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QUARTERLY

The effects of combined antifolates on inhibition of growth of murine leukemia cells cultured *in vitro**

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The synergistic effect of trimetrexate (TMTX) and sulphonamide derivatives of quinazoline on the cultured 5178Y murine leukemia cells was examined. On exposure to the slightly inhibitory concentrations of TMTX (0.1 nM) in combination with 2-desamino-2-methyl-10-propargyl-5,8-dideaza-pteroyl-sulphoglycine (DMPDDSF) (0.02 μM) a synergistic inhibitory effect of the antifolates on cell growth was observed. These two drugs in the same combination caused also synergistic inhibition of *de novo* synthesis of thymidylate in intact cells as measured by tritium release from [5-^{3}H]deoxyuridylate. This was accompanied by a marked reduction in intracellular concentration of 5,10-methylenetetrahydro-pteroyl-polyglutamate (5,10CH_2H_4PteGlu$_n$) (0.2 μM) and dihydropteroyl-polyglutamate (0.12 μM). In these conditions *de novo* biosynthesis of purine was decreased by 50%.

These observations show that growth inhibition by combined antifolates is mediated by intracellular depletion of the substrate of thymidylate synthase — 5,10CH_2H_4PteGlu$_n$. The results obtained strongly suggest that under certain conditions inhibition of thymidylate synthesis by DMPDDSF is intensified by prior application of TMTX — an inhibitor of dihydrofolate reductase.

Inhibitors of folate metabolism have long been known to suppress the cell-growth. For this reason structural analogues of folic acid, antifolates, have been approved for use in the chemotherapy of neoplastic diseases [1–4] and a number of disorders related to immune and inflammatory systems [5, 6]. Most of these compounds that have been developed, represented by 4-amino-10-methyl analogue of folic acid, methotrexate (MTX), are the tight-binding inhibitors of dihydrofolate reductase (DHFR, EC 1.5.1.3) [7, 8]. In addition, intracellular metabolites of MTX — its γ-glutamate derivatives, which, besides dihy-

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Abbreviations: AICAR, aminoimidazolecarboxyamide ribonucleotide; 5,10CH_2H_4PteGlu$_n$, 5,10-methylenetetrahydro-pteroyl-polyglutamate; DHFR, dihydrofolate reductase; DMPDDSF, 2-desamino-2-methyl-10-propargyl-5,8-dideaza-pteroyl-sulphoglycine; FdUMP, fluorodeoxyuridine-5′-monophosphate; GAR, glycinamide ribonucleotide; H_2PteGlu$_n$, dihydropteroyl-polyglutamate; MTX, methotrexate (4-amino-10-methylpteroylglutamic acid); n, indicates the number of γ-glutamate residues; PDDF, 10-propargyl-5,8-dideazafolic acid; TMTX, trimetrexate; TS, thymidylate synthase.

drofolate reductase, inhibit also other enzymes involved in folate metabolism: thymidylate synthase (TS, EC 2.1.1.45), glycinamide ribonucleotide and aminoimidazolecarboxyamide ribonucleotide formyltransferases (GAR and AICAR formyl transferases, EC 2.1.1.21 and EC 2.1.1.22) [9–11]. That is why MTX is a very powerful inhibitor with a low specificity. Recently developed analogues [12] have enhanced specificity and ability to inhibit also other enzymes of folate metabolism. Dihydrofolate reductase is a sole target of lipophilic antifolate trimetrexate (TMTX) [13]. The derivatives of 10-propargyl-5,8-dideazafolic acid (PDDF) and their intracellular metabolites, γ-glutamates, are very potent inhibitors of TS [13, 14]. Both TMTX and PDDF have been shown to be active as cell-growth inhibitors in many *in vitro* and *in vivo* models [12, 13]. However, much less data are available concerning the effect of combined action of TMTX and non-polyglutamable derivatives of PDDF with a low cytostatic activity [15–17].

In our study on the synergistic effect of antifolates on cell growth inhibition we used two inhibitors which can not be converted to polyglutamates — TMTX and a newly synthesized sulphonamide derivative of 10-propargyl-5,8-dideazafolate (PDDF) 2-desamino-2-methyl-10-propargyl-5,8-dideazapteroyl- sulphoglycine (DMPDDSF) (Fig. 1). Either inhibitor exhibits a much weaker effect than MTX or PDDF, respectively, and enters the cell independently [18]. Our study has been concentrated on the ability to predict the mechanism of effective synergistic action of low doses of these antifolates on cell-growth inhibition.

MATERIALS AND METHODS

Materials

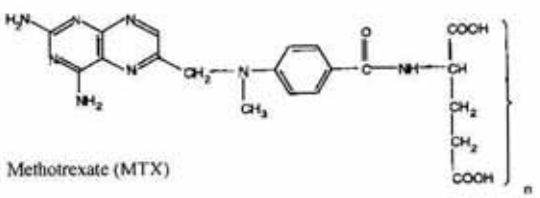
Fisher's medium, Dulbecco medium, bovine fetal serum, bovine new born serum and trypsin were purchased from Grand Island Biochemical Company, Life Technologies Ltd., Paisley, Scotland.

10-Propargyl-5,8-dideazafolic acid (PDDF) and its *p*-aminosulphonyl derivative (DMPDDSF) were purchased or synthesized as described previously [18, 19].

Trimetrexate (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.). Dihydrofolate was prepared according to the method of Blakley [20]. pABAGlu$_n$ was a gift from Professor Rzeszotarska and Dr. Krzyżanowski from the Opole University.

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

All other chemicals were of reagent grade.



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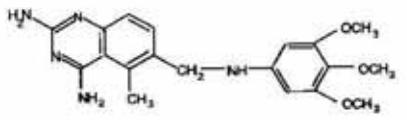
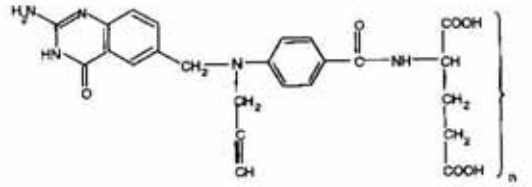
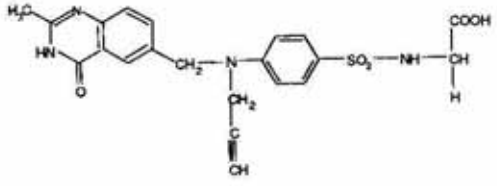


Trimetrexate (TMTX)



10-Propargyl-5,8-dideazafolic acid (PDDF)



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

Figure 1. Chemical structures of antifolates.

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 [18, 19, 21].

Growth inhibition study. The 5178Y cells in logarithmic phase of growth 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 indicated concentration and the cells were counted in Neubauer camera using the trypan-blue staining method.

Enzyme assays. For enzyme assays cells were collected by centrifugation, washed twice in ice cold phosphate buffer-saline, sonicated in Branson Sonifier 250 sonicator in 100 mM phosphate buffer (pH 7.4) containing 10 mM mercaptoethanol and centrifuged for 20 min at 20000 × ***g***. The supernatant was used for enzyme assay.

Folylpolyglutamate synthetase was assayed according to McGuire *et al.* [22] using tetrahydrofolate (250 µM) as a substrate.

γ-Hydrolase was assayed in cell extracts according to Sikora *et al.* [23], 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.

Protein was estimated by the method of Bradford [24].

Thymidylate biosynthesis. Deoxyuridylate incorporation was used to measure thymidylate biosynthesis *de novo* by the cells cultured *in vitro*. Following the exposure of the cells to a drug the culture was exposed to 1 µCi of [5-^{3}H]dUrd for 40 min and radioactivity incorporation was measured as described previously [17]. The results were expressed as pmol/well.

Purine biosynthesis. Incorporation of 20 µM [2-^{14}C]glycine into DNA was measured as described previously [15, 17, 18].

Estimations of 5,10CH_2H_4PteGlu$_n$ and H_2PteGlu$_n$. Cells after exposure to the drugs were extracted by boiling with 50 mM Tris, 1 mM EDTA and 50 mM ascorbate (pH 7.4) and 5,10CH_2H_4PteGlu$_n$ was measured in the supernatant as described by Priest and coworkers [25, 26]. Then the samples were incubated with 10 pmol of [^{3}H]FdUMP and 10 pmol of bovine thymidylate synthase for 30 min in the 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$_n$ was quantitated following separation of unreacted [^{3}H]FdUMP by G-25 Sephadex filtration. H_2PteGlu$_n$ was reduced 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 by using thymidylate synthase reaction. The amount of dihydrofolate was calculated by subtraction of the amount of 5,10CH_2H_4PteGlu$_n$ present in the sample prior to the treatment from the amount following dihydrofolate reductase addition.

Standard curves were generated using 5,10CH_2H_4PteGlu$_7$ and H_2PteGlu$_7$. The results were expressed as nmol/g cell protein, which was converted to cell volume by a factor of 2.97 [17, 18].

Functional interactions between drugs. The combined drug effect was evaluated by using the Chou & Talalay [27] analysis based on median effect principle and the concentration-effect analysis statistical program.

RESULTS AND DISCUSSION

Growth inhibition studies

Inhibitory activities of antifolates, inhibitors of DHFR — MTX, TMTX, and inhibitors of TS — PDDF and DMPDDSF are shown in Table 1.

Inhibition by MTX was the highest and showed I_{50} approximately of 0.5 nM after continuous exposure to the drug. PDDF and DMPDDSF exhibited the weakest inhibitory effect — having I_{10} of 0.5 nM and 20 nM, respectively. The relative toxicity of MTX, TMTX, PDDF is consistent with their activity *in vitro* [17, 21, 26, 28].

To examine a possible drug interaction of the two antifolates acting at different enzymes growth inhibition by TMTX and DMPDDSF at low inhibitory concentration was tested alone and in combination (Table 2).

Table 1. Growth inhibition of 5178Y cells by MTX, TMTX, PDDF and DMPDDSF.

The cells were plated at a density of 1×10^4 cells/well. After 2 h the drugs were added for 48 h, than the cells were counted *in situ*. The results are the mean ± S.D. from 4–6 independent experiments.

Antifolate	I_{50} (μM)	I_{10} (μM)
MTX	0.0005 ± 0.00005	0.00001 ± 0.00001
TMTX	0.008 ± 0.0005	0.0001 ± 0.00001
PDDF	0.5 ± 0.01	0.02 ± 0.007
DMPDDSF	20.0 ± 0.1	1.0 ± 0.01

The former is a weaker inhibitor of DHFR than MTX and the latter a weaker inhibitor of TS than PDDF.

Continuous exposure of 5178Y cells successively to TMTX and DMPDDSF at low (I_{10}) concentration caused a higher inhibition than expected from the sum of their separate effects. Evaluation of the data by median effect suggested a synergistic effect [27]. Addition of DMPDDSF for 48 h at low concentration after 2 h exposure to TMTX resulted in an even greater synergy than when the drugs were added together at the same time (Table 2).

De novo thymidylate and purine biosynthesis

A synergistic inhibition by the antifolates would be expected to result from simultaneous action of the drugs on more than one folate dependent pathway [15, 18]. It is known that these inhibitors can inhibit both biosynthesis of purine and thymidylate. One of the ways to test thymidylate synthesis in intact cells is to evaluate *de novo* synthesis of thymidylate from [5-^{3}H]deoxyuridylate, this being the relative measure of the thymidylate synthase activity. Cells were grown in the presence of the drugs at concentrations shown in Table 2, followed by 4 h exposure to [5-^{3}H]deoxyuridylate. The data demonstrated (Table 3) that each drug alone exerted only modest inhibition (< 35%), while the combination of TMTX and DMPDDSF resulted in synergistic inhibition of TS in intact 5178Y cells. These results indicate that the synergistic effect of the combined action of these drugs consists in enhancement of the inhibitory effect of either antifo-

Table 2. Effect of TMTX and DMPDDSF on cell growth.

The cells were plated and counted as described for Table 1. Results are the mean ± S.D. from 4–6 independent experiments.

Additions (μM)		Number of living cells (% of control)	
TMTX	DMPDDSF	Observed	Expected
–	–	100 ± 6	
0.0001	–	91 ± 3	
–	0.02[a]	90 ± 7	
0.0001[a]	0.02[a]	27 ± 6	62.5[a]
0.0001[b]	0.02[b]	7 ± 4	60.5[d]
0.0001[c]	0.02[c]	22 ± 2	60.5[d]

[a]Drugs were added at the same time, 2 h after the cells were plated; [b]DMPDDSF was added 2 h after the cells were plated, and TMTX was added after further 4 h; [c]TMTX was added 2 h after the cells were plated and DMPDDSF was added after further 4 h; [d]Expected cell number is predicted by the product of fractional inhibition of two agents assuming summation [27].

late. However, the assay gives no information concerning the mechanism of this effect.

Measurement of incorporation of [2-^{14}C]glycine into DNA is used to estimate *de novo* purine biosynthesis and is to some extent relative to the activity of GAR and AICAR transformylases. The results show (Table 3) that the inhibition of *de novo* synthesis of purine was much less sensitive than thymidylate synthesis to either drug, and demonstrated a lack or a very low synergistic effect of the drugs on this process.

tamylation process of endogenous folates were performed in extracts of the cells treated with the drugs (Table 4). Only a slight inhibitory effect (about 25%) was observed in the cells treated with both drugs; this is probably connected with a depletion of folates in the cells treated with combined drugs (Table 5) [17, 18].

Effect of TMTX and DMPDDSF on cellular concentration of 5,10CH$_2$H$_4$PteGlu$_n$ and H$_2$PteGlu$_n$. To elucidate the mechanism responsible for the synergistic in-

Table 3. Effect of TMTX and DMPDDSF on *de novo* thymidylate synthesis and purine synthesis in 5178Y cells.

The cells were cultured in the presence of drugs as described in Table 2. *De novo* thymidylate synthesis was measured by incorporation [5-^{3}H]deoxyuridylate and *de novo* purine synthesis was estimated by incorporation of [2-^{14}C]glycine into DNA as described in Material and Methods. The results are the mean ± S.D. from 4–6 independent experiments.

Addition (μM)		Incorporation (%)	
TMTX	DMPDDSF	[2-^{14}C]Gly	[5-^{3}H]dUrd
–	–	100 ± 4	100 ± 2
0.0001	–	–	80 ± 4
–	0.02	110 ± 7	65 ± 4
0.0001[a]	0.02[a]	75 ± 5	24 ± 7
0.0001[b]	0.02[b]	52 ± 3	8 ± 1

[a]The drugs were added at the same time; [b]DMPDDSF was added 2 h after the cells were plated and TMTX was added after further 4 h.

Effect of TMTX and DMPDDSF on activity of γ-folylpolyglutamate synthetase and γ-glutamyl hydrolase. The activities of these two enzymes crucial for polyglu-

hibitory effect on DHFR and TS we have examined the intracellular concentration of the folate substrate (5,10CH$_2$H$_4$PteGlu$_n$) and the product (H$_2$PteGlu$_n$) nf thymidylate

Table 4. Effect of TMTX and DMPDDSF on activity of folylpolyglutamate synthetase (FPGS) and γ-hydrolase in 5178Y cells.

The cells were cultured in the presence of drugs as described in Table 2. Enzyme activity was performed in cell extracts as described in Materials and Methods. Results are the mean ± S.D. from 4 independent experiments.

Addition (μM)		Enzyme activity (nmoles/mg protein per h)	
TMTX	DMPDDSF	FPGS	γ-Hydrolase
–	–	0.32 ± 0.07	14.0 ± 2
0.0001	–	0.32 ± 0.08	17.2 ± 1
–	0.02	0.28 ± 0.04	19.1 ± 3
0.0001[a]	0.02	0.20 ± 0.09	12.3 ± 3

[a]The drugs were added to the cell culture in the same time.

synthase. When the cells were exposed to 0.1 nM TMTX the cellular pool of $5,10CH_2H_4$-$PteGlu_n$ was reduced approximately by 50% with only 20% reduction in deoxyuridine incorporation (Tables 3, 5). Increased concentration of TMTX caused a marked reduction of the $5,10CH_2H_4PteGlu_n$ pool (not shown). The intracellular pool of $H_2PteGlu_n$ under the same conditions was increased (Table 5), consistently with the inhibition of DHFR [17, 25].

The capacity of DMPDDSF to reduce the pools of $5,10CH_2H_4PteGlu_n$ and $H_2PteGlu_n$ is shown in Table 5.

When the cells were exposed to 0.02 μM DMPDDSF, the cellular pool of $H_2PteGlu_n$ decreased by about 20% and the $5,10CH_2H_4$-$PteGlu_n$ pool increased by 35%, parallelly to [5-^{3}H]deoxyuridylate incorporation.

The inhibitory effect of combined drugs on intracellular pools of the two folates depends on the sequence of their addition. When the cells were exposed first to 0.02 μM DMPDDSF and later to the 0.1 μM TMTX, at least 50% inhibition was observed. In comparison, when 4 h exposure of the cells to 0.1 nM TMTX was followed by incubation with 0.02 μM DMPDDSF, the inhibitory effect on intracellular pools of $5,10CH_2H_4PteGlu_n$ and $H_2PteGlu_n$ reached 93% (Table 4).

These results show that two antifolates, TMTX and DMPDDSF, which have different enzyme targets in tumor cells [15], act synergistically.

It is hoped that utilization of this and similar combinations of drugs suggested by Diddens *et al.* [16] and others authors [15, 17, 18, 25, 26] may lead to a better understanding of the interaction between folates and their cellular targets and to efficient use of these agents in therapy of neoplastic diseases. It is apparent from these results that concentration and sequence of addition of two antifolates acting on different enzymes is extremely important in studies *in vivo*, since low concentration of DMPDDSF can enhance inhibition of growth by TMTX. Thus, the agents which can reduce thymidylate synthase activity in cells exert a synergistic effect on the cell growth preventing the activity of dihydrofolate reductase inhibitors [15–17]. This is due to the reduction of the activity of thymidylate synthase causing a rapid depletion of tetrahydrofolate pool, e.g. $5,10CH_2H_4PteGlu_n$ which have reached approximately the level of 0.2 μM (Table 5), [14, 15] and to the loss of ability to synthesize thymidylate by 5178Y cells treated with TMTX [26]. On the other hand, as suggested by Goldman's group [28, 29], increased concentration of $H_2PteGlu_n$ also can cause inhibition of thymidylate synthase, but this seems to play a less important role in the inhibitory process. However, it should be

Table 5. Effect of TMTX and DMPDDSF on cellular $5,10CH_2H_4PteGlu_n$ and $H_2PteGlu_n$ concentration.

The cells were cultured in the presence of drugs as described in Table 2. The cells were washed twice with PBS and extracted with 50 mM Tris, 1 mM EDTA, 50 mM ascorbate buffer, pH 7.4. The concentrations of $5,10CH_2H_4PteGlu_n$ and $H_2PteGlu_n$ were measured as described in Methods. The results are the mean ± S.D. from 3–4 independent experiments.

Additions (μM)		Intracellular concentration (μM)	
TMTX	DMPDDSF	$5,10CH_2H_4PteGlu_n$	$H_2PteGlu_n$
–	–	3.6 ± 0.47	1.6 ± 0.38
0.0001	–	1.7 ± 0.32	2.1 ± 0.01
–	0.02	4.8 ± 0.17	1.4 ± 0.1
0.0001[a]	0.02[a]	1.8 ± 0.42	0.75 ± 0.3
0.0001[b]	0.02[b]	0.2 ± 0.01	0.12 ± 0.01

[a]The drugs were added at the same time; [b]DMPDDSF was added 2 h after the cells were plated and TMTX was added after further 4 h.

noted that numerous parameters including altered cellular drug transport or retention, affects the target enzyme levels, the folate pools, and the cell cycle kinetics. Presumably other factors which are not yet understood could also be involved in the observed synergy. Irrespectively of the mechanism that is operating in these processes the results obtained suggest that the growth inhibitory activity of DMPDDSF can be increased nearly by two orders of magnitude (DMPDDSF alone — 20 μM, in combination with TMTX — 0.1 μM) in the presence of the dihydrofolate reductase inhibitor.

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