhibitory effect of combined drugs on cellular pools of the two folates depended on the sequence of their addition. When the cells were exposed first to 0.001 μM FdUrd and then to 0.02 μM or 0.003 μM DMPDDSFGly or DMPDDSFGlu, respectively, the inhibitory effect on cellular pool of $5,10CH_2H_4PteGlu_n$ was as high as 80% (Table 3). In comparison, a 2 h exposure of the cells to antifolates followed by incubation with FdUrd (at I_{10} concentration) resulted in a 65% decrease of the $5,10CH_2H_4PteGlu_n$ intracellular pool.

These results suggest that TS inhibitors, FdUrd and dideazafolates (DMPDDSFGlu or DMPDDSFGly), can act synergistically and effect of synergy does not depend on TS level in the cells either in 5178Y or 5178Y/F.

It is hoped that application of combined drugs-TS inhibitors may lead to better understanding of the interactions between intracellular folates, DNA and thymidylate synthesis and to a successful use of these drugs in cancer therapy.

REFERENCES

1. Myers, C.E. (1985) The pharmacology of the fluoropyrimidines. *Pharmacol. Rev.* **331,** 1–15.
2. Langeback, R.J., Danenberg, P.V. & Heidelberger, C. (1972) Thymidylate synthase mechanism of inhibition by 5-fluoro-2-deoxyuridylate. *Biochem. Biophys. Res. Commun.* **48,** 1565–1571.
3. Danenberg, P.V. & Lockshin, A. (1982) Thymidylate synthase-substrate complex formation. *Mol. Cell. Biochem.* **43,** 49–57.
4. Danenberg, P.V., Heidelberger, C., Mulkins, M. & Petterson, A.R. (1981) The incorporation of 5-fluoro-2′-deoxyuridine into DNA of mammalian tumor cells. *Biochem. Biophys. Res. Commun.* **102,** 654–658.
5. Cory, J.G., Berland, J.C. & Carter, G.C. (1979) Effect of fluorouracil on RNA metabolism of Novikoff hepatoma cells. *Cancer Res.* **39,** 4905–4913.
6. Akazava, S., Kumai, R., Yoshida, K., Ayusawa, D., Shimizu, K. & Seno, T. (1986) The cytotoxicity of 5-fluorouracil is due to its incorporation into RNA not its inhibition of thymidylate sythase evidenced by the use of a mouse cell mutant deficient in thymidylate synthase. *Jpn J. Cancer Res.* **77,** 620–624.
7. Jones, T.R., Calvert, A.H., Jackman, A.L., Brown, S.J. & Harrap, K.R. (1981) A potent antitumor quinazoline inhibitor of thymidylate synthase: Synthesis, biological properties and therapeutic results in mice. *Eur. J. Cancer* **17,** 11–19.
8. Pawełczak, K., Makowski, M., Kempny, M., Dzik, J.M., Balińska, M. & Rode, W. (1993) Sulphonamide antifolates inhibiting thymidylate synthesis, enzyme inhibition and cytotoxicity. *Adv. Exp. Med. Biol.* **338,** 625– 627.
9. Jacewicz, D., Kaczorowska, K., Szablewska, I. & Balińska, M. (1995) Effect of antifolates on intracellular level of folates and folate enzymes in 5178Y murine leukemia cells. *J. Exp. Clin. Cancer Res.* **14,** 24–26.
10. Bretner, M., Kulikowski, T., Dzik, J.M., Balińska, M., Rode, W. & Shugar, D. (1993) 2-Thioderivatives of dUrd and 5-fluoro dUrd and their 5′monophosphates, synthesis, interaction with tumour thymidylate synthase and *in vitro* antitumour activity. *J. Med. Chem.* **36,** 3613–3617.
11. McGuire, J.J., Hsieh, P., Coward, J.K. & Bertino, J.R. (1980) Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. *J. Biol. Chem.* **255,** 5776–5788.
12. Sikora, E., Krzyżanowski, L. & Rzeszotarska, B. (1989) A rapid, sensitive, nonradioactive assay for serum γ-glutamyl hydrolase using synthetic *N*(4-aminobenzoyl)-γ-oligo(L-glutamic acids) as substrates. *Pteridines* **1,** 175–178.
13. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.
14. Hryniuk, W.M., Fisher, G.A. & Bertino, J.R. (1969) S-phase cells of rapidly growing and

resting populations. Differences in response to methotrexate. *Mol. Pharmacol.* **5,** 557–564.

15. Bunni, M., Doig, M.T., Donato, H., Kesaran, V. & Priest, D.G. (1988) Role of methylenetetrahydrofolate depletion in methotrexate-mediated intracellular thymidylate synthesis inhibition in cultured L1210. *Cancer Res.* **48,** 3398–3404.

16. Chou, T.C. & Talalay, P. (1984) Quantitative analysis of dose-effect relationship: The combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22,** 27–55.

17. Sikora, E., Jackman, A.L., Newell, D.R. & Calvert, A.H. (1988) Formation and retention and biological activity of N^{10}-propargyl-5,8-dideazafolic acid (CB3717) polyglutamates in L1210 cells *in vitro*. *Biochem. Pharmacol.* **37,** 4047–4054.

18. Balińska, M. (1986) Synthesis and retention of methotrexate polyglutamates in Ehrlich ascites carcinoma cells. *Drugs Exptl. Clin. Res.* **12,** 551–554.

19. Moran, R.G. (1989) Leucovorin enhancement of the effect of the fluoropyrimidines on thymidylate synthesis. *Cancer* **63,** 1008–1012.

20. Carreras, C.W. & Santi, D.V. (1995) The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **64,** 721–762.

21. Monfort, W.R., Perry, K.M., Fauman, E.B., Fiszer-Moore, J.S., Maley, G.F., Hardy, L., Maley, F. & Stroud, R.M. (1990) Structure, multiple site binding and segmental accomodation in thymidylate synthase on binding dUMP and an antifolate. *Biochemistry* **29,** 6964–6976.

22. Fiszer-Moore, J.S., Monfort, W.R. & Stroud, R.M. (1990) Pairwise specificity and sequential binding in enzyme catalysis: Thymidylate synthase. *Biochemistry* **29,** 6977–6986.

23. Perry, K.M., Carreras, C.W., Chang, L.C. & Santi, D.V. (1993) Structures of thymidylate synthase with C-terminal deletion. Role of the C-terminus in alignment of 2'deoxyuridine-5'-monophosphate and 5,10-methylenetetrahydrofolate. *Biochemistry* **32,** 7116–7125.

24. Pogolotti, A., Dannenberg, P.V. & Santi, D.V. (1986) Kinetics and mechanism of interaction of 10-propargyl-5,8-dideazafolate with thymidylate synthase. *J. Med. Chem.* **29,** 478–482.

25. Fernandes, D.J. & Cranford, S.K. (1986) Dissociation of thymidylate biosynthesis from DNA biosynthesis by 5-fluoro-2'-deoxyuridine and dideazaisofolic acid. *Cancer Res.* **46,** 1741–1747.