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# A Potent Antitumour Quinazoline Inhibitor of Thymidylate Synthetase: Synthesis, Biological Properties and Therapeutic Results in Mice*

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**Abstract**—*We describe the synthesis and some biological properties of* N-(4-{N-[(2-*amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino}benzoyl)-*L-*glutamic acid* (CB 3717), *a potent antifolate inhibitor of thymidylate synthetase* (EC 2.1.1.45). *This compound inhibited thymidylate synthetase from* L1210 *cells competitively with respect to the substrate methylene-tetrahydrofolate. The inhibitor constant* ($K_i$) *was approximately* 1nM. *Toxicity to* L1210 *cells in suspension culture could be reversed more effectively by co-incubation with thymidine than with folinic acid. A cultured subline of* L1210 *cells resistant to methotrexate by virtue of increased cellular dihydrofolate reductase* (EC 1.5.1.4) *content was not cross-resistant to* CB 3717. *Treatment of animals bearing the* L1210 *tumour with* CB 3717 *at* 125 *or* 200 mg/kg/day *for* 5 days *resulted in 'cures'* (>120 day *survival) in* 90% *of animals. These doses did not cause obvious toxicity or significant weight loss in animals. The studies in tissue culture and the enzymology suggest that thymidylate synthetase is the cytotoxic locus of action of this compound. The high therapeutic efficacy of* CB 3717 *may be due to preservation of* de novo *purine synthesis, which is inhibited by other antifolates. The* in vitro *results further suggest that* CB 3717 *could be active against methotrexate-resistant tumours.*

## INTRODUCTION

METHOTREXATE, a classical antifolate drug, is used widely in the treatment of human malignant disease, particularly in the treatment of choriocarcinoma [1] and osteosarcoma [2] and in the maintenance of acute lymphatic leukaemia [3]. It acts by the inhibition of the enzyme dihydrofolate reductase (DHFR, EC 1.5.1.4) which in turn causes a depletion of the one-carbon carrying tetrahydrofolate pools resulting in the inhibition of the *de novo* synthesis both of thymidine and the purine nucleotides necessary for DNA synthesis [4]. Although the "purineless" state induced by methotrexate has been shown to cause toxicity to the gastro-intestinal tract of mice [5], it does not contribute to the cytotoxic effect of methotrexate against some cultured tumour cell lines [6] and may antagonise it in others [7]. Methotrexate does not require metabolic activation, and does not undergo significant metabolic degradation in the majority of cell types [4]. Known causes of methotrexate resistance include reduced membrane transport [8–10] and increased cellular DHFR [11–13]. In this latter case the effective target of methotrexate may become thymidylate synthetase (TS, EC 2.1.1.45), the enzyme catalysing the terminal step in the *de novo* synthesis of thymidine nucleotides required exclusively for DNA synthesis [14]. Direct inhibition of TS may be achieved by an active metabolite of 5-fluorouracil, 5-fluorodeoxyuridine monophosphate. However, 5-fluorouracil resistance is frequently accompanied by a reduction in the appropriate activating enzymes [15] and the drug may also have toxic effects which are due to its incorporation into RNA [16].

We therefore sought an effective folate inhibitor of TS since such a compound may be expected to have activity equal to or superior

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to methotrexate not only in methotrexate-sensitive tumours, but also in those resistant by virtue of raised cellular DHFR. It should also be less toxic to the host since the synthesis of purines would not be affected. Further, folate analogues should be more effective than pyrimidines since they do not require metabolic activation and are poor substrates for degradation.

Several classical quinazoline antifolates have been shown to be inhibitors of TS. Compounds with the 2,4-diamino configuration have been shown to be reasonably effective inhibitors of TS [17–19] but these compounds also bind extremely tightly to DHFR [18–21]. In the 2-amino-4-hydroxy series of quinazolines it has been demonstrated that a methyl group introduced into the 5-position impairs the inhibition of thymidylate synthetase [18, 22], but introduced into the 10-position enhances it [23, 24]. A study of a series of compounds with aliphatic chains of increasing length at the 10-position showed that optimal TS inhibition was found with the ethyl substituent [25, 26]. The proposed structure of the modified Friedkin intermediate proposed by Danenberg [27] for the ternary complex thought to occur during TS catalysis (Fig. 1) suggested the possibility of irreversible

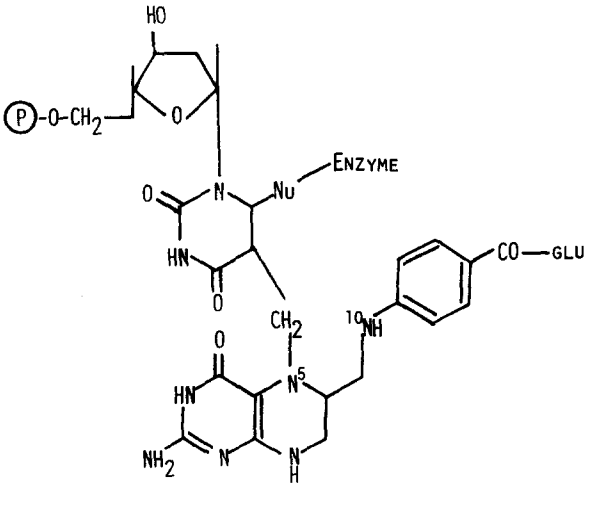



*Fig. 1. Friedkin's proposed intermediate during TS catalysis.*

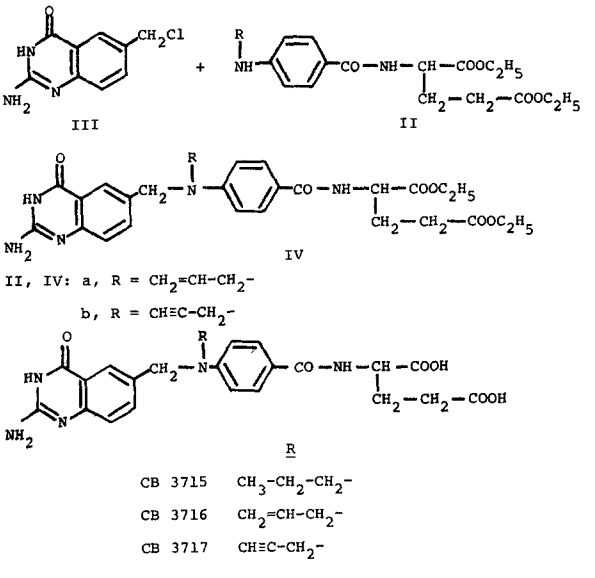
inhibition of TS by alkylation of its catalytic nucleophile. This is almost certainly a cysteine sulphydryl group [28–36]. From Fig. 1 a three-carbon chain is seen to be needed at N10 or N5. The potentially reactive allyl and propargyl groups were therefore introduced at the 10-position giving two new compounds, CB 3716 and 3717, respectively. Although these groups would not normally be considered reactive to sulphydryl addition, their proximity to this group in the enzyme–inhibitor complex might favour such a reaction.

The compounds were evaluated *in vitro* as inhibitors of TS and cell growth and *in vivo* against the L1210 tumour. Since methotrexate (MTX) resistance is an important problem in cancer therapy these quinazolines were also investigated with this in mind; the cross-resistance of an MTX-resistant cell line L1210/R71 [37], which is known to have elevated DHFR but normal MTX transport, was measured in culture, and the ability of a quinazoline to compete with labelled MTX or folic acid for transport into L1210 cells was also assessed.

## MATERIALS AND METHODS

### *Chemistry*

The desired antifolates, CB 3716 and CB 3717, having, respectively, the allyl and propargyl groups at N-10, were synthesised using the method reported elsewhere [25]. Thus the allylamine IIa and the propargylamine IIb, each prepared by the direct alkylation of diethyl *N*-(4-amino-benzoyl)-L-glutamate [38], were conjugated with 2-amino-6-chloromethyl-4-hydroxyquinazoline (III) [39] yielding the diesters IVa and IVb, respectively. Mild saponification of these diesters gave the finished antifolates (see Scheme).




### *Experimental*

The methods used for the determination of melting points, elemental analyses, preparative h.p.l.c. and t.l.c. have been described elsewhere [25]. Every compound had a satis-

factory (±0.4%) microanalysis for C, H and N. N.M.R. spectra were taken on a Perkin–Elmer R12B 60MHz spectrometer except those for compounds IVa and IVb which were taken at 50°C on a Bruker HFX 90 MHz Fourier transform instrument. Centrifugation was performed at 20,000 ***g***.

Diethyl *N*-[4-(prop-2-enylamino)benzoyl]-L-glutamate (IIa) and diethyl *N*-[4-(prop-2-ynylamino)benzoyl]-L-glutamate (IIb) were prepared by treating diethyl *N*-(4-aminobenzoyl)-L-glutamate [38] (5.00 g) in ethanolic solution (100 ml) with the appropriate bromide in the presence of potassium carbonate (2.14 g). The reaction conditions are collected in Table 1. Following removal of the solvent *in vacuo* the residue was dissolved in chloroform and the solution washed with water. The extract was dried ($MgSO_4$) and concentrated to give the crude product. This was purified by h.p.l.c. on a Jobin-Yvon Chromatospac Prep 10 containing silica gel (Merck, Art 11695) and using 50:50 chloroform:petroleum ether as eluent. The structures were confirmed by elemental analysis and n.m.r. spectroscopy.

*Table* 1. *Preparation of chemical intermediates*

| Amine | IIa | IIb |
|---|---|---|
| Reagent | Allyl bromide | Propargyl bromide |
| Amount | 5.4 ml | 10 ml* |
| Reaction temp., °C | 60 | 70 |
| Reaction time, hr | 8 | 4 |
| Yield, % | 76.5 | 55.5 |
| m.p., °C | 60–61† | 98–99† |

*Volume of an 80% solution in toluene.
†Not recrystallised.

Diethyl *N*-(4-{*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl] prop-2-enylamino}-benzoyl)-L-glutamate (IVa) and diethyl *N*-(4-{*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino}-benzoyl)-L-glutamate (IVb) were prepared by alkylation of the respective amines IIa and IIb with 2-amino-6-chloromethyl-4-hydroxyquinazoline III using exactly the method previously described [25]. Details of the reactions are collected in Table 2. The structures were confirmed by elemental analysis and Fourier n.m.r. spectroscopy.

*N*-(4-{*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-enylamino}benzoyl)-L-glutamic acid (CB 3716) and *N*-(4-{*N*-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino}-benzoyl)-L-glutamic acid (CB 3717) were obtained from the corresponding diesters IVa and IVb by the saponification method described elsewhere [25]. The structures were upheld by elemental analysis and u.v. spectroscopy (spectra determined in 0.1 N NaOH and presented in Table 3).

*Table* 2. *Preparation of antifolate diesters*

| Diester | IVa | IVb |
|---|---|---|
| Reaction temp., °C | 100 | 105 |
| Reaction time, hr | 5 | 6 |
| Yield, % | 21.5 | 27.5 |
| m.p., °C | 107–112 | 143–147 |

*Table* 3. *Physical properties of antifolates*

| Diacid | CB 3716 | CB 3717 |
|---|---|---|
| Yield, % | 46 | 81 |
| m.p., °C | 207–209 | 232–235 |
| u.v. $\lambda$ (nm), ($\varepsilon$) | | |
| Maxima | 311.5 (29,100) | 301.5 (26,600) |
| | 276 (17,600) | 279 (23,900) |
| | 228.5 (49,300) | 229 (50,700) |
| Minima | 282 (16,900) | 284 (23,700) |
| | 252 (9060) | 251.5 (9800) |

*Enzymology*

Quinazolines were dissolved in N/10 NaOH and neutralised before use. TS was partially purified from the cytosol of log phase L1210 cells harvested from animals and assayed as described before [18]. The $I_{50}$ was defined as the inhibitor concentration necessary to reduce the reaction rate by 50%. DHFR was purified by affinity chromatography and $K_i$ determinations performed using published methods [40, 41].

*Cell culture*

L1210 and L1210/R71 cells were grown in continuous suspension culture in RPMI 1640 medium containing 10% horse serum (Flow Laboratories, Irvine, Scotland, U.K.). Cells were diluted to $10^5$/ml and additions (diluted in medium and sterilised by Millipore filtration) made prior to starting each incubation. Cell counts were performed after 24 and 48 hr using an improved Neubauer haemocytometer. Under these conditions the doubling time of untreated cells was 12 hr. The $ID_{50}$ was defined as that concentration of

drug nessary to reduce the 48 hr count to 50% of control.

Transport studies were conducted as previously described [9]. Briefly, cells were incubated in the presence of a labelled substrate, aliquots removed, the cells separated and washed by centrifugation and the pellet counted. Radiochemicals were supplied by The Radiochemical Centre (Amersham, Bucks, U.K.).

*Animal experiments*

The L1210 tumour was carried routinely in DBA2 mice. For experiments $5 \times 10^{-4}$ cells were implanted i.p. into female C57Bl $\times$ DBA2 $F_1$ hybrid mice 3 days prior to treatment, giving an established tumour of about $2 \times 10^6$ cells at the time of treatment. Quinazolines were dissolved in N/10 NaOH, diluted to the appropriate volume and the pH adjusted to 8.5 before i.p. injection.

## RESULTS

*Inhibition of DHFR and TS*

This is shown in Table 4. The published results obtained with the 10-propyl compound (CB 3715) [25, 26] are included for comparison.

The substitution of an allyl or a propargyl group for the propyl group at the 10-position improved the inhibition of TS by about 3 and 30-fold, respectively, while that of DHFR was less affected. The inhibition of TS by CB 3717 was characterised and found to be competitive with 5,10-methylenetetrahydrofolate with a $K_i$ of 1.14 nM.

*Cell cultures*

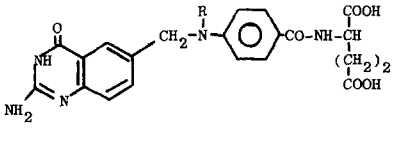
The $ID_{50}$ concentrations in cell culture for L1210 and L1210:R71 are shown in Table 5. Not only did the toxicity of the drugs to both cell lines increase with progressive unsaturation of the 10-substituent but also the degree of cross-resistance of the L1210:R71 became less marked. The L1210:R71 is about 600-fold resistant to MTX [37].

Reversal of the toxicity of CB 3717 could be achieved by co-incubation with thymidine (10 $\mu$M) but only partial reversal was achieved with folinic acid at any concentration up to 100 $\mu$M. These results are shown in Fig. 2.

*Transport studies*

The effect of varying concentrations of CB 3717 in the medium upon the transport of tritiated methotrexate and tritiated folic acid is shown in Fig. 3. The transport of both of

*Table 4. Inhibition of DHFR and TS by quinazolines*



| House number | R = | Thymidylate synthetase $I_{50}$ (nM) | Dihydrofolate reductase $K_i$ (nM) |
|---|---|---|---|
| CB 3715 | $-CH_2-CH_2-CH_3$ | 170 | 20 |
| CB 3716 | $-CH_2-CH=CH_2$ | 69 | 27 |
| CB 3717 | $-CH_2-C\equiv CH$ | 5 | 14 |

*Table 5.* In vitro *toxicity of quinazolines*

| House number | $ID_{50}$ values ($\mu$M) L1210 | L1210/R71 | Cross resistance ($ID_{50}$ L1210:R71/ $ID_{50}$ L1210) |
|---|---|---|---|
| CB 3715 | 33.0 | 315 | 10 |
| CB 3716 | 8.8 | 100 | 11 |
| CB 3717 | 5.0 | 14 | 3 |

these compounds was inhibited by the quinazoline.

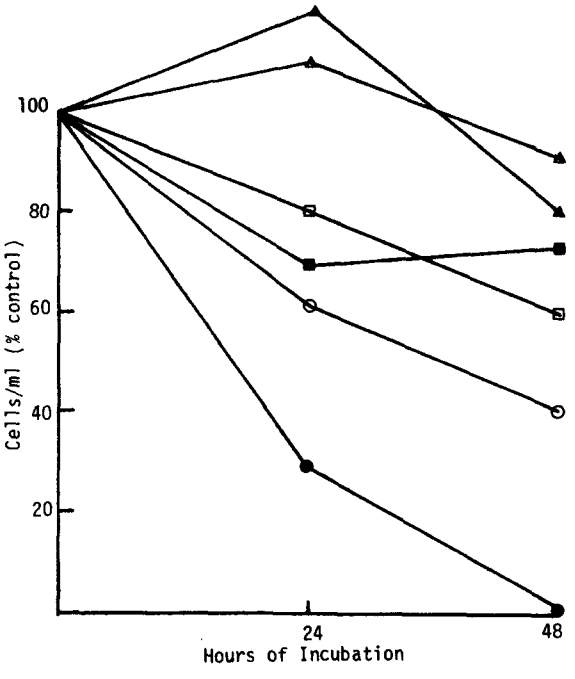



*Fig. 2. Protection of* L1210 *cells in culture from* 20 μM CB3717. ●, CB 3717 *alone;* ○, CB 3717 + *folinic acid* 5 μM; □, CB 3717 + *folinic acid* 50 μM; ■, CB 3717 + *folinic acid* 500 μM; ▲, CB 3717 + TdR 1 μM; △, CB 3717 + TdR 10 μM.

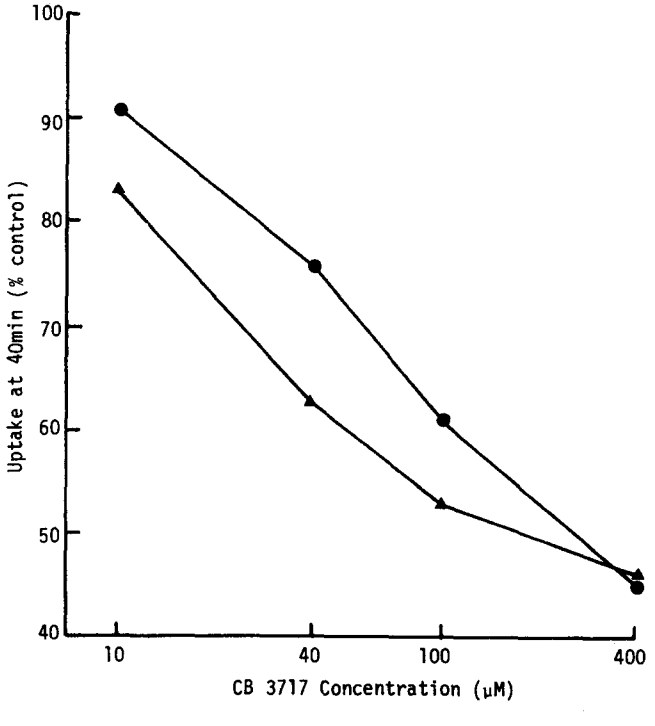



*Fig. 3. The effect of* CB 3717 *on the transport of* [$^3$H] *folic acid and* [$^3$H] *methotrexate into* L1210 *cells.* ●, 4 μM *MTX;* ▲, 4 μM *folic acid.*

*Treatment of* L1210 *tumour-bearing animals*

These results are summarised in Table 6 and Fig. 4. Single doses of CB 3717 were not toxic and produced very little therapeutic effect. However, a 5 daily dose schedule produced increases in mean survival time which were dose related (Table 6). At doses of 125 or 200 mg/kg 9 out of 10 long-term survivors (> 120 days) were seen (Fig. 4). These animals did not lose a significant amount of weight or show other signs of toxicity while undergoing treatment with the drug (Fig. 5). Drug availability and solubility precluded the use of higher doses. As the injection solutions could only be neutralised to pH 8.5 while preserving solubility, the appropriate controls were injected with bicarbonate at pH 8.5. None of the control groups showed any increase in life span. For comparative purposes further groups of animals were treated with various doses of methotrexate given on the same schedule. The optimal dose of methotrexate, 3 mg/kg, gave an increase in median lifespan of 71%, but only 2/10 long-term survivors.

## DISCUSSION AND CONCLUSIONS

The 10-substituent of a 2-amino-4-hydroxy quinazoline antifolate is clearly an important determinant of its activity against thymidylate synthetase. The studies reported here show that progressive unsaturation of the terminal C–C bond of the three-carbon substituent at N10 improves the inhibition of TS. Although our objective was to form a covalent bond to the active site of the enzyme producing an irreversible inhibition, the fact that this inhibition is competitive with methylene-tetrahydrofolate supports the alternative possibility that the propargyl substitution acts simply by greatly increasing the affinity of the drug for the catalytic site on the enzyme. CB 3717 is thus the tightest binding folate inhibitor of thymidylate synthetase reported at the time of writing. The hypothesis that this drug acts on TS as the chief locus is supported by several considerations. Firstly, its affinity for DHFR is 10-fold less than that for TS. Since DHFR is usually present in about 20-fold excess of TS [42–44] it is unlikely to become rate limiting. Secondly, it was possible to afford virtually complete protection to L1210 cells in culture by the addition of thymidine to the medium. Since methotrexate toxicity in the same system may only be reversed by the simultaneous addition of thymidine and a purine [6] it is likely that CB

*Table 6. Therapeutic results of* CB 3717 *treatment in mice bearing the* L1210 ascitic tumour

| CB 3717 dose (mg/kg) | Animals per group | Protocol | % Increase in mean survival time | Long term survivors (>80 days) |
|---|---|---|---|---|
| 80 | 5 | Single dose | 14 | 0 |
| 150 | 5 | Single dose | 32 | 0 |
| 8 | 5 | Daily × 5 | 8 | 0 |
| 16 | 5 | Daily × 5 | 13 | 0 |
| 16 | 5 | Daily × 5 | 29 | 0 |
| 32 | 5 | Daily × 5 | 62 | 0 |
| 64 | 5 | Daily × 5 | 72 | 0 |
| 128 | 5 | Daily × 5 | >1200 | 4 |
| 125 | 10 | Daily × 5 | >1200 | 9* |
| 200 | 10 | Daily × 5 | >1200 | 9* |

The bracketed groups indicates individual experiments.
*See Figure 4.

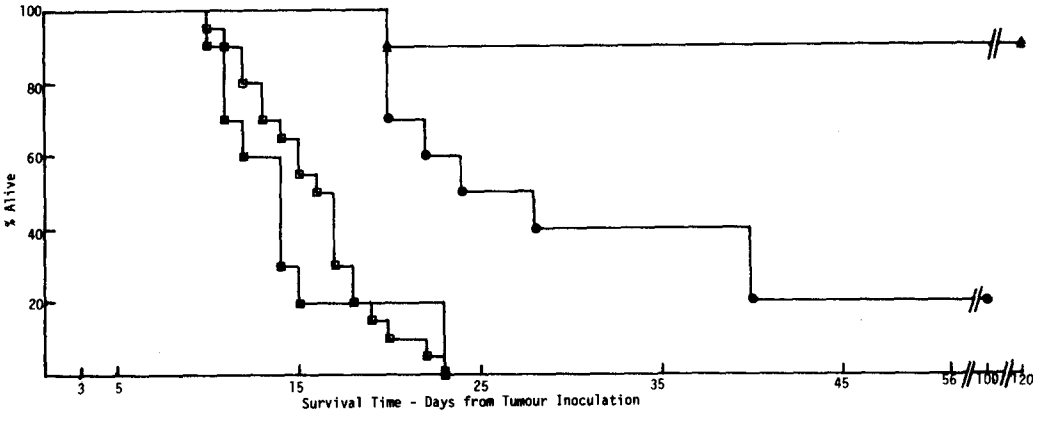



*Fig. 4. Survival of* L1210*-bearing mice injected with* CB 3717. ■, *No treatment* (10 *animals*)—*MTX control;* □, *No treatment* (20 *animals*)—CB 3717 *control.* (*Animals were injected with* 20 mM $NaHCO_3$ *buffer* 0.4 ml *daily* × 5 days.) ●, *MTX* 3 mg/kg *daily* × 5 (10 *animals*); ▲, CB 3717 125 mg/kg *daily* × 5 (10 *animals*);* △, CB 3717 200 mg/kg *daily* × 5 (10 *animals*).* *The difference in the survival of quinazoline- and MTX-treated animals is highly significant,* $P<0.001$ *using the log rank test* [45]. **Survivals from these treatments were identical, hence the data points are superimposed.*

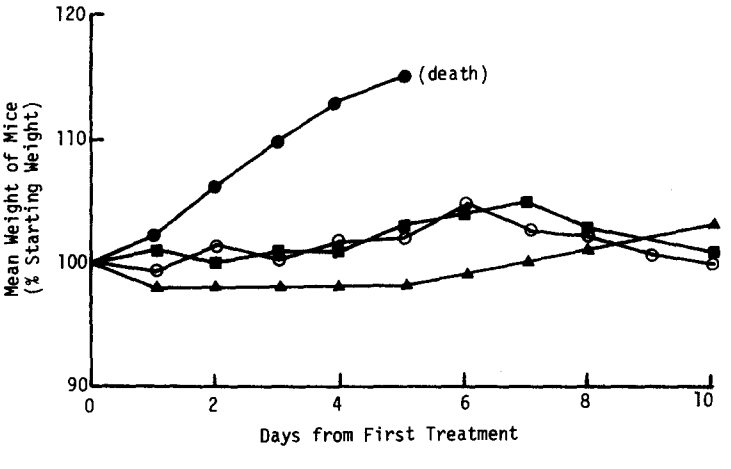



*Fig. 5. Body weight changes in groups of* 10 *mice treated with* 5 *daily doses of* CB 3717 *at* 125 mg/kg/day (■); 200 mg/kg/day (▲); *untreated tumour-bearing controls* (●): *untreated controls without tumour* (○).

3717 had no significant effect on *de novo* purine synthesis. Thirdly, folinic acid reversal of CB 3717 in cell culture was incomplete even when a high concentration (100 $\mu$M) was used. This concentration is 200 times higher than that needed to reverse methotrexate (unpublished observations). The partial reversal seen with folinic acid may be attributed to expansion of the intracellular methylenetetrahydrofolate pool and the competition of this substrate with the inhibitor.

Methotrexate resistance has been documented as due to an increased cellular DHFR content and a reduction in the cell membrane transport by the mechanism responsible for carrying both methotrexate and tetrahydrofolate derivatives. An alternative antifolate is clearly advantageous if it can circumvent these causes. Increased cellular DHFR will be expected not to confer resistance to a TS inhibitor unless TS is also raised. Mammalian cell lines in which DHFR is reported to be raised have not had concurrently raised TS [14]. Thus CB 3717 may be expected to be active against such DHFR-mediated methotrexate-resistant cell lines. As it was not possible to measure the transport of CB 3717 directly owing to the lack of availability of a suitable assay, the indirect method was used. CB 3717 caused a dose-dependent reduction of the transport of both methotrexate and folic acid in L1210 cells. As methotrexate and folic acid are transported by separate routes, this suggests that the quinazoline may utilise either pathway. This is in contrast to a 2-4-diaminoquinazoline which appears to be transported by the same mechanism as methotrexate [18].

The achievement of 90% long-term survival in the L1210 tumour is a remarkable result since a high proportion of cures is not usually obtained with single drug therapy in this system. The fact that a group of animals treated optimally with methotrexate contained only 2 long-term survivors suggests that this result is not an artifact of the system. No long-term survivors were seen in groups of animals treated with lower or higher doses of methotrexate. Clearly a much more extensive evaluation of this compound is necessary, but the available evidence suggests that it will be a useful alternative antifolate with possible activity against methotrexate resistant tumours.

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