Biochemical Pharmacology, Vol. 32, No. 24, pp. 3783–3790, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

BIOCHEMICAL EFFECTS OF A QUINAZOLINE INHIBITOR OF THYMIDYLATE SYNTHETASE, *N*-(4-(*N*-((2-AMINO-4-HYDROXY-6-QUINAZOLINYL)METHYL)PROP-2-YNYLAMINO)BENZOYL)-L-GLUTAMIC ACID (CB3717), ON HUMAN LYMPHOBLASTOID CELLS*

ROBERT C. JACKSON,† ANN L. JACKMAN and A. HILARY CALVERT

Laboratory for Experimental Oncology, Indiana University, Indianapolis, IN 46223, U.S.A. (R.C.J.), and Department of Biochemical Pharmacology, Institute of Cancer Research, Sutton, Surrey SM2 5PX, U.K. (A.L.J., A.H.C.)

(*Received* 23 *June* 1982; *accepted* 25 *May* 1983)

Abtract—The biochemical effects of the antitumor agent *N*-(4-(*N*-((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynylamino)benzoyl)-L-glutamic acid (CB3717) were studied in WI-L2 cultured human lymphoblastoid cells. CB3717 was a potent inhibitor of human thymidylate synthetase; the inhibition was competitive with 5,10-methylenetetrahydrofolate ($K_i = 4.9 \times 10^{-9}$ M). CB3717 also inhibited human dihydrofolate reductase, competitively with dihydrofolate ($K_i = 2.3 \times 10^{-8}$ M). The growth-inhibitory effect of CB3717 could be prevented completely by 10 μM thymidine. Administration of thymidine could be delayed for up to 8 hr after CB3717 treatment without cytotoxicity but, if thymidine was delayed for 24 hr, severe toxicity resulted. Incubation for 16 hr in the presence of a growth-inhibitory concentration of CB3717 did not result in the appearance of dihydrofolate in WI-L2 cells. These results indicate that, in the presence of CB3717, thymidylate synthetase, rather than dihydrofolate reductase, became rate-limiting for the cycle of dihydrofolate oxidation and reduction. Treatment of cells for 16 hr at an IC_{50} concentration of CB3717 caused a decrease of 88% in cellular dTTP and a 2,300% increase in dUMP. The level of dUDP also increased, and traces of dUTP appeared in treated cells. No large changes were seen in ribonucleotide pools. A kinetic analysis was made, by computer simulation, of predicted consequences of metabolic effects of compounds that inhibit both dihydrofolate reductase and thymidylate synthetase. It was concluded that, even if the K_i of the inhibitor for thymidylate synthetase were 3 orders of magnitude higher (weaker) than the K_i for dihydrofolate reductase, thymidylate synthetase could still become rate-limiting.

Most of the folic acid analogues that are used as anticancer drugs act through inhibition of dihydrofolate reductase. These inhibitors often have a 2,4-diamino structure [1, 2]. Inhibition of dihydrofolate reductase results in depletion of cellular pools of both dTTP and purines [3, 4]. Methotrexate, in addition to being a tight-binding inhibitor of dihydrofolate reductase, also inhibits thymidylate synthetase [5, 6], and there are indications in methotrexate-resistant cells which over-produce dihydrofolate reductase that thymidylate synthetase may become rate-limiting for growth in the presence of large amounts of methotrexate [7]. Certain quinazoline antifolates have been demonstrated to give inhibition of thymidylate synthetase; those quinazolines with a 2,4-diamino structure bound more tightly to dihydrofolate reductase, while compounds with a 2-amino-4-hydroxy structure were more effective as thymidylate synthetase inhibitors [8–11]. Recently a new 2-amino-4-hydroxy-quinazoline antifolate was described that was a potent inhibitor of the thymidylate synthetase of mouse leukemia L1210 cells, with a K_i of about 4 nM [12–14]. This figure is sufficiently low that an appropriate kinetic characterization may require a zone B analysis for tight-binding inhibition [15]. This new compound, which has a 10-propargyl sidechain, has the house number CB3717‡; its structure is shown in Fig. 1. In addition to inhibiting thymidylate synthetase, it also inhibited dihydrofolate reductase of L1210 cells. However, the cytotoxicity to L1210 cells in culture could be prevented more effectively by thymidine than by folinic acid. A subline of L1210 cells resistant to methotrexate because of dihydrofolate reductase

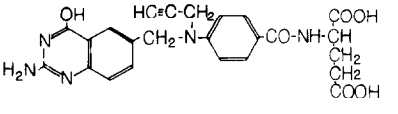

Fig. 1. Structure of CB3717 (*N*-(4-(*N*-((2-amino-4-hydroxy - 6 - quinazolinyl)methyl)prop - 2 - ynylamino)benzoyl)L-glutamic acid).

* This research was supported by grants from the USPHS (CA-18129) and from the Medical Research Council and the Cancer Research Campaign (U.K.).

† Address correspondence to: Dr. R. C. Jackson, Waner-Lambert/Parke-Davis Pharmaceutical Research Division 2800 Plymouth Road, Ann Arbor, MI 48105, U.S.A.

‡ Abbreviations: CB3717, *N*-(4-(*N*-((2-amino-4-hydroxy - 6 - quinazolinyl)methyl)prop - 2 - ynylamino)-benzoyl)-L-glutamic acid, DHFR, dihydrofolate reductase; TS, thymidylate synthetase, MTX-methotrexate; and FH_2, dihydrofolate.

overproduction was not cross-resistant to CB3717. Treatment of mice bearing L1210 leukemia with CB3717 at a dose of 125 mg per kg per day for 5 consecutive days resulted in long-term survivors (>120 days) in 9/10 animals [11].

The relative therapeutic relevance of the thymineless and purineless states induced by various antimetabolites has long been a subject of debate [3, 4, 16, 17]. CB3717 was designed to be a specific inhibitor of thymidylate synthetase. Fluorinated pyrimidine analogues, although they may be activated to metabolites that inhibit thymidylate synthetase, may also be incorporated into nucleic acids [18–22]. Thus, effects following treatment with such compounds are not necessarily attributable to an uncomplicated thymineless state. The present report therefore describes experimental and mathematical studies designed to identify the principal site of action of CB3717 in human lymphoblastoid cells *in vitro*. In particular, we sought biochemical evidence that inhibition of thymidylate synthetase is the rate-determining site of action of this double inhibitor.

EXPERIMENTAL

Cell culture. The WI-L2 lymphoblastoid cell line was originally cultured from human spleen by Levy *et al.* [23]. Cells were maintained in stationary suspenions culture by inoculating at a density of 4×10^4 cells/ml in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum, penicillin G (100 units/ml) and streptomycin (100 μg/ml). Because cultures have occasionally become contaminated and overgrown by rodent cells, the lactate dehydrogenase isozyme pattern of the cells was examined by cellulose acetate electrophoresis [24] to eliminate this possibility. Cultures were regularly screened for mycoplasma contamination by the method of Levine [25]. Colony assays were performed by placing single cells into wells of 96-well, flat bottom microtitre plates (Costar, Inc., Cambridge, MA, type 3596). Each well contained 0.1 ml of fresh RPMI 1640 medium (+ serum) and 0.1 ml of RPMI 1640 medium that had been conditioned by growth of WI-L2 cells to a density of 6×10^5 cells/ml, followed by centrifugation and ultrafiltration. The cloning efficiency under these conditions was 20%.

Enzyme kinetics. WI-L2 thymidylate synthetase was purified by affinity chromatography based on the method of Rode *et al.* [26] using a different spacer and elution conditions as previously described [14]. The column material consisted of the 10-ethylquinazoline analogue of folic acid, covalently attached to AH-Sepharose 4B. The eluant was 0.2 M potassium phosphate buffer, pH 7.4, containing 0.1% Triton X-100 and 0.01 M 2-mercaptoethanol. Following elution, the enzyme preparation was made 20% with respect to sucrose and 50 μM with respect to dUMP, to aid stability, and stored at −40°. This procedure gave a purification factor of <2000 and specific activity of >2 μmole per hr per mg protein. Thymidylate synthetase activity was assayed isotopically using the principle of tritium release from the 5-position of deoxyuridylate originally described by Roberts [27]. Separation of the products was by ion exchange columns [11]. The purified enzyme was free of contaminating phosphatases that interfere with the assay. Modifications to the reaction mixture were made, to adapt the system for use with purified enzyme [14]. The 0.5 ml reaction mixture contained 50 μM [5-^{3}H]dUMP (24 mCi/mmole), 10 mM dithiothreitol, 5,10-methylenetetrahydrofolate and CB3717, and 0.1 ml of the buffered enzyme preparation described above. In all rate determinations, less than 2% of the substrates were converted to products.

Dihydrofolate reductase was purified to homogeneity by affinity chromatography and assayed spectrophotometrically as previously described [28]. For both enzymes, values of K_i were calculated by fitting rate measurements to the zone B tight-binding inhibition equation by nonlinear regression [28].

Dihydrofolate assays. Dihydrofolates in cell extracts were assayed by using the extract as limiting substrate in spectrophotometric dihydrofolate reductase determinations. Cells (5×10^8) were suspended in 2 ml of 0.05 M potassium phosphate buffer, pH 6.0, containing 1% ascorbic acid. This buffer was gassed before use with oxygen-free nitrogen. The cell suspension was boiled for 90 sec, then cooled in ice and freeze-dried, and redissolved immediately before use in 0.3 ml of distilled water. Reaction cuvettes contained 2.5 ml of 0.05 M Tris–chloride buffer, pH 7.0, with 0.12 μmole of NADPH and 0.1 ml of either a cell extract or standard dihydrofolate. After equilibration at 37°, reaction was initiated by addition of 0.01 I.U. of purified L1210 DHFR [28]. The absorbance change was monitored on a Cary 118CX spectrophotometer at 340 nm, using a full-scale chart deflection of 0.02 absorbance units. The reaction was allowed to go to completion, and the absorbance change was corrected for a blank that contained no enzyme, and also for the small absorbance change caused by the addition of enzyme. Under these conditions 5 nmoles of standard dihydrofolate (92% pure) gave a corrected absorbance change at 340 nm of 0.022. Recovery of dihydrofolate added to cells extracts averaged 90%, and the limit of sensitivity of the method was 0.5 nmole/sample. This method measures the total of all folate forms with substrate activity for DHFR, i.e. dihydrofolate, folate, and their polyglutamates. If samples were prepared from cells grown in the presence of methotrexate or CB3717, drug residues slowed down the reaction, but so long as purified DHFR was present in excess the total extent of reaction was not affected.

Deoxyuridylate assays. About 5×10^7 cells were extracted in 0.6 ml of 0.7 M perchloric acid at 0°. Following centrifugation at 50,000 *g* for 20 min, extracts were neutralized by addition of 45 mg of potassium bicarbonate and centrifuged to remove the precipitated potassium perchlorate. The pH was adjusted to 5.0 by addition of 10 μl of 4 N HCl. A 200-μl aliquot of this extract was loaded onto a 25 × 0.46 cm column of Whatman Partisil-SAX anion exchange resin. The initial elution buffer was 10 mM ammonium acetate, pH 5.0, and the final elution buffer was 3.5 M ammonium acetate, pH 5.0; after a gradient delay of 4 min, the proportion of final buffer was increased at 4%/min for 20 min. The UMP, UDP and UTP peaks were collected. The

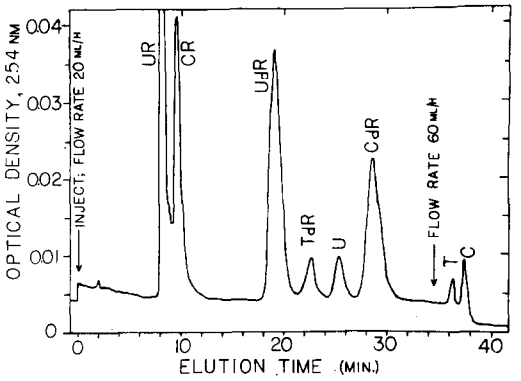

Fig. 2. Cation exchange chromatographic separation of pyrimidine bases, ribonucleosides and deoxyribonucleosides on Aminex-A6. Elution conditions are described in the text.

Table 1. Prevention of CB3717- or MTX-induced growth inhibition of thymidine*

Time of addition of thymidine (10 μM)	Cell counts at 48 hr (% of control) CB3717 (2×10^{-5} M)	MTX (2×10^{-7} M)
None	6	3
2 Hr before antifolate	89	58
Same time as antifolate	81	33
8 Hr after antifolate	79	2
24 Hr after antifolate	14	2

* Cultures of WI-L2 cells were maintained as described in the Experimental section. Values shown are mean cell counts for triplicate cultures, expressed as percentages of quadruplicate control counts.

thymidine and deoxyuridine nucleotides migrated with the corresponding uridine phosphates. The nucleotide fractions were then hydrolyzed to the corresponding nucleosides as follows. Fractions were adjusted to pH 8.5 with 4 N potassium hydroxide. Then, to 1 ml of sample were added 0.2 ml of Tris–chloride, pH 8.5 (containing 2 mM magnesium chloride), 0.1 ml snake venom (20 mg/ml; from *Crotalus adamanteus*) and 5 units of alkaline phosphatase (from *Escherichia coli*). After 60 min at 37°, reaction was stopped with perchloric acid, and following potassium bicarbonate treatment the mixture was concentrated by flash evaporation and redissolved in 0.3 ml of distilled water. Nucleosides were separated by cation-exchange chromatography on a 25 × 0.9 cm column of Aminex A-6, isocratically eluted with 0.02 M sodium borate (adjusted to pH 8.9 with formic acid). Separation of standards on this column is shown in Fig. 2.

Ribonucleotide analysis. Cells were extracted in 0.7 M perchloric acid, followed by neutralization with KOH to remove perchlorate. Analysis was by high pressure liquid chromatography on Whatman Partisil-SAX columns, using buffer conditions as previously described [29, 30]. Peaks were electronically integrated using a Varian model CDS-111L integrator which had been calibrated against known quantities of standard nucleotides.

Enzymatic assay of deoxyribonucleoside triphosphates. Cell pellets were extracted in 60% aqueous methanol, and samples were prepared for analysis by the procedure of Harrap and Paine [31]. dCTP and dGTP were assayed using the method of Solter and Handschumacher [32] with calf thymus DNA as template/primer. dATP and dTTP were measured by the modified DNA polymerase procedure of Lindberg and Skoog [33], using poly(deoxyadenylate-deoxythymidylate) as template/primer.

RESULTS

Reversal of CB3717-induced growth inhibition by thymidine. Figure 3 shows the growth-inhibitory effect of CB3717 towards WI-L2 cells measured following 72 hr of continuous exposure. The IC_{50} concentration determined under these conditions was

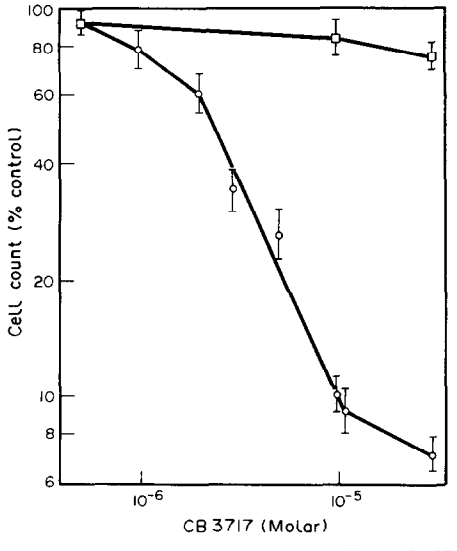

Fig. 3. Inhibition of growth of WI-L2 cells by CB3717 in the presence and absence of 10 μM thymidine. Key: (○) CB3717; and (□) CB3717 + thymidine.

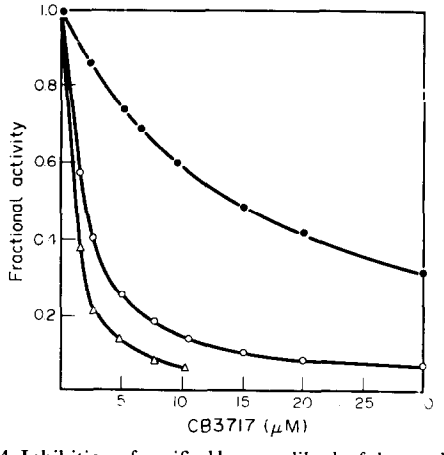

Fig. 4. Inhibition of purified human dihydrofolate reductase by CB3717. Reaction conditions were as described in the text. Key: (●) dihydrofolate, 80 μM; (○) dihydrofolate, 10 μM; and (△) dihydrofolate, 4 μM.

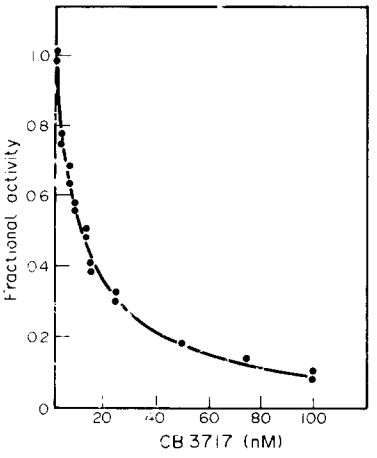

Fig. 5. Inhibition of purified human thymidylate synthetase by CB3717. Reaction conditions were as described in the text. Concentrations of (+)5,10-methylenetetrahydrofolate was 75 μM.

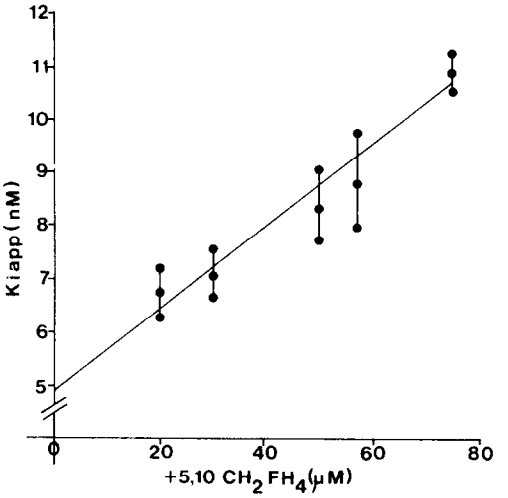

Fig. 6. Replot of estimates of K_{iapp} for inhibition of thymidylate synthetase by CB3717. Interpretation of this plot is considered in Results.

2.5×10^{-6} M. Addition of thymidine (10 μM) to the cultures at 0, 24 and 48 hr resulted in almost complete protection of the cells. This result is similar to observations made with mouse L1210 cells [12]. Good protection of WI-L2 cells from CB3717 cytotoxicity was obtained even if administration of thymidine was delayed up to 8 hr after addition of antifolate; however, addition of thymidine 24 hr after CB3717 did not protect (Table 1). These results are in contrast to results obtained with methotrexate (MTX), where protection by simultaneous or previous thymidine treatment was less complete, and delayed thymidine "rescue" was nonexistent (Table 1).

Inhibition of dihydrofolate reductase. Dihydrofolate reductase was inhibited by CB3717, to an extent that varied with concentration of dihydrofolate (Fig. 4). Preincubation of the inhibitor with enzyme plus NADPH for periods of 10 and 20 min did not increase the extent of inhibition. There was thus no indication of irreversible inactivation. Values of $K_{i,app}$ were calculated from the data of Fig. 4 by nonlinear regression to the equation of Morrison [15]. Estimates of $K_{i,app}$ for the different concentrations of dihydrofolate, when divided by the factor $(1 + [FH_2]/K_m)$, where $K_m = 0.13$ μM [28], gave near-constant estimates of K_i (21.8, 20.5 and 23.4 nM, respectively, when $[FH_2] = 4$, 10 and 80 μM), indicating that inhibition of dihydrofolate reductase by CB3717 was competitive with dihydrofolate. The mean estimate of K_i from the data shown was 2.3×10^{-8} M.

Inhibition of thymidylate synthetase. Inhibition by CB3717 of a purified WI-L2 thymidylate synthetase is shown in Fig. 5. Because the inhibition was comparatively potent, it was necessary to analyze the data by a method appropriate for tight-binding inhibitors. The line in Fig. 5 represents a computer generated best fit, using the Morrison equation [15]. This experiment was repeated over a range of five concentrations of the substrate, 5,10-methylenetetrahydrofolate, from 22 to 82.5 μM. Figure 6 shows a replot of estimates of $K_{i,app}$ as a function of (+)5,10-methylenetetrahydrofolate concentration. The fitted line was obtained by linear regression weighted according to the reciprocals of the variances for the individual points. The line intercepts the ordinate at a value of 4.9 nM, which thus represents the K_i value. From these data we conclude that inhibition of thymidylate synthetase by CB3717 was competitive with 5,10-methylenetetrahydrofolate over the range of substrate concentrations studied. Preincubation of inhibitor with enzyme for periods up to 60 min did not increase the degree of inhibition, again suggesting that there was no irreversible inhibition of enzyme.

Kinetic simulation of dual inhibitors. A compound that inhibits both DHFR and TS competitively will be expected to behave similarly to either a pure DHFR inhibitor or a pure TS inhibitor, since, in the cycle of dihydrofolate oxidation and reduction, one of the enzymes must become rate-limiting and, in the inhibited steady state, inhibition of the non-rate-limiting enzyme will be irrelevant. The biochemical consequences of the two cases will differ. If DHFR is rate-limiting, dihydrofolate will be produced faster than it can be reduced, and it will accumulate, with a corresponding decrease in the tetrahydrofolate pools. Thus, *de novo* purine biosynthesis will be inhibited, and reversal of the effect of the agent will require thymidine plus a purine. However, if TS is rate-limiting, dihydrofolate will not accumulate, tetrahydrofolates will not be depleted, and purine *de novo* biosynthesis will remain uninhibited. Growth inhibition should then be reversible by thymidine (without a purine). It would be helpful to be able to predict, from kinetic data, which enzyme will become rate-limiting in the presence of a dual inhibitor. We have attempted to do this by kinetic simulation, using a mathematical model of folate metabolism [34]. Figure 7 shows, for a given inhibition constant (K_i) for DHFR, the K_i for TS below which this enzyme will become rate-limiting in the presence of the dual inhibitor (this treatment assumes that K_i values obtained for the isolated enzymes are the same when the enzymes are present together). The conclusion was that (for

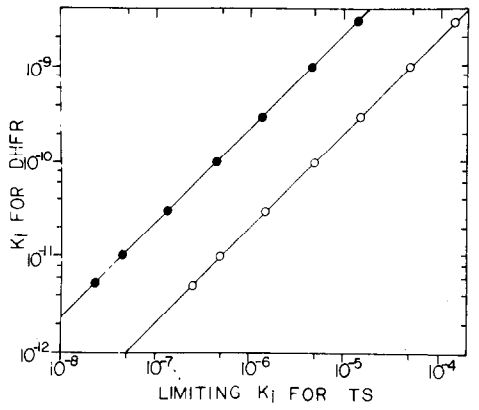

Fig. 7. Prediction of the rate-limiting enzyme of thymidylate biosynthesis in the presence of compounds that inhibit both DHFR and TS, as determined by kinetic simulation. For a given double inhibitor, if the point representing the pair of K_i values (for DHFR and TS) falls above and to the left of the appropriate line, then TS becomes rate-limiting at the steady state; if the point is below and to the right of the line, then DHFR is rate-limiting. Key: (●) wild-type WI-L2 cells; and (○) WI-L2 cells with 10-fold overproduction of DHFR.

this particular cell line), even if binding of inhibitor to TS was up to 3.5 orders of magnitude weaker than binding to DHFR, TS would still become rate-limiting. For example, if K_i for DHFR is 10^{-11} M, then (from the abscissa of Fig. 7) any value of K_i for TS equal to or less than 4.5×10^{-8} M will make TS rate-limiting in the inhibited steady state. There are two main reasons for this. First, in WI-L2 lymphoblasts, as in most mammalian cells, DHFR is over 20-fold more active than TS, so it must be inhibited to a much greater extent before it becomes rate-limiting. Second, when DHFR is inhibited, its substrate, dihydrofolate, which has no other known routes of metabolic utilization, will accumulate, by 30-fold or more [35]. In contrast, 5,10-methylenetetrahydrofolate, the folate cofactor of TS, does not (according to the simulation) accumulate in the presence of a TS inhibitor, because there are several other reactions that utilize this cofactor. Other predictions of the modelling studies were: (a) The greater the DHFR activity (or the lower the TS activity), the greater the tendency for TS to become rate-limiting. A 10-fold increase in V_{max} of DHFR caused a predicted increase of 1 order of magnitude in the limiting K_i for TS to become rate-determining (Fig. 7) and, as shown in a previous study [7] with a 230-fold overproduction of DHFR, even methotrexate becomes functionally a TS inhibitor. (b) DHFR overproducing mutants selected for resistance to methotrexate may show a minor degree of cross-resistance to dual inhibitors, even those that act principally as TS inhibitors. This is because the DHFR will bind intracellular inhibitor, thus decreasing the amount available for binding to TS. This conclusion assumed that exposure of cells to inhibitor was for a brief time period, so that total cell content of inhibitor was limited by uptake rate. However, if exposure time is long, or uptake is rapid, cells should reach a state in which the cellular free inhibitor content equilibrates with extracellular inhibitor so that amount of inhibitor bound to thymidylate synthetase should be unaffected by inhibitor bound to DHFR. In this situation MTX-resistant DHFR-overproducing cells should not be cross-resistant to CB3717. If a steady state is never reached, because of synthesis of DHFR faster than drug uptake, some cross-resistance would be predicted, and is observed.

Table 2. Dihydrofolates in WI-L2 cells treated with methotrexate or CB3717*

Treatment	Dihydrofolates (nmoles/10^2 cells)
None	<0.2
MTX (50 nM, 16 hr)	6.8 ± 1.6
CB3717 (3 μM, 16 hr)	<0.2

* Values are means for triplicate cultures. Extraction and assay were as described in the Experimental section.

Dihydrofolate accumulation in the presence of methotrexate and CB3717. A comparison of the inhibition of purified enzyme preparations indicated that CB3717 inhibited thymidylate synthetase more powerfully than dihydrofolate reductase. However, the actual degree of inhibition of each enzyme within the cell will depend upon concentrations of the relevant substrates and cofactors. The mathematical modelling described above suggested that CB3717 should act primarily as a thymidylate synthetase inhibitor. We attempted to confirm this prediction experimentally by looking for accumulation of dihydrofolate within treated cells. Results are summarized in Table 2. Treatment of WI-L2 cells with a growth-inhibitory dose of MTX resulted in accumulation of measurable dihydrofolate, indicating that the activity of DHFR within the inhibited cells was less than the activity of TS. In cells treated with CB3717, however, no accumulated dihydrofolate was detectable, indicating that DHFR activity remains greater than TS activity. This result was consistent with TS being rate-limiting for the dihydrofolate cycle in cells treated with CB3717.

Ribonucleotide pools in CB3717-treated cells. MTX and other DHFR inhibitors cause accumulation of dihydrofolate, with corresponding depletion of tetrahydrofolates [28, 35]. Since tetrahydrofolate

Table 3. Effect of antifolates on cellular ribonucleotide contents*

Treatment	Nucleotide pool sizes, as % of control†			
	ATP	GTP	UTP	CTP
MTX (0.2 μM)	56‡	67‡	86	78
CB3717 (20 μM)	116	123‡	139‡	128‡

* Values are means for triplicate cultures. Treatment was for 4 hr.

† Nucleotide contents of untreated control cultures were (nmoles/10^9 cells): ATP, 2890 ± 180; GTP, 960 ± 75; UTP, 1730 ± 110; and CTP, 80 ± 7 (mean ± S.E.M. for quadruplicate cultures).

‡ Significantly different from untreated control ($P < 0.05$).

Table 4. Effect of antifolates on cellular pools of deoxyribonucleoside triphosphatates*

Treatment	Nucleotide pool sizes, as % of control†			
	dATP	dGTP	dTTP	dCTP
MTX (0.2 μM)	55	42	22	135
CB3717 (20 μM)	139	72	17	67

* Values are means for triplicate cultures. Treatment was for 4 hr. All values were significantly different from control ($P > 0.05$).

† Nucleotide contents of untreated control cultures were (nmoles/10^9 cells): dATP, 22 ± 3; dGTP, 15 ± 1; dTTP, 48 ± 5; and dCTP, 29 ± 2 (mean ±S.E.M. for quadruplicate cultures).

cofactors are required for the purine *de novo* biosynthetic pathway, DHFR inhibitors have an antipurine effect [4]. Our results, presented above, show that dihydrofolate does not accumulate after CB3717 treatment, so presumably tetrahydrofolates are not depleted. However, it is possible that antifolate drugs may exert an antipurine effect by direct inhibition of one of the folate-requiring transformylase enzymes of the purine *de novo* pathway. Table 3 shows the effect of a 4-hr incubation with lethal concentrations of MTX and CB3717. In MTX-treated cells, significant decreases were observed in concentrations of ATP and GTP. In contrast, pools of all four ribonucleotides were slightly increased in cells treated with CB3717.

Deoxyribonucleoside triphosphates in WI-L2 cells. The effects of MTX and CB3717 on cellular contents of deoxyribonucleoside triphosphates, as measured by the DNA polymerase procedure, are shown in Table 4. Both antifolates gave marked decreases in dTTP and a lesser decrease in dGTP. However, the two agents had qualitatively different effects on the pools of dATP and dCTP, with MTX giving decreased dATP and increased dCTP, whereas CB3717 gave increased dATP and decreased dCTP.

Deoxyuridylates in antifolate-treated WI-L2 cells. Using the two-stage chromatographic procedure described in the Experimental section, uridylates, deoxyuridylates and thymidylates were measured in cells treated with MTX or CB3717. The results of dTTP measured confirmed the extensive decrease shown by the enzymatic assay, and we also found decreases in dTMP and dTDP in cells treated with either MTX or CB3717. Both agents gave very large increases in both dUMP and dUDP. In addition, small but measurable amounts of dUTP were detected in the antifolate-treated cells (Table 5).

In vitro *effects of combinations of CB3717 with deoxyuridine and antipyrimidines.* Because of suggestions that deoxyuridylate misincorporation into DNA may contribute to cytotoxicity of antifolates [36–39], we studied the growth-inhibitory effect of CB3717 with added deoxyuridine and also with

Table 5. Effect of antifolates on cellular pools of pyrimidine nucleotides*

Nucleotide	Cellular content (nmoles/10^9 cells)		
	Control	MTX (0.5 μM)	CB3717 (3 μM)
dUMP	11	323	490
dUDP	7	170	185
dUTP	0	1.2	2.0
dTMP	2	0	0
dTDP	13	5	2
dTTP	57	9	7
UMP	390	403	199
UDP	550	623	545
UTP	1080	1230	1070

* WI-L2 cells were treated for 16 hr and then extracted and assayed chromatographically as described in the Experimental section. Values shown are means from triplicate determinations.

Table 6. Growth-inhibitory effects of combinations of CB3717 with 2′-deoxyuridine and antipyrimidines*

Inhibitors added	Number of cell doublings in 72 hr	Treated as % of control	Predicted for summation
None	3.7	(100)	
CB3717 (3 μM)	2.0	54	
dUrd (0.3 mM)	3.5	95	
Pyrazofurin (30 nM)	1.9	51	
PALA (80 μM)	2.6	69	
CB3717 (3 μM) + dUrd (0.3 mM)	1.8	49	51
CB3717 (3 μM) + pyrazofurine (30 nM)	0.88	24	28
CB3717 (3 μM) + PALA (80 μM)	1.7	47	37

* Cultures were set up as described in the experimental section, and grown for 72 hr in the continuous presence of the inhibitors.

two antipyrimidines, *N*-phosphonacetyl-L-aspartate (PALA) and pyrazofurin, both of which prevent biosynthesis of uridylate and deoxyuridylate. Results are given in Table 6. Deoxyuridine did not significantly potentiate the CB3717, and combined effects of CB3717 with both antipyrimidines were approximately additive.

DISCUSSION

It has been reported that CB3717 is an inhibitor of both DHFR and TS extracted from mouse L1210 cells [12]. The present study has confirmed that finding, using purified enzymes from human lymphoblastoid cells, and has provided evidence that CB3717 functions primarily as a TS inhibitor. Evidence for this conclusion was provided by a number of approaches. Reversibility of the drug effect by thymidine alone, without a requirement for purines, was consistent with TS being the primary site of action, but was not conclusive. In some cell lines, effects of DHFR inhibitors may be partially prevented by concurrent thymidine, particularly in the presence of traces of reduced folates [16, 17, 40, 41]. However, the fact that delayed administration of thymidine several hours after CB3717 gave "rescue" provides strong evidence that TS was the rate-limiting enzyme in the presence of the inhibitor, since delayed thymidine does not rescue from DHFR inhibitors *in vitro* [41]. The modelling studies predicted from consideration of the kinetic parameters for the system, and from the concentrations of the competing substrates, that TS should be rate-limiting in the presence of CB3717, and this prediction was supported experimentally by the observation that dihydrofolate did not accumulate in the inhibited cells.

Unlike MTX, CB3717 did not have antipurine activity. Thymidine prevented or reversed growth inhibition in CB3717-treated cells, and measurement of ribonucleotide pools in treated cells showed no decrease in ATP or GTP. However, a small but significant decrease was found in the content of dGTP in treated cells. Since dTTP is an obligatory activator for the GDP reductase activity of rodent and human ribonucleotide reductase [42, 43], and the GTP level went up, the decreased dGTP is probably a secondary consequence of decreased dTTP, rather than a direct antipurine effect of CB3717.

The initial report of increased deoxyuridylate in MTX-treated cells [44] has been confirmed repeatedly [37, 41]. The suggestion has been made that excessive production of deoxyuridylate following treatment of cells with antifolates could lead to misincorporation of uracil into DNA [36–38]. Early studies using thin-layer chromatographic techniques indicated increased dUTP in cells treated with MTX [36, 44] and the first quantitative study of dUTP in MTX-treated cells was reported recently [39]. Our present work shows that CB3717 may also lead to appreciable accumulation of dUTP in cells. At present it is uncertain to what extent uracil misincorporation into DNA contributes to antifolate cytotoxicity. It is possible that this effect is restricted to cells in which activity of dUTPase is relatively low. To date, the only detailed reports of dUTP accumulation, uracil misincorporation, or DNA damage following antifolate treatment have been in human lymphoid cells (present study and Refs. 36–39).

If uracil misincorporation into DNA and resulting genetic damage is indeed an important contributor to antifolate cytotoxicity, then it would be predicted that added deoxyuridine should potentiate cytotoxicity, and inhibitors of uridylate and deoxyuridylate accumulation, such as pyrazofurin or PALA, should antagonize antifolates. Table 6 summarizes experiments designed to test this possibility. The combination of CB3717 + PALA was less than additive (though not strongly antagonistic), and combinations of CB3717 plus deoxyuridine or pyrazofurin gave approximately additive inhibition. Thus, in general, these results do not support the concept of uracil misincorporation into DNA as the primary lethal result of antifolate treatment. It is clear that this important hypothesis requires further evaluation. The availability of a potent folate analogue inhibitor of TS should be of great value in future studies of the biochemical pharmacology of antifolates.

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