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Biochemical effects of folate-based inhibitors of thymidylate synthase in MGH-U1 cells

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Abstract The TS-inhibitory effects induced by a 24-h exposure to the folate-based TS inhibitors CB3717, C2-desamino analogs of CB3717 including D1694, and BW1843U89 were quantitated using the MGH-U1 human bladder carcinoma. The effects of D1694 on the time course of TS inhibition and on intracellular deoxyuridine monophosphate (dUMP) accumulation and deoxyuridine (dUrd) production were evaluated. D1694 and BW1843U89 were the most active TS inhibitors with IC_{50} values of 2.4 and 0.5 n*M*, respectively. The C2-desamino C2-methyl dideazafolates were 27–292 times more potent than the parent CB3717 as TS inhibitors. A methyl group at the C2 position of CB3717 had the most dramatic effect, whereas a thiazole substitution for a benzyl added a small benefit and N10 substitution had a limited impact on TS-inhibitory potency and clonogenic survival. There was a significant correlation between the IC_{50} values for TS inhibition and those for cytotoxic potency obtained for these drugs. LV and thymidine protected cells from these folate-based TS inhibitors. Intracellular dUMP levels following 24 h D1694 (IC_{50}) exposure increased 7-fold. Levels of dUrd effluxing into the media increased up to 4.5 μ*M* following a 24-h exposure to D1694 (IC_{90}). We conclude that (a) C2-desamino C2-methyl dideazafolates are potent TS inhibitors, (b) TS inhibition requires prolonged exposure with these folate TS inhibitors, (c) survival is correlated with inhibition of TS for the folate-based TS inhibitors and (d) the biochemical consequences of TS inhibition include increased dUMP and dUrd levels.

Key words Thymidylate synthase · Inhibitors · Antifolate

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Abbreviations TS thymidylate synthase · *D1694* *N*-{5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl}-L-glutamic acid · *BW1843U89* (S)-2-(5-[{[1,2-dihydro-3-methyl-1-oxobenzo(F)quinazolin-9-yl]-methyl}amino]1-oxo-2-isoindolinyl) glutamic acid · *CB3717* N^{10}-propargyl-5,8-dideazafolic acid · *dCB3717* 2-desamino-N^{10}-propargyl-5,8-dideazafolic acid · *LV* leucovorin · *FPGS* folylpolyglutamate synthetase · IC_{50}, IC_{90} concentrations necessary to inhibit function by 50% and 90%, respectively

Introduction

The biosynthesis of thymidine monophosphate requires 5,10-methylenetetrahydrofolate, which serves as a cofactor in the TS-catalyzed transfer of a one-carbon unit to deoxyuridine monophosphate. Thus, TS is an attractive target for inhibition of DNA synthesis. Due to the limited clinical success of fluoropyrimidine substrate analogs in the treatment of colorectal and other cancers [1], analogs of the folate cofactor were developed with TS as the primary target. Although CB3717, one of the first quinazoline folate-based TS inhibitors was active clinically [2–6], it was withdrawn because of a low therapeutic index resulting from limited solubility and nephrotoxicity. As an improvement on CB3717, novel water-soluble analogs modified at the C2, N10 and benzyl regions were synthesized (Fig. 1). Changes at the C2 position of CB3717 have resulted in greater biological activity with a decrease in apparent toxicity [7]. This has been attributed to the utilisation of the reduced folate carrier, which does not seem to be utilised by CB3717 [8].

D1694 was identified as one of the most potent TS inhibitors with significant preclinical antitumour activity amongst the series of CB3717 analogs. Rapid intracellular uptake of D1694 and its retention in the form of more active polyglutamated metabolites contribute to its antitumour potency [9]. The mode of action appears to be specific and is based on competition with 5,10-methylenetetrahy-

drofolate for TS. BW1843U89, a more recently synthesized folate-based TS inhibitor, differs from the dideazafolates in its 3-methyl benzoquinazoline chemical backbone [10]. Both D1694 and BW1843U89 are soluble, act primarily as TS inhibitors, are very potent in vivo and in vitro with IC_{50} values in the nanomolar range and seem to be transported by the reduced-folate carrier. BW1843U89 and D1694 are good substrates for FPGS and polyglutamation of these drugs seems to be essential to their cytotoxicity. BW1843U89 is a non-competitive inhibitor of TS, whereas D1694 is a mixed non-competitive inhibitor [10]. LV protects cells from BW1843U89 and D1694 toxicity, but higher LV concentrations are required to protect cells from BW1843U89. Since D1694 is more dependent on polyglutamation for its activity than is BW1843U89, LV competes more effectively. BW1843U89 is probably less dependent on polyglutamation and, therefore, not so dependent on LV. This may explain the need for higher LV concentrations to protect cells from BW1843U89. Thymidine is capable of rescuing cells from the cytotoxic action of either drug [8, 10], supporting TS as the primary site of action.

We undertook these studies in MGH-U1 cells (a) to determine the relationship between TS inhibition and modification to the structure of CB3717, (b) to relate clonogenic survival to TS inhibition and (c) to define the biochemical effects of D1694, including TS inhibition, intracellular deoxyuridine monophosphate (dUMP) formation and production of dUrd.

Materials and methods

Chemicals

CB3717 and dCB3717, provided by Dr. V. Narayanan (Drug Synthesis and Chemistry Branch, NCI, Bethesda, Md.), were dissolved in 0.15 *M* $NaHCO_3$. The C2-desamino C2-methyl analogs of CB3717 were a gift from ICI Pharmaceuticals (Alderly Park, Macclesfield, Cheshire, UK). They were dissolved in 0.4 *M* $NaHCO_3$. BW1843U89, a gift from Burroughs Wellcome (Research Triangle Park, N.C., USA), was made in a 2:1 molar ratio of NaOH:BW1843U89 and adjusted to pH 7. Drugs were protected from light and dilutions were made in phosphate-buffered saline (PBS). 5-[^{3}H]-dUMP (18 Ci/mmol) and ACS scintillant were obtained from Amersham (Arlington Heights, Ill.). 5-[^{3}H]-2′-Deoxyuridine (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif.). *Lactobacillus casei* TS (53.4 μmol h^{-1} ml^{-1}) from Biopure (Boston, Mass.) was diluted in the following buffer: 310 mg dithiothreitol, 310 mg magnesium chloride, 1.2 g TRIS and 0.2 g bovine serum albumin in a total volume of 100 ml at pH 7.4. Media, PBS, antibiotics and trypsin were purchased from Gibco (Grand Island, N.Y.). All other chemicals were obtained from Sigma (St. Louis, Mo.).

Cell line and clonogenic survival

The growth conditions for the MGH-U1 cells and the drug-induced inhibition of colony formation were determined as reported previously [11]. Drug-exposure periods varied from 6 to 24 h. The IC_{50} and IC_{90} values for clonogenic survival resulting from a 24-h exposure to D1694 were 6.4 and 10 n*M*, respectively.

TS assays

Exponentially growing MGH-U1 cells in nucleoside-free α-minimum essential medium (α-MEM) supplemented with 10% dialysed fetal calf serum were incubated with various concentrations of TS inhibitors (Fig. 1) for a maximal period of 24 h. Following drug incubation, TS activity was determined by a whole-cell assay. TS activity in intact MGH-U1 cells was determined by a modification of the method published by Yalowich and Kalman [13] and has been reported previously [12, 14]. The data are presented as the rate of tritium release expressed as a percentage of the untreated control value. Correlation between cytotoxicity and TS inhibition for the series of TS inhibitors was determined by the Spearman rank correlation of IC_{50} values for cytotoxicity and those for inhibition of TS after 24-h exposures.

Quantitation of intracellular dUMP

A modification of the tritium-release assay of Roberts was used to measure free dUMP levels within cells [15, 16]. To cell pellets, 8% trichloroacetic acid was added to obtain a concentration of 5×10^7 cells/ml. This was incubated on ice for 15 min, sonicated and centrifuged for 15 min at 14,000 *g* and 4° C. The acidic supernatant was neutralized with 1:2 parts (v/v) of tri-*n*-octylamine and Freon (1:4, v/v). The mixture was shaken for 2 min and centrifuged at 14,000 *g* for 5 min, and the top layer was used to measure free cytosolic dUMP. To 100 μl cell extract was added 25 μl (5 pmol) *L. casei* TS, 1.4 pmol 5-[^{3}H]-dUMP (50 μl) and 25 μl of the 2-m*M* cofactor solution. The reaction was allowed to proceed for 30 min at 37° C. The amount of dUMP in cell extracts was determined from a standard curve constructed using known amounts of cold dUMP in competition with 5-[^{3}H]-dUMP.

Quantitation of extracellular deoxyuridine

After a 24-h exposure to drug doses resulting in 10% cell survival (IC_{90}), culture media were analyzed for effluxing deoxyuridine (dUrd) by reverse-phase high-performance liquid chromatography (HPLC) [17]. Medium (200 μl) was injected onto a reverse-phase column (Bio-Rad C_{18}, 250 × 4 mm, 5 μm), on a Waters Model 440 HPLC system using isocratic elution with 1% acetonitrile in 0.1 *M* ammonium acetate (pH 4). A flow rate of 1.2 ml/min was used. Peaks of dUrd were detected at 254 nm and peak areas were integrated by computer (Shimadzu 510C, Japan). A standard curve ranging from 0 to 10 μ*M* dUrd was constructed having a sensitivity of 1 μ*M*. The retention times for uridine, dUrd and thymidine were 3.4, 4.5 and 8.7 min, respectively.

Results

Inhibition of TS and clonogenic survival

Inhibition of TS increased in a dose-dependent manner for all compounds. The potency of the inhibition as defined by the IC_{50} for each compound is summarized in Table 1. The most potent TS inhibitors were D1694, a quinazoline-based TS inhibitor, and BW1843U89, a benzoquinazoline-based TS inhibitor, with IC_{50} values of 2.4 and 0.5 n*M*, respectively. Doses of drug that caused cell killing were found to be very similar to those that diminished TS activity after a 24-hour exposure for all the folate-based TS inhibitors [18]. There was a significant relationship between the rank of cytotoxic potency and the rank of TS inhibition between drugs (Spearman coefficient, 0.95; $P < 0.05$).

Table 1 Inhibition of TS and cell survival following a 24-h drug exposure

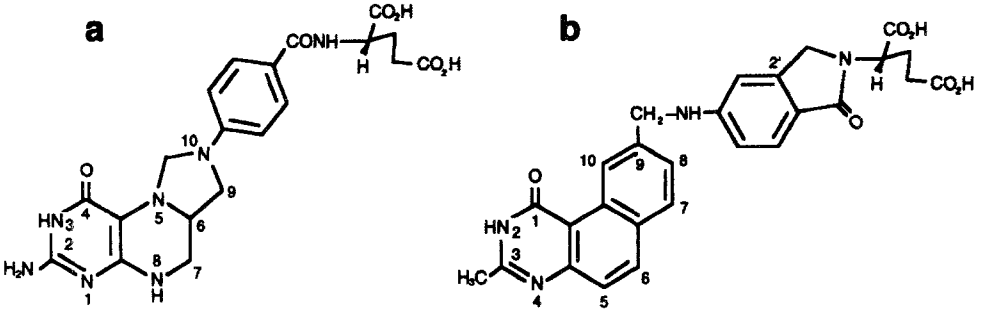
Compound	Cytotoxicity IC_{50} (n*M*)		TS Inhibition[a] IC_{50} (n*M*)	
CB3717	1600[b]		700	
dCB3717	3700	(0.43x)[d]	750	(0.93x)
1[c]	60	(27x)	20	(35x)
2	200	(8x)	26	(27x)
3	10	(160x)	4	(175x)
4	9	(178x)	4	(175x)
5	20	(80x)	5	(140x)
D1694	6.4	(250x)	2.4	(292x)
BW1843U89	2.5	(640x)	0.5	(1400x)

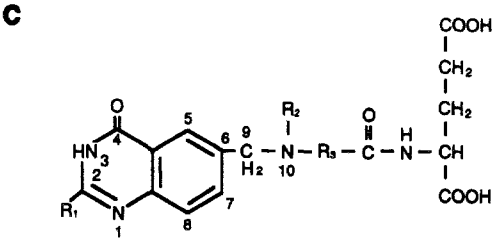
[a] Whole-cell TS assay (see Materials and methods). All assays were performed twice and each point was assayed in duplicate
[b] From Erlichman and Mitrovski [18]
[c] For chemical structures, refer to Fig. 1
[d] Fold improvement over CB3717

Structure-activity relationship

With the exception of dCB3717, the dideazafolate analogs were 27–292 times more active than the parent CB3717 as TS inhibitors. Of the modifications to the CB3717 compound studied, replacing the C2 amino with a methyl moiety increased the TS-inhibitory effect the most (35-fold). Substitution of a hydrogen in place of the amino group at the C2 position appeared to decrease the cytotoxic potency. Placement of a pyridine group at the benzyl position did not enhance TS inhibition. Substitution of a thiazole in place of a benzyl gave a further enhancement (5-fold), and modifications at the N10 position had no effect. Analogs 3, 4 and 5 and D1694 were between 4 and 8 times more potent than compound 1.

Fig. 1 a–c Chemical structures of **a** the folate cofactor 5,10-methylenetetrahydrofolate, **b** BW1843U89 and **c** CB3717 and analogs





Compound	R_1	R_2	R_3
CB3717	NH_2	$CH_2C \equiv CH$	
dCB3717	H	$CH_2C \equiv CH$	
1	CH_3	$CH_2C \equiv CH$	
2	CH_3	$CH_2C \equiv CH$	
3	CH_3	$CH_2C \equiv CH$	
4	CH_3	C_2H_5	
5	CH_3	CH_3	
D1694	CH_3	CH_3	

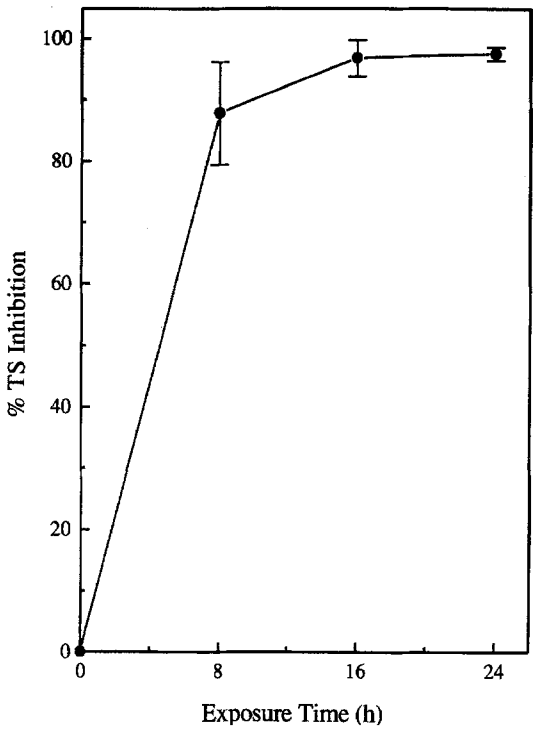
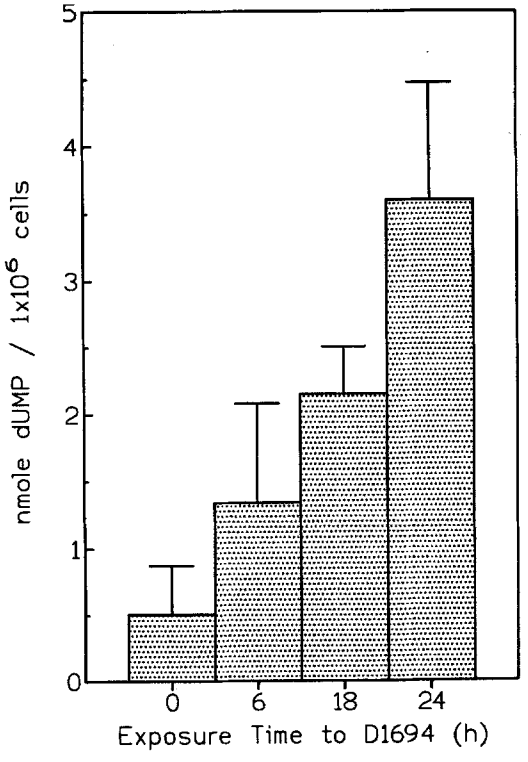


Fig. 2 Kinetics of TS inhibition following exposure to 10 n*M* (IC_{90}) D1694. TS activity was assayed by the whole-cell TS assay as described in Materials and methods. Experiments were done in triplicate

Fig. 3 Intracellular dUMP levels following exposure to 6.4 n*M* (IC_{50}) D1694 for 24 h. Each point represents the mean value for three separate experiments



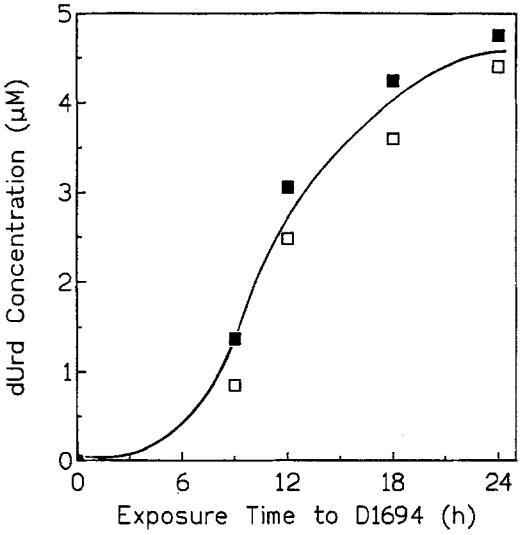


Fig. 4 Time course of dUrd accumulation in media following exposure to 10 n*M* (IC_{90}) D1694. Experiments were done in duplicate

Time course of TS inhibition

MGH-U1 cells were exposed to 10 n*M* D1694 (IC_{90}) and the TS activity in whole cells was measured over a 24-h period (Fig. 2). TS activity was affected at 8 h following drug exposure and remained inhibited for up to 24 h.

Intracellular dUMP and extracellular dUrd in media

Intracellular dUMP levels were measured for up to 24 h after exposure of cells to 6.4 n*M* of D1694 (Fig. 3). A steady increase in dUMP levels was observed initially at 6 h. At 24 h, levels were 7 times greater than the untreated control values. The dUMP kinetics observed at an IC_{90} dose of D1694 were comparable with those of an IC_{50} dose. Similar results were seen with CB3717 (data not shown). The dUrd concentration in media of cells that had been exposed for 24 h to an IC_{90} concentration of D1694 increased linearly during the 24-h exposure period (Fig. 4). The concentrations ranged from 1 to 4.5 μ*M*. dUrd was first detected at 9 h. Similarly, concentrations of dUrd present in the media after a 24-h exposure to IC_{90} concentrations of the other dideazafolates ranged from 2 to 6.5 μ*M*. Control cells were negative for dUrd.

Discussion

The development of novel folate-based TS inhibitors offers the opportunity for a better understanding of the biochemical consequences of such inhibition and the development of more effective treatment of cancer. Whereas 5-fluorouracil combined with folinic acid can mediate its cytotoxicity via a TS-based mechanism, the effects on RNA and incorporation of 5-fluorouracil into DNA makes interpreta-

tion of this effect less clear. The availability of several TS inhibitors with differences in drug uptake and intracellular metabolism allows further dissection of the various factors influencing TS inhibition.

A comparison of the potencies for cytotoxicity and inhibition of TS by CB3717, dCB3717 and compounds 1–5 gives some insight into the chemical determinants that are important in mediating drug activity. The addition of a C2 methyl group (compounds 1–5, D1694) increases cell killing and inhibition of TS significantly. These results are consistent with the structure-activity findings previously reported for L1210 cells [19–22]. This increased potency may in part be related to the use of the reduced-folate carrier by such compounds, whereas the parent compound CB3717 is not transported by this carrier [8]. Different potencies amongst the analogs can also be attributed to increased substrate specificity for FPGS. The formation of polyglutamated forms of the TS inhibitors is associated with a greater affinity for TS [19]. CB3717, dCB3717, compound 1 and D1694 are good substrates for FPGS and their polyglutamated derivatives are up to 200 times more potent as TS inhibitors [9, 23, 24]. Similarly, Duch et al. [10] have reported that BW1843U89 is also a substrate for FPGS and is polyglutamated to the diglutamate, which is not more inhibitory to TS. Modification at the benzene ring of CB3717 (R_3 in Fig. 1) appears to increase cytotoxicity and TS inhibition further (compounds 3, 5) when a five-membered ring is substituted. Modification at the N10 position (compounds 3, 5) has less effect on TS inhibition and cell killing than does the benzyl replacement. In the case of BW1843U89, the combination of a 3-methyl benzoquinazoline moiety with an isoindolinyl replacement of the benzyl ring in 5,10-methylenetetrahydrofolate leads to more potent TS inhibition and cytotoxicity than do all of the CB3717 analogs. Whereas part of this may be attributed to increased drug transport, it is likely that the three-dimensional structure of BW1843U89 is also associated with its greater binding affinity to TS.

There was an association between TS inhibition and cytotoxicity for each of the drugs examined, supporting the contention that TS is the primary target for the drugs studied. The strong association found between cytotoxic potency and TS inhibition (Table 1) further supports this mechanism of drug action. Previous studies of TS inhibition and cytotoxicity in L1210 cells [19, 22] did not reveal an association between these two parameters. These investigators used growth inhibition as an indicator of cytotoxicity and measured TS inhibition by parent compounds in TS extracted from cells. The impact of polyglutamation in this approach could not be assessed, nor could the effects of intracellular pools of dUMP and 5,10-methylenetetrahydrofolate. Thus, the association that we saw between clonogenic survival and TS inhibition suggests that the use of the whole-cell TS assay can predict clonogenic cell killing by folate-based TS inhibitors in MGH-U1 cells at least.

The time-course studies of TS inhibition by D1694 showed a concomitant increase in intracellular dUMP levels and extracellular dUrd concentrations. Furthermore, marked increases in the two metabolites and TS inhibition occurred with drug-exposure periods longer than 12 h. This is consistent with polyglutamation increasing TS inhibition of D1694. The production of dUrd as a result of TS inhibition has previously been reported with fluoropyrimidines. dUrd levels have been measured as a surrogate for TS inhibition in vivo following treatment with CB3717 [25]. Despite the increase in extracellular dUrd levels observed in these cells, there was a 7-fold increase in dUMP levels (Fig. 4) over 24 h. Production of dUMP after TS inhibition has been reported for FdUrd, methotrexate and CB3717 [15, 25–27]. As reported by Curtin et al. [28], this accumulation of dUMP may lead to formation of dUTP, and subsequent DNA strand breaks.

We conclude that inhibition of TS is the primary mechanism responsible for cell death induced in MGH-U1 cells by the folate-based TS inhibitors we studied. Structure-activity analysis supports the importance of a C2 methyl and a five-ring thiazole requirement for potency of TS inhibition. The benzoquinazoline BW1843U89 is the most potent inhibitor of TS. The biochemical consequences of TS inhibition by D1694 were increased intracellular dUMP levels and increased extracellular dUrd concentrations.

References

1. Rustum YM, Creaven PJ (1988) Symposium on the expanding role of folates and fluoropyrimidines in cancer chemotherapy. J Natl Cancer Inst 80: 1524–1525
2. Cantwell BM, Macaulay V, Harris AL, Kaye SB, Smith IE, Milsted RA, Calvert AH (1988) Phase II study of the antifolate *N*10-propargyl-5,8-dideazafolic acid (CB 3717) in advanced breast cancer. Eur J Cancer Clin Oncol 24: 733–736
3. Harding MJ, Cantwell BM, Milstead RA, Harris AL, Kaye SB (1988) Phase II study of the thymidylate synthetase inhibitor CB3717 (*N*10-propargyl-5,8-dideazafolic acid) in colorectal cancer. Br J Cancer 57: 628–629
4. Calvert AH, Newell DR, Jackman AL, Gumbrell LA, Sikora E, Grzelakowska-Sztabert B, Bishop JA, Judson IR, Harland SJ, Harrap KR (1987) Recent preclinical and clinical studies with the thymidylate synthase inhibitor *N*10-propargyl-5,8-dideazafolic acid (CB 3717). Natl Cancer Inst Monogr 5: 213–218
5. Bassendine MF, Curtin NJ, Loose H, Harris AL, James OF (1987) Induction of remission in hepatocellular carcinoma with a new thymidylate synthase inhibitor, CB3717. A phase II study. J Hepatol 4: 349–356
6. Calvert AH, Alison DL, Harland SJ, Robinson BA, Jackman AL, Jones TR, Newell DR, Siddik ZH, Wiltshaw E, McElwain TJ, et al (1986) A phase I evaluation of the quinazoline antifolate thymidylate synthase inhibitor, *N*10-propargyl-5,8-dideazafolic acid, CB3717. J Clin Oncol 4: 1245–1252
7. Jackman AL, Taylor GA, Oconnor BM, Bishop JA, Moran RG, Calvert AH (1990) Activity of the thymidylate synthase inhibitor 2-desamino-*N*-10-propargyl-5,8-dideazafolic acid and related compounds in murine (L1210) and human (W1L2) systems in vitro and in L1210 in vivo. Cancer Res 50: 5212–5218
8. Jackman AL, Newell DR, Gibson W, Jodrell DI, Taylor GA, Bishop JA, Hughes LR, Calvert AH (1991) The biochemical pharmacology of the thymidylate synthase inhibitor, 2-desamino-2-methyl-*N*10-propargyl-5,8-dideazafolic acid (ICI 198583). Biochem Pharmacol 42: 1885–1895

9. Jackman AL, Taylor GA, Gibson W, Kimbell R, Brown M, Calvert AH, Judson IR, Hughes LR (1991) ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo: a new agent for clinical study. Cancer Res 51: 5579–5586
10. Duch DS, Banks S, Dev IK, Dickerson SH, Ferone R, Heath LS, Humphreys J, Knick V, Pendergast W, Singer S et al (1993) Biochemical and cellular pharmacology of 1843U89, a novel benzoquinazoline inhibitor of thymidylate synthase. Cancer Res 53: 810–818
11. Erlichman C, Vidgen D (1984) Cytotoxicity of Adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids, and xenografts in immune-deprived mice. Cancer Res 44: 5369–5375
12. Aschele C, Romanini A, Nicolin A, Rosso R, Sobrero A (1990) Comparison of in vitro drug sensitivity by inhibition of tritium release from [5-^{3}H]-2′-deoxyuridine and a clonogenic assay. Anticancer Res 10: 759–764
13. Yalowich JC, Kalman TI (1985) Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 leukemia cells in vitro. Biochem Pharmacol 34: 2319–2324
14. Pressacco J, Erlichman C (1994) Combination studies with 3′-azido-3′-deoxythymidine (AZT) plus ICI D1694. Biochem Pharmacol (in press)
15. Grem JL, Mulcahy RT, Miller EM, Allegra CJ, Fischer PH (1989) Interaction of deoxyuridine with fluorouracil and dipyridamole in a human colon cancer cell line. Biochem Pharmacol 38: 51–59
16. Roberts D (1966) An isotopic assay for thymidylate synthetase. Biochemistry 5: 3546–3548
17. Frick LW, Nelson DJ, St Clair MH, Furman PA, Krenitsky TA (1988) Effects of 3′-azido-3′-deoxythymidine on the deoxynucleotide triphosphate pools of cultured human cells. Biochem Biophys Res Commun 154: 124–128
18. Erlichman C, Mitrovski B (1994) Comparative cytotoxicity of folate-based inhibitors of thymidylate synthase and 5-fluorouracil ± leucovorin in MGH-U1 cells. Cancer Chemother Pharmacol 34: 51–56
19. Marsham PR, Hughes LR, Jackman AL, Hayter AJ, Oldfield J, Wardleworth JM, Bishop JA, O'Connor BM, Calvert AH (1991) Quinazoline antifolate thymidylate synthase inhibitors: heterocyclic benzoyl ring modifications. J Med Chem 34: 1594–1605
20. Jones TR, Thornton TJ, Flinn A, Jackman AL, Newell DR, Calvert AH (1989) Quinazoline antifolates inhibiting thymidylate synthase: 2-desamino derivatives with enhanced solubility and potency. J Med Chem 32: 847–852
21. Jones TR, Betteridge RF, Neidle S, Jackman AL, Calvert AH (1989) Quinazoline antifolates inhibiting thymidylate synthase: computer modelling of the N10 substituent. Anticancer Drug Des 3: 243–248
22. Hughes LR, Jackman AL, Oldfield J, Smith RC, Burrows KD, Marsham PR, Bishop JA, Jones TR, O'Connor BM, Calvert AH (1990) Quinazoline antifolate thymidylate synthase inhibitors: alkyl, substituted alkyl, and aryl substituents in the C2 position. J Med Chem 33: 3060–3067
23. Pawelczak K, Jones TR, Kempny M, Jackman AL, Newell DR, Krzyzanowski L, Rzeszotarska B (1989) Quinazoline antifolates inhibiting thymidylate synthase: synthesis of four oligo(L-gamma-glutamyl) conjugates of *N*10-propargyl-5,8-dideazafolic acid and their enzyme inhibition. J Med Chem 32: 160–165
24. Jackman AL, Taylor GA, Moran RG, Bishop JAM, Bisset G, Pawelczak K, Balmanno K, Hughes LR, Calvert AH (1988) Biologic properties of 2-desamino-2-substituted-5,8-deazafolates that inhibit thymidylate synthase (TS) (abstract). Proc Am Assoc Cancer Res 29: 287
25. Jackman AL, Taylor GA, Calvert AH, Harrap KR (1984) Modulation of anti-metabolite effects: effects of thymidine on the efficacy of the quinazoline-based thymidylate synthetase inhibitor, CB3717. Biochem Pharmacol 33: 3269–3275
26. Berger SH, Hakala MT (1984) Relationship of dUMP and free FdUMP pools to inhibition of thymidylate synthase by 5-fluorouracil. Mol Pharmacol 25: 303–309
27. Curtin NJ, Harris AL (1988) Potentiation of quinazoline antifolate (CB3717) toxicity by dipyridamole in human lung carcinoma, A549, cells. Biochem Pharmacol 37: 2113–2120
28. Curtin NJ, Harris AL, Aherne GW (1991) Mechanism of cell death following thymidylate synthase inhibition: 2′-deoxyuridine-5′-triphosphate accumulation, DNA damage, and growth inhibition following exposure to CB3717 and dipyridamole. Cancer Res 51: 2346–2352