Biochemical Pharmacology, Vol. 25, pp. 681–685. Pergamon Press, 1976. Printed in Great Britain.

LIMITED ABILITY OF THYMIDINE TO RELIEVE MITOTIC INHIBITION BY PYRIMETHAMINE IN HUMAN FIBROBLASTS

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(*Received* 27 *June* 1975; *accepted* 22 *September* 1975)

Abstract—Pyrimethamine (PY), an antimalarial drug with antileukaemic properties, is a folate analogue which inhibits the formation of tetrahydrofolic acid (THFA). As a result it blocks the biosynthesis of glycine, methionine, purines, pantothenate and thymidine. It also prevents the formylation of methionyl-tRNA and hence precludes mitochondrial protein synthesis. In human fibroblast cultures treated with PY at concentrations of 30, 100 or 200 μg/ml in the presence of the essential metabolites listed above, except thymidine, DNA synthesis and mitosis were arrested. The ability of thymidine to prevent this depended on the PY concentration and the duration of exposure to the drug. Thus, in the presence of thymidine, cells cultured in 30 μg/ml PY exhibited normal growth even after 2 days in contact with the drug; at 100 μg/ml PY, DNA synthesis continued but there was a reduction in the mitotic rate, which became more marked as the exposure time to the drug increased; at 200 μg/ml PY, there was no DNA synthesis or mitosis. These results indicate that the antimitotic effect of PY cannot be wholly accounted for by thymidine starvation. Oxygen uptake measurements showed a markedly decreased respiratory activity in fibroblasts which had been in contact with 100 μg/ml PY for 24 hr in the presence of the above metabolites, including thymidine. It is proposed that a major factor in the antimitotic activity of PY is the inhibition of mitochondrial protein synthesis.

Pyrimethamine (PY) is in current use as an antimalarial drug and has also been shown to be of value in the treatment of meningeal leukaemia [1]. This compound, as well as the two related anti-tumor agents in clinical use, 2:4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine (BW5097) [2] and methotrexate [3], is a folate analogue which inhibits dihydrofolate reductase and thereby blocks the conversion of dihydrofolate to tetrahydrofolate (THFA). There is also evidence that folate analogues may directly inhibit thymidylate synthetase and at least one enzyme involved in purine biosynthesis [4]. The net result of these effects is to block the biosynthesis of glycine, methionine, purines, pantothenate, thymine, and the formylation of methionyl-tRNA, all of these requiring the transfer of C_1-fragments from the THFA coenzymes. These metabolites, with the exception of the tRNA, can be supplied exogenously in culture media. The significance of formylmethionyl-tRNA is seen in recent findings that mitochondrial protein synthesis, in common with bacterial protein synthesis, requires this molecule for its initiation [5, 6]. (Cytoplasmic protein synthesis in eukaryotic cells does not have this requirement.) In studies with yeast it was demonstrated that blockage of THFA formation by PY affects both DNA synthesis and mitochondrial protein synthesis [7]. The first effect is relieved by the addition of thymidine monophosphate (TMP) to the growth medium of those yeast strains capable of assimilating this compound, but the cells still fail to grow unless conditions permit glycolysis [8]. In human cells, blockage of folate reductase would likewise be expected to lead to the arrest of mitochondrial protein synthesis, which has been shown to be a prerequisite for normal mitotic division in fibroblasts [9]. In the present work we have attempted to study the antimitochondrial effect of pyrimethamine in human fibroblast cultures in the presence of an exogenous supply of those small molecules whose biosynthesis requires THFA.

MATERIALS AND METHODS

Cells and growth media. All fibroblasts used for these studies were normal human diploid cells derived from skin biopsies, none having undergone more than 22 culture passages. Cells were grown in glass medicine bottles. All experiments utilised cells from confluent cultures which were plated in Eagle's Minimum Essential Medium (MEM), supplemented with human serum (20%), Hepes buffer (10 mM), glutamine (0·02 mM), gentamycin (48 μg/ml), pencillin (100 International Units/ml) and incubated at 37°. This medium is referred to hereafter as the basal growth medium. Eagle's MEM contains methionine and pantothenate. Glycine (0·66 mM) was always added to the medium unless otherwise stated. Hypoxanthine, present in serum [10], provided a readily available purine source.

Scoring for mitosis. Cells were plated at 10^5/ml in 1·5 ml of basal growth medium in 3 cm Nunclon petri-dishes containing a glass coverslip. Cells were harvested by removing a coverslip, rinsed in 0·9% saline, fixed in 95% methanol and stained by the Feulgen procedure. Mitoses were scored using an oil-immersion objective.

Feulgen microdensitometry of nuclear DNA. DNA values of Feulgen stained interphase nuclei, as scored

for mitoses in Figs. 1 and 2, were measured with a Barr and Stoud integrating microdensitometer. The readings were converted into logarithms to the base 2 and delineated into the 3 major DNA classes, 2C, 4C and intermediate as described previously [11]. Nuclei with the 2C amount of DNA were classed as G_1, those with twice this amount as G_2, while nuclei with intermediate values were regarded to be in the S phase of DNA synthesis. A small proportion of cells had DNA values greater than 4C and these must have been polyploid.

Growth curves. Growth curves of fibroblasts were obtained essentially as described by Raff and Houck [12]. Fibroblasts were seeded into Leighton tubes at a density of approx 35×10^2 cells/cm^2. These conditions permit logarithmic growth to continue for at least 4 days. Cell counts were made using an inverted microscope and an eyepiece graticule (E_{35}, Graticules Ltd.). Tubes were set up in duplicate and average cell counts per tube were calculated from 10 random samples along the median of the tube, edge effects being avoided. Each point plotted is the mean of 2 replicates.

Oxygen uptake. Oxygen uptake of fibroblasts was measured polarographically in a Clark type oxygen electrode. Approximately 6×10^6 cells in the logarithmic phase of growth were used for both the control and the treatment. Control and PY treated mono-

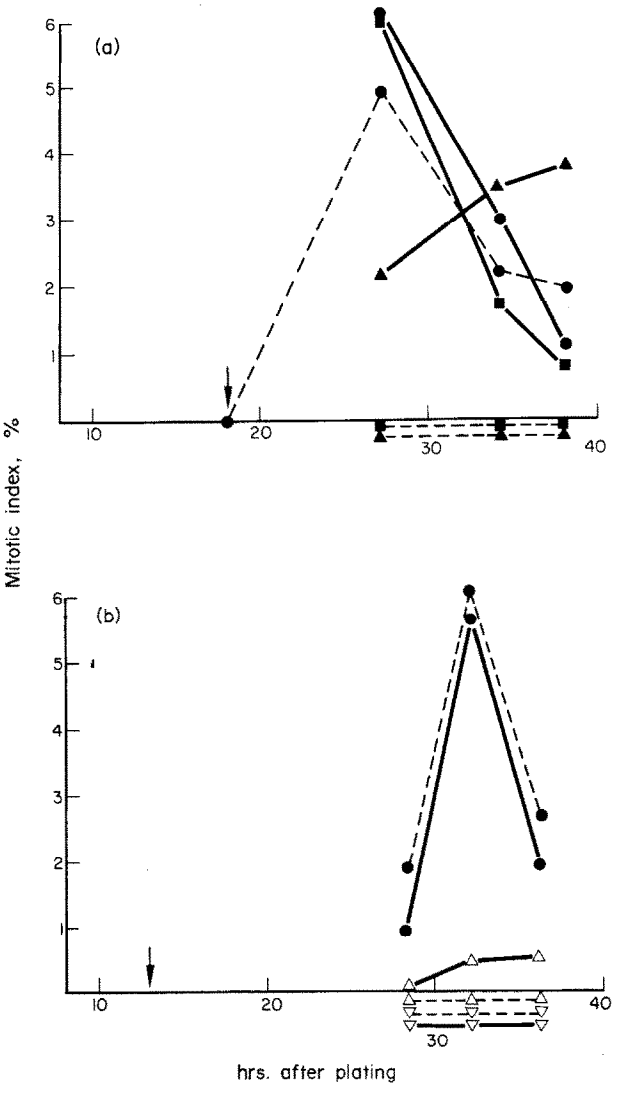

Fig. 1. The effect of exogenous thymidine on the anti-mitotic action of pyrimethamine (PY). (a) Effects of 30 and 100 μg/ml PY on cell growth in the presence (+T) and absence (−T) of exogenous thymidine (0·05 mM). The arrow indicates addition of drug in late S (18 hr after plating). Control − T (●----●), control + T(●——●), 30 μg/ml PY − T (■----■), 30 μg/ml PY + T (■——■), 100 μg/ml PY − T (▲ ▲), 100 μg/ml PY + T (▲——▲). (b) Effects of 100 and 200 μg/ml PY on cell growth in the presence (+T) and absence (−T) of exogenous thymidine (0·15 mM). The arrow indicates the addition of drug in late G_1/early S (13 hr after plating). Control − T (●----●), control + T (●——●), 100 μg/ml PY − T (△----△), 100 μg/ml PY + T (△——△), 200 μg/ml PY − T (▽----▽), 200 μg/ml PY + T (▽——▽).

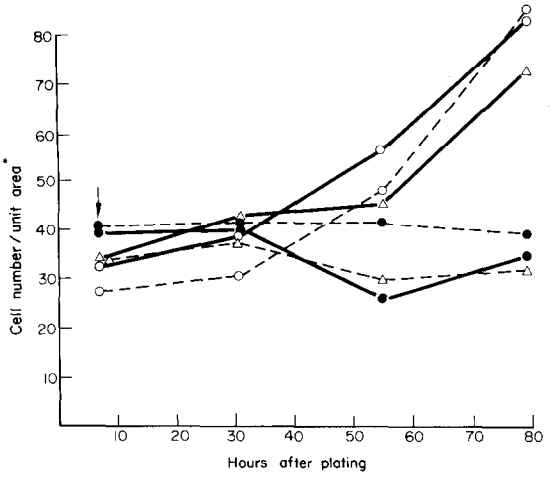

Fig. 2. The effect of exogenous thymidine on growth inhibition by methotrexate (MTX). Effect of 40 and 150 μg/ml MTX on fibroblast growth in basal growth medium containing glycine (0·66 mM) and adenine (0·5 mM) with (+T) and without (−T) exogenous thymidine (0·15 mM). Arrow indicates addition of drug in G_1 (7 hr after plating). Control − T (O----O), control + T (O——O), 40 μg/ml MTX − T (△----△), 40 μg/ml MTX + T (△——△), 150 μg/ml MTX − T (●----●), 150 μg/ml MTX + T (●——●). *Unit area = 1·42 mm².

layers were scraped from the glass surface, centrifuged, resuspended in 1 ml of Hank's Balanced Salt Solution and the oxygen uptake measured.

Pyrimethamine sulphate (PY). This was the gift of Dr. A. H. Griffith, Wellcome Research Laboratories, Beckenham, Kent.

Methotrexate (MTX) was purchased from Lederle Laboratories, Cyanamide (GB), Gosport, Hants.

RESULTS

Inhibition by pyrimethamine and its prevention by thymidine

The addition of 30 or 100 μg/ml of pyrimethamine (PY) to cells partially synchronised in late S (18 hr after plating) prevented any cells from reaching mitosis (Fig. 1a). Figure 1a also shows that exogenous thymidine (0·05 mM) abolished the antimitotic effect of 30 μg/ml of drug and permitted a delayed partial recovery (approx 50%) in cells treated with 100 μg/ml.

Investigation of the amount of nuclear DNA (Table 1a) showed that the addition of PY at these levels had an immediate effect of blocking DNA synthesis and hence prevented any progession of cells from G_1 to S or from S to G_2 during the experiment. Under these conditions, blockage of DNA synthesis was prevented by the addition of thymidine (Table 1). As a check that the amount of hypoxanthine present in the serum was adequate for DNA synthesis, the experiment was repeated with adenine included in the medium. Table 2 confirms that the addition of adenine had no effect on DNA synthesis, either on its inhibition by PY or the reversal of inhibition by thymidine.

The effect of increasing the dose and duration of treatment is illustrated in Fig. 1b and Table 1b, where higher drug concentrations (100 and 200 μg/ml) were added earlier in the growth cycle, at approximately the G_1/S boundary (13 hr after plating). Predictably, both mitosis and DNA synthesis were prevented. However, under these conditions, exogenous thymidine failed to overcome the antimitotic effect of 100 μg/ml PY (Fig. 1b), in contrast to the experiment described above, in which exposure to the drug was shorter. Thymidine did, nevertheless, permit the continuation of DNA synthesis (Table 1b).

With 200 μg/ml PY, DNA synthesis was almost totally inhibited and no mitoses occurred even in the presence of thymidine (Fig. 1b; Table 1b).

Oxygen uptake of PY treated cells. Cells were grown for 24 hr in the presence of 100 μg/ml PY in Eagle's MEM supplemented with glycine (0·66 mM) and thymidine (0·15 mM). Experiments performed on three different occasions using different cultures showed a reduction in the average oxygen uptake rate from 17± 3 nmole O_2/min per 10^6 cells for control cells to 5 ± 1 nmole O_2/min per 10^6 cells for the 100 μg/ml PY-treated cells. Hence the respiratory activity of PY-

Table 1. Effects of pyrimethamine (PY) on DNA synthesis in the presence and absence of exogenous thymidine

PY concentration (μg/ml)	Thymidine (0·15 mM)	Av duration of culture (hr)	Av time in contact with drug (hr)	% cells in G_1	S	G_2	Polyploidy	No. cells counted
(a) Addition of drug in late S (18 hr after plating)								
0	−	5·5	0	88	0	10	2	50
0	−	18	0	68	22	10	0	50
0	−	33	0	54	17	26	3	150
0	+	33	0	55	13	30	3	150
30	−	33	15	63	25	11	1	150
30	+	33	15	53	11	30	7	150
100	−	33	15	67	19	11	2	150
100	+	33	15	53	11	33	3	150
(b) Addition of drug in late G_1/early S (13 hr after plating)								
0	−	13	0	88	0	12	0	50
0	−	32	0	51	15	33	1	150
0	+	32	0	49	11	37	3	150
100	−	32	19	86	3	11	0	150
100	+	32	19	50	11	34	4	150
200	−	32	19	91	0	9	0	150
200	+	32	19	83	1	14	1	150

Table 2. Effects of exogenous thymidine on the inhibition of DNA synthesis by pyrimethamine (PY) added in G_1 (9 hr after plating) in the presence of supplementary adenine (0·5 mM)

PY concentration (μg/ml)	Thymidine (0·1 mM)	Duration of culture (hr)	Time in contact with drug (hr)	% cells* in G_1	S	G_2	Polyploidy
0	–	9	0	78	4	18	0
0	–	30	0	68	16	16	0
0	+	30	0	54	16	26	0
30	–	30	21	82	0	18	0
30	+	30	21	68	20	10	2
100	–	30	21	84	4	12	0
100	+	30	21	82	4	14	0
0	–	46	0	54	10	36	2
0	+	46	0	42	14	38	6
30	–	46	37	78	4	18	0
30	+	46	37	50	6	44	0
100	–	46	37	80	4	16	0
100	+	46	37	80	0	20	0

* No. of cells counted = 50.

treated cells was approximately 1/3 that of control values.

By contrast, PY had no immediate effect on respiratory activity when added to the cells even at 150 μg/ml, indicating that the drug does not mediate its inhibitory effect by direct interaction with mitochondria.

Reversibility of methotrexate (MTX) inhibition of fibroblasts with thymidine. It was of interest to compare PY with MTX, a folate reductase inhibitor whose overall structure is similar to that of folic acid but differs appreciably from that of PY. Fibroblasts grown in basal medium supplemented with glycine and adenine were treated with MTX with and without thymidine. The growth curves shown in Fig. 2 illustrate that the inhibition caused by MTX at 40 μg/ml (equimolar with 30 μg/ml PY) is prevented by exogenous thymidine, whereas that produced with 150 μg/ml MTX is not. The results obtained with MTX are very similar, therefore, to those produced by PY in spite of marked structural differences (Fig. 3).

DISCUSSION

These findings show that the inhibitory effects of pyrimethamine on human fibroblasts in the presence of adequate supplies of essential metabolites, including thymidine (see introduction), depend on the concentration of the drug and the length of time the cells are in contact with it. Thymidine can reverse the inhibitory effect of PY only when the drug is present in low concentrations (30 μg/ml); at higher concentrations (100 μg/ml) the reversal by thymidine is only during short exposure times.

It is significant that cells treated with a high dose of PY (100 μg/ml for 24 hr) in the presence of thymidine show a marked reduction in respiration. Recently it has been shown that yeast cells grown in the presence of this drug possess defective mitochondria as a result of the suppression of mitochondrial protein synthesis caused by a lack of formylmethionyl-tRNA [7, 8]. In yeast, when mitochondrial protein synthesis is blocked, there is a loss, over several hours, of cytochrome oxidase, the terminal component of the respiratory chain [13]. If this also applies to human fibroblast cultures, it would explain why PY (100 μg/ml) added early in S allows DNA synthesis to proceed (when exogenous thymidine is supplied) but prevents subsequent mitosis, there being, by then, no means of generating ATP. With 200 μg/ml PY, the inhibition of dihydrofolate reductase is presumably more complete, so that a shortage of THFA and its products are sooner apparent. Hence DNA synthesis is blocked as well as mitosis. The absence of mitosis under such conditions could, on the other hand, reflect an action of PY other than inhibition of the reductase. For example, the drug is very lipophilic and may have an inhibitory effect directly at the cell membrane. However, the fact that 100 μg/ml PY does not prevent mitosis if added later in the cell cycle makes this unlikely. Furthermore, although MTX differs from PY in molecular structure and lipophilicity it has a very similar effect on fibroblasts and the response to exogenous thymidine. The idea of mitochondrial involvement is further supported by the finding that aminopterin, another inhibitor of folate reductase, also suppresses mitochondrial protein synthesis in HeLa cells [14].

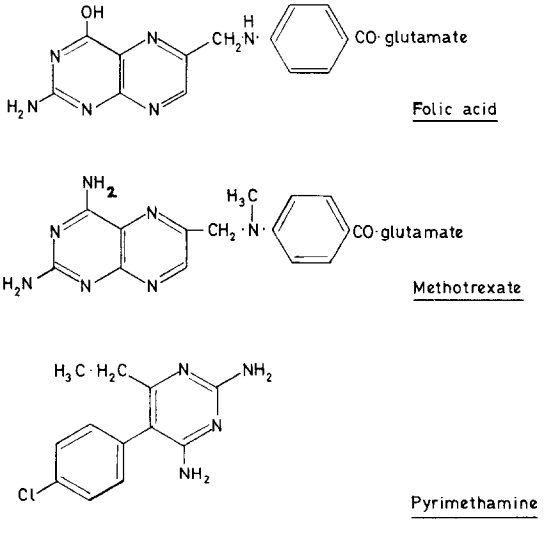

Fig. 3. Comparative structures of folic acid, methotrexate and pyrimethamine.

Acknowledgements—This work was supported by a grant from the Cancer Research Campaign. ABS was in receipt of a grant from the Medical Research Council. We thank George Barret and Richard James for maintaining the cell cultures and assisting with experiments.

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