Biochemical Pharmacology, Vol. 33, No. 20, pp. 3269–3275, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

MODULATION OF ANTI-METABOLITE EFFECTS

EFFECTS OF THYMIDINE ON THE EFFICACY OF THE QUINAZOLINE-BASED THYMIDYLATE SYNTHETASE INHIBITOR, CB3717

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(*Received* 19 *January* 1984; *accepted* 1 *June* 1984)

Abstract—CB3717 (*N*-(4-(*N*-((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynylamino)benzoyl)-L-glutamic acid) is an antitumour agent that inhibits thymidylate synthetase (TS). A dose-dependent fall in plasma thymidine (dThd) (1.43 μM to 0.47 μM) occurred in non-tumour-bearing mice following the administration of CB3717. Further, in mice carrying the L1210/CBRI tumour, the drug's antitumour properties were ablated by co-administration of dThd, an effect consistent with TS being the cytotoxic locus. *In vitro* studies of protection by dThd against CB3717 cytotoxicity were carried out in an attempt to quantify this reversal. The metabolism of [^{14}C]-dThd was measured in cultures of L1210 cells (10^4/ml) exposed to a completely cytotoxic dose of CB3717 (50 μM). The cytotoxicity of the drug was only expressed when the dThd concentration (0.5–2 μM) had fallen to <0.1 μM in the media. This reduction was due to: (1) dThd incorporation into DNA, (2) catabolism of dThd to thymine. By reducing the initial cell concentration to 10^3/ml the depletion of dThd was substantially reduced and consequently cells continued to grow for a longer period. The critical concentration of dThd, below which growth in the presence of CB3717 could not be supported was estimated to be between 0.026 and 0.1 μM. Thus even the minimum level of dThd achieved *in vivo* was still in excess of that required for protection from CB3717 toxicity *in vitro*. There was a small accumulation of deoxyuridine (dUrd) (~2-fold) in mouse plasma 24 hr after completion of a 5-day course of CB3717 (200 mg/kg) but *in vitro* studies demonstrated that this was unlikely to modulate CB3717 toxicity in the presence of dThd. We caution against the use of rodent tumour models (or human tumour xenografts) for antitumour or toxicity testing of compounds designed to inhibit the *de novo* synthesis of thymidylate; they may be misleading because the high dThd levels found in these animals compared with man may mask the cytotoxic effects of these drugs.

Folate analogues have been synthesized for many years as potential antimetabolites. In the late forties a number of diaminopteridines, for example methotrexate (MTX), were shown to possess antitumour activity which was later found to be associated with the inhibition of dihyrofolate reductase, EC 1.5.1.4. (DHFR) [1]. The widespread use of MTX in various clinical protocols prompted more detailed studies of folate and nucleotide biochemistry. This led to the realization that salvage of preformed purines and/or pyrimidines may circumvent the block on their *de novo* synthesis resulting from inhibition of DHFR [2–7 and reviewed in 8–11]. This phenomenon has been exploited clinically by the use of thymidine (dThd) [12–14] or dThd/purine rescue regimens [15] to overcome the toxicity of MTX. However, there is no good evidence to suggest that such a rescue is selective for the normal tissues at the expense of the tumour. The possibility that the presence of plasma nucleosides could prejudice the antitumour effects of antifolates therefore exists.

We have previously described the design, synthesis, antitumour and biochemical properties of a novel quinazoline antifolate, CB3717 (Fig. 1), that has thymidylate synthetase EC 2.1.1.45 (TS) as its cytotoxic locus [16–19].

We were aware of the apparent paradox that certain inhibitors of thymidylate synthesis (e.g. MTX, CB3717) have antitumour properties despite the presence of salvageable thymidine. Fluorinated pyrimidines may inhibit TS but are unsuitable compounds with which to study salvage, because they require metabolic activation by the enzymes involved in normal pyrimidine metabolism and their cytotoxicity may, at least in part, be due to their incorporation into nucleic acids (reviewed in [20, 21]). It is therefore more appropriate to approach the problem by investigating the mode of action of folate analogues which neither require activation nor are incorporated into nucleic acid. As CB3717 is an antifolate devoid of effects on purine synthesis we set out to investigate the reversibility of its effects by dThd both *in vivo* and *in vitro*.

Reduction of *de novo* thymidylate synthesis might be expected to result in a reduction of the plasma dThd level. This has been shown to occur in man following high-dose MTX treatment [25]. In addition, the inhibition of TS by CB3717 results in a rise in the level of 2′-deoxyuridylate (dUMP) behind the block [19]. We have previously demonstrated that the level of the corresponding deoxynucleoside, dUrd, is elevated (~5-fold) in the plasma of mice for several hours following a curative dose of CB3717 (120 mg/kg) [23].

In the current study we have first demonstrated

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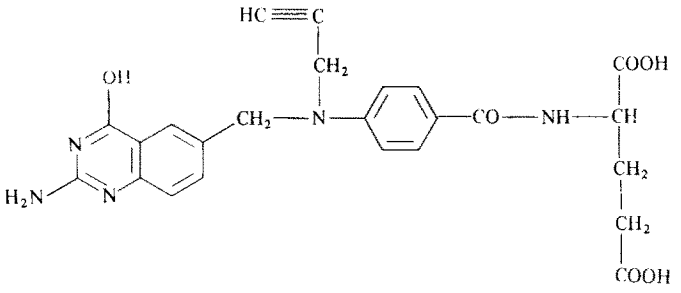

Fig. 1. Structure of CB3717 (*N*-(4-(*N*-((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynylamino)-benzyol))-L-glutamic acid.

the ability of dThd to prevent the antitumour effect of CB3717 *in vivo*. In non-tumour-bearing mice we measured the levels of dThd 24 hr after each dose of a 5-day course of CB3717 in plasma and followed these levels for several days. In addition, we measured plasma dThd and dUrd in individual mice which had completed their 5-day course of CB3717 24 hr earlier so that variability between mice could be established and the mean level compared with controls. We then investigated whether the changes observed had any significance as determinants of cytotoxicity during CB3717 treatment using L1210 cells grown in culture as a model. As cells grown *in vitro* are not maintained in homeostatic conditions we found it necessary to monitor the metabolism of dThd in the medium during these studies. The rapid depletion of dThd in the medium of L1210 cells exposed to MTX and inosine had previously been reported [22].

Finally we discuss the possible relevance of these results in relation to patient response to CB3717, and in the development of new drugs targeted at the same area of metabolism.

MATERIALS AND METHODS

All chemicals were of analytical grade. 2′-deoxyuridine was obtained from Sigma (London, U.K.) and thymidine from Cambrian Chemicals (Croydon, U.K.). Tissue culture medium and horse serum were purchased from Flow Laboratories (Irvine, Scotland, U.K.). [^{14}C]-thymidine was supplied by The Radiochemical Centre (Amersham, Bucks, U.K.) and Cocktail T from BDH (Poole, Dorset, U.K.). Jones Chromatography (Llanbradach, Mid-Glamorgan, U.K.) supplied the Apex 5 μ C18 packing material.

Anti-tumour testing. The L1210 tumour was carried routinely in male DBA2 mice. For experiments, 5×10^4 cells were implanted i.p. into male C57B1 × DBA2 F_1 hybrid mice on day 0. Treatment with CB3717 (100 mg/kg/day × 5 i.p.) started on day 3. The drug (10 mg/ml) was dissolved in 0.15 M $NaHCO_3$ and adjusted to pH 9.0 with NaOH. Treatment with dThd was 500 mg/kg t.d.s. and was given for either eight days starting with the first CB3717 injection or for four days starting with the last CB3717 injection. Control animals received solvent(s) alone. All groups contained ten mice.

Plasma dThd *and* dUrd. Male C57B1 × DBA2 F_1 hybrid mice were injected with CB3717 (100 mg or 200 mg/kg daily i.p.) for up to five days. Controls received either nothing or 0.15 M $NaHCO_3$ pH 9.0. Exsanguination was done by cardiac puncture. Plasma separation was performed immediately and frozen at −20° for up to one month. No deterioration in plasma nucleosides occurred as determined by radioactively spiked plasma samples. Neutralized perchlorate extracts of plasma from either individual mice or pooled from four mice were assayed for dThd and dUrd by the HPLC method previously described [23, 26].

Tissue culture. L1210 cells were grown in suspension culture in RPM1 1640 with 20 mM HEPES buffer and supplemented with 10% horse serum. Cell suspensions were diluted to either 10^3/ml or 10^4/ml as indicated in the text. CB3717 in RPM1 1640 was adjusted to pH 9.0 with 1 N NaOH prior to millipore filtration and then neutralized with sterile 1 N HCl. The final concentration of CB3717 in all experiments was 50 μM ($ID_{50} = 5\ \mu M$). In the absence of rescue agents this concentration gives a cell count at 48 hr of <1% of control. All sterile components were added to the tissue flasks prior to the addition of the cells. dThd was added to give the final concentrations shown with a specific activity of ~20 μCi/μmole. Cell counts were performed daily using an improved Neubauer haemocytometer. However, because of the low cell numbers involved when inoculated at 10^3/ml, separate, non-radioactive 10 ml cultures were spun for 5′ at 600 *g* and the cell pellet resuspended in 1 ml before the cell count was determined. This was performed at the 44 hr and 68 hr time points. At the times indicated 1 ml aliquots were removed from each of the duplicate cultures into tubes on ice containing 20 μl of 12.4 M perchloric acid. After standing for 15 min on ice, acid-insoluble material was removed by centrifugation at 2000 *g* for 5 min. The supernatant was neutralized with solid potassium hydrogen carbonate and the resulting potassium perchlorate removed by centrifugation. dThd and its metabolites were separated by HPLC on an Apex 5 μ C18 reverse-phase column (15 cm × 4.6 mm) running isocratically in 90% ammonium acetate pH 5.0, 10% methanol. Following the injection of a 500 μl sample, fourteen 1 ml fractions were collected directly into scintillation vials in a LKB 2111 Multirak fraction collector. Ten millilitres of cocktail T scintillant were added to each vial which was then counted on an Intertechnique Scintillation Counter Model SL30.

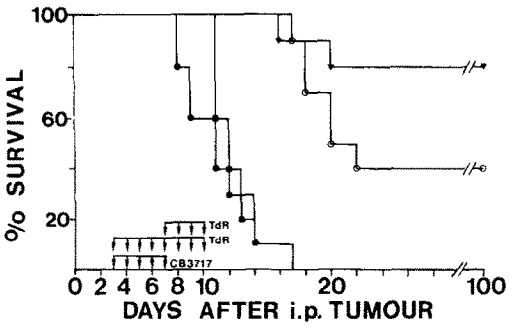

Fig. 2. Prevention of antitumour activity (L1210/CBRI) of CB3717 by thymidine. ●—● controls; ▼—▼ CB3717 100 mg/kg/day × 5; ■—■ CB3717 100 mg/kg/day × 5 + dThd 500 mg/kg t.d.s. × 8 days; ○—○ CB3717 100 mg/kg/day × 5 + dThd 500 mg/kg t.d.s. × 4 days.

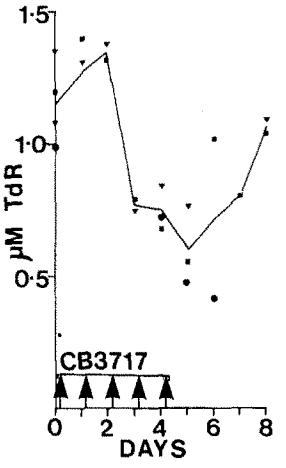

Fig. 3. Plasma thymidine levels during and following a 5 day course of CB3717 (100 mg/kg/day) in non-tumour-bearing mice: ●, experiment 1; ▼, experiment 2; ■ experiment 3. Each point represents the pooled plasma of four mice (24 hr after injection) and the line (——) is the mean of the three separate experiments.

RESULTS

Reversal of CB3717 *antitumour activity by* dThd *in vivo.* Figure 2 demonstrates the effectiveness of CB3717 (100 mg/kg daily × 5) against this L1210/CBRI tumour grown i.p. where 80% of the mice were 'cured' (>100 days). The mean day of death of the control mice was 11.3 days and was unaffected by injection of dThd alone (data not shown). The mice that received dThd (500 mg/kg t.d.s.) for the duration of the CB3717 treatment plus an additional 3 days showed no significant increase in lifespan. Therefore, dThd completely ablated the antitumour activity of CB3717 thus providing good evidence that TS is the cytotoxic locus for the drug *in vivo.* However, if dThd was given as a 'rescue' after completion of the 5-day course of CB3717 then 40% of the mice were 'cured' while the remaining mice had a 70% increase in lifespan. Thus, 60% of the mice still had viable L1210 tumour on day 7 which was capable of being 'rescued' from the effects of the drug.

The effects of CB3717 *on plasma* dThd *and* dUrd *in non-tumour bearing mice.* During CB3717 treatment (100 mg/kg/day × 5) the plasma dThd fell after the second injection reaching a minimum 24 hours after the fifth injection (day 5). Recovery to normal values occurred by day 8 (Fig. 3). Although each point represents the pooled plasma of four mice, wide variation was observed between experiments. We therefore measured plasma dThd in individual mice on day 5 in order to quantitate the level at the

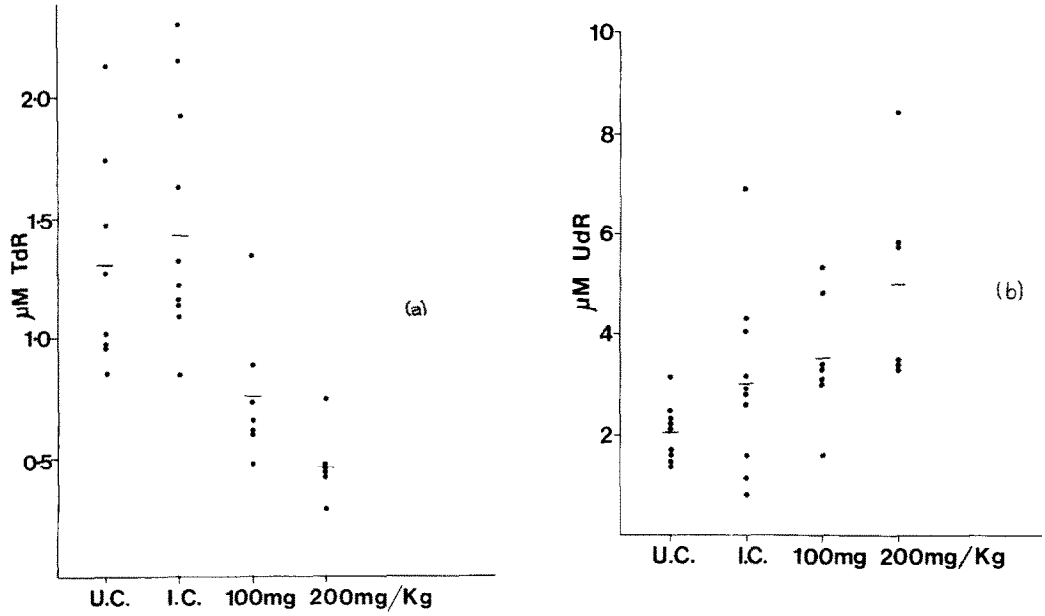

Fig. 4(a), (b). Plasma thymidine and deoxyuridine levels 24 hr (day 5) after completion of CB3717 treatment (100 mg/kg/day × 5) in non-tumour-bearing mice. (●) Each point represents the thymidine level in the plasma of one mouse; (——) mean. See text for details. U.C., uninjected controls; I.C., injected controls.

minimum (Fig. 4a). No significant differences were found in the plasma dThd levels between the solvent injected (1.43 ± 0.17 μM) and uninjected controls (1.3 ± 0.16 μM). Mice treated with 100 mg/kg/day × 5 of CB3717 had a mean dThd level of 0.76 ± 0.11 μM while those treated with 200 mg/kg/day × 5 ($\sim LD_{10}$) had a mean level of 0.47 ± 0.15 μM. Both these levels were significantly reduced when compared with controls ($P < 0.005$). The effect was dose-related as the 200 mg/kg/day × 5 treated group had a dThd level significantly lower than the 100 mg/kg group ($P = 0.04$).

Plasma dUrd was measured in the same samples (Fig. 4b). Again no significant differences were observed between solvent injected and uninjected controls ($P = 0.12$), the mean values being 3.04 ± 0.56 μM and 2.07 ± 0.19 μM, respectively. dUrd in the 100 mg/kg/day × 5 treated group was not significantly increased over either set of controls. The 200 mg/kg treated mice had a mean plasma dUrd level of 5.01 ± 0.82 μM which was significantly raised in comparison with pooled controls ($P = 0.026$), but did not quite achieve significance in comparison with the injected controls ($P = 0.07$).

The prevention of CB3717 *cytotoxicity to* L1210 *cells in culture by* dThd. In Fig. 5 (starting cell No. = 10^4/ml) the protection of a cytotoxic dose of CB3717 by 0.5, 1.0 and 2.0 μM dThd is shown to be complete after 24 hr exposure to these compounds. By 48 hr only those cultures given 2 μM dThd had cell counts equal to control values. After this time the cytotoxic effects of CB3717 were expressed even in these cultures. This is illustrated in Figs. 6a and 6b where the level of dThd and its catabolite thymine were followed in cultures with initial cell concentrations of 10^3 and 10^4 cells/ml. At the higher cell concentration (Fig. 6a) 0.5 μM dThd was rapidly depleted to

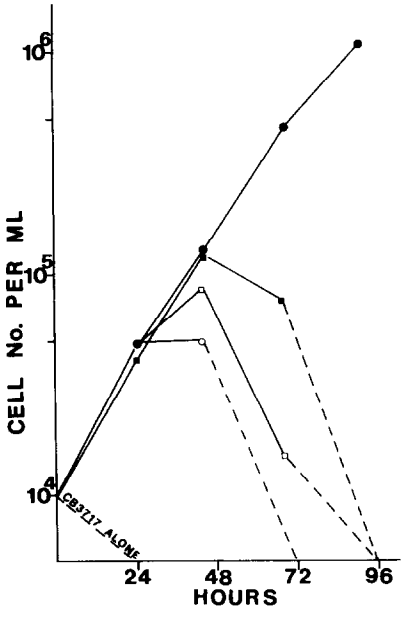

Fig. 5. Prevention of CB3717 cytotoxicity by thymidine in tissue culture (L1210 cells): ●, control; ○, 50 μM CB3717 + 0.5 μM dThd; □, 50 μM CB3717 + 1 μM dThd; ■, 50 μM CB3717 + 2 μM dThd.

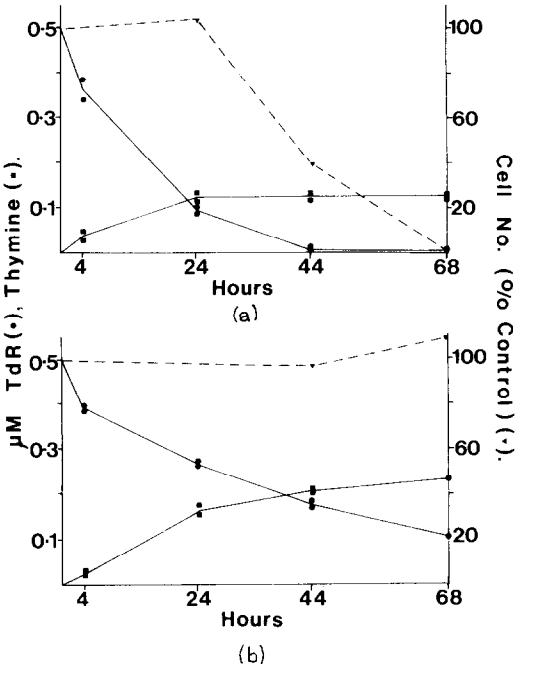

Fig. 6(a), (b). Utilization and catabolism of 0.5 μM thymidine in L1210 cultures exposed to a completely cytotoxic dose of CB3717 (50 μM). Initial cell concentration was 10^4 cells/ml (a) and 10^3 cells/ml (b): ●, dThd, μM; ■, thymine, μM; ▼, cell number as % control.

~0.1 μM by 24 hr due both to its incorporation into DNA (0.27 μmole dThd/l) and its catabolism to thymine. Although the cell counts were similar to control values at 24 hr, as the dThd levels continued to fall the onset of cytotoxicity was revealed.

The calculation of the amount of dThd necessary as a substrate for DNA synthesis to allow the replication of 10^4 L1210 cells is approximately 100 pmoles. Thus, if a population of cells starts at 10^4/ml and doubles twice the expected fall in the dThd concentration in the medium would be 0.3 μM. This is in good agreement with the observed results (Fig. 6a), where by 24 hr (two doublings) the fall in dThd concentration was ~0.4 μM but 0.1 μM of this was accounted for by catabolism to thymine.

Cultures with an initial cell concentration of 10^3/ml obviously incorporated less dThd into DNA (<0.08 μmoles/l in the first 24 hr) (Fig. 6b) so that dThd declined more slowly to only 0.27 μM by 24 hr and 0.17 μM by 44 hr. As the cells continued to grow at the control rate for at least a further 24 hr we concluded that the minimum level of dThd for protection must be <0.17 μM. The higher dThd levels left in the medium led to enhanced catabolism of dThd to thymine. In this system (10^3/ml) it was possible to add as little as 0.1 μM dThd to cultures without complete utilization of the dThd within a few hours (Fig. 7). This amount of dThd (0.1 μM) should theoretically be sufficient for at least three rounds of replication as this would reduce the dThd concentration to ~0.03 μM. However, the cells only doubled in number by 44 hr (29% of control) and

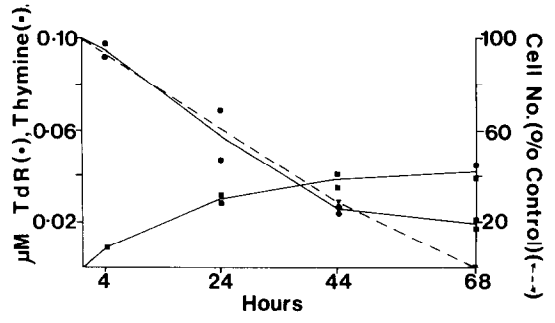

Fig. 7. Utilization and catabolism of 0.1 μM thymidine in L1210 cultures exposed to a completely cytotoxic dose of CB3717 (50 μM). Initial cell concentration was 10^3 cells/ml: ●, dThd, μM; ■, thymine, μM; ▼, cell number as % of control.

dThd was clearly not protecting the cells from the cytotoxic effects of CB3717 (an earlier cell count was not possible because of the low cell numbers involved). The dThd level at this time was 0.026 μM. After this, dThd levels fell slightly (due to catabolism to thymine) and complete cell death had occurred by 68 hr. However, because of the obvious time delay in the dThd level falling below a required minimum and cell death following, it is not possible to be precise about the minimum concentration required to maintain adequate dThd salvage except to say that it probably lies in the range 0.026–0.1 μM.

Figure 8 demonstrates how as much as 100 times excess of dUrd (100 μM) over dThd fails to diminish (or affect) the complete protection of L1210 cells from CB3717 by dThd. This is supported by the data which show that utilization of [^{14}C]-dThd is unaffected by this concentration of dUrd.

DISCUSSION

CB3717 is a quinazoline antifolate that is a tight-binding inhibitor of mammalian TS ($K_i \sim 4$ nM) [18, 19]. *In vitro* studies have demonstrated that this enzyme is the cytotoxic locus of the drug, as shown by dThd reversal studies and measurement of intracellular nucleotide pools [16, 19]. For this reason CB3717 is active against cells resistant to MTX [16, 27].

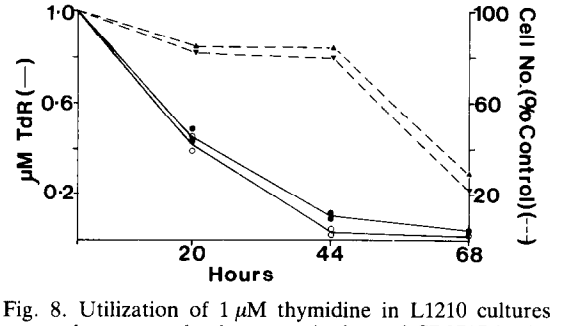

Fig. 8. Utilization of 1 μM thymidine in L1210 cultures exposed to a completely cytotoxic dose of CB3717 in the presence of 100 μM deoxyuridine. CB3717 concentration was 50 μM and that of dUrd 100 μM: ○, dThd, μM in the absence of dUrd; ●, dThd, μM in the presence of dUrd; cell count as % control, ▼ (minus dUrd), ▲ (plus dUrd).

The complete prevention of the antitumour activity of CB3717 in mice bearing the L1210/CBRI tumour, by co-administration of thymidine is consistent with the intracellular locus responsible for the antitumour effect of the drug being thymidylate synthetase. The dose-limiting toxicity of CB3717 in mice is renal due to precipitation of the drug in the tubules rather than to the antimetabolite effect of the agent [28]. Thus the use of dThd in the experiment described here was to demonstrate the antitumour locus of CB3717, and not as a normal tissue rescue agent.

These tumour cells which have the capacity to salvage injected dThd are apparently unable adequately to circumvent the block on TS using the substantial levels of dThd (~1.3 μM) normally circulating in mouse plasma (see Fig. 4a and ref. [23]). In clinical studies, using dThd alone, it has been demonstrated that adequate rescue from MTX toxicity is achieved with plasma dThd levels of less than 1 μM [12]. If dThd is administered together with a purine then rescue is achieved with doses of dThd that do not significantly increase plasma dThd (median value for patients prior to treatment = 0.17 μM; 5/12 patients had undetectable levels of <0.1 μM [15]). We investigated the possibility that the inhibition of TS during the course of CB3717 treatment in mice reduced the circulating level of dThd. At a curative dose of CB3717 (100 mg/kg) a significant reduction in plasma dThd (~50%) was observed.

Tissue culture experiments designed to determine the minimum level of dThd required to sustain cell growth in the presence of a TS inhibitor are inherently difficult to interpret. This is illustrated by our experiments describing the utilisation and catabolism of [^{14}C]-dThd in L1210 cells.

The cell counts shown in Fig. 5a would suggest that high levels of dThd (>2 μM) were required to overcome the cytotoxicity of CB3717 at 48 hr and even this level was insufficient at later times. However, by monitoring the metabolism of the dThd we found that cytotoxicity was only expressed when the concentration of dThd in the medium was depleted due to incorporation into DNA and catabolism to thymine. The latter has subsequently been found to be due to thymidine phosphorylase activity in the horse serum and consequently was independent of cell number [29]. By reducing the initial cell inoculum so that dThd was depleted less rapidly, we deduced that ~0.1 μM dThd was sufficient to maintain cell growth in the presence of CB3717, but a value less than this was insufficient. This value is at least an order of magnitude lower (three orders in one case) than other published estimates using MTX together with a purine as the inhibitor of *de novo* thymidylate synthesis in a number of different mammalian cells [22, 24, 30, 31]. Although it is possible that the metabolism of different tissues/tumours could result in these differences, the experimental design is a more likely explanation. Indeed, Leyva *et al.* [22] have demonstrated the depletion of [^{14}C]-dThd in the media of L1210 cells exposed to MTX and inosine.

Thus experiments using suspension cultures or colony assays designed to esimate minimal dThd

requirements for antimetabolite rescue may be subject to errors unless certain criteria are met. Firstly, the number of viable cells must be low enough and exposure time short enough so that dThd in the media is not significantly reduced either by incorporation into DNA or by catabolism. Secondly, the plating efficiency is also important as lysis of non-viable cells may release salvageable precursors capable of modulating cytotoxic protocols. Finally, if cells are exposed to the cytotoxic agent together with the salvage precursor(s) for the whole period of the experiment, the latter may fall below the critical concentration and cytotoxicity will then be expressed. This could result in a number of small colonies that may or may not be scored depending on the chosen colony size cut-off. Thus, as a general rule, the fewer the cells inoculated (or plated) the fewer the problems, and the easier it is to correlate dThd levels in the media with cytotoxicity.

If we try to correlate the decrease in plasma dThd during CB3717 treatment in mice with expression of cytotoxicity in the presence of dThd *in vitro* we must conclude that plasma dThd never reaches a critically low concentration. The 5-fold elevations in plasma dUrd levels observed at 4 hr [17, 23] do not result in a significant accumulation of this nucleoside throughout treatment, and are unlikely to affect the tumour cells' ability to salvage dThd by, for example, competing for uptake and phosphorylation [32, 33].

In summary, fluctuations in plasma dThd and dUrd observed in mice following treatment with CB3717 were the result of inhibition of TS, but our *in vitro* system failed to demonstrate how they could contribute towards the effectiveness of the drug. The high levels of deoxypyrimidines found in mice compared to man could explain the inactivity of CB3717 against some mouse tumours (e.g. the TLX/5 lymphoma [17]) but does not explain the excellent activity against the L1210/CBRI i.p. or i.v. tumour [16, 17]. The metabolism of this L1210 tumour may be sufficiently different *in vivo* to account for this activity although the cytotoxic locus is clearly TS since the antitumour activity was prevented by injection of dThd. There must be a minimal level of extracellular dThd, below which the salvage pathway is unable to provide the cell with TTP at a rate necessary to support normal growth. We conclude that this level is $<0.1\ \mu M$. This level is similar to that found in human plasma [15, 23, 30] suggesting that reduction in plasma dThd resulting from drug treatment in patients may prejudice the possibility of its salvage. Howell *et al.* have reached a similar conclusion in the case of MTX, where they suggest that it may increase its own (marrow) toxicity by decreasing plasma dThd and that variability in plasma dThd between patients may account for the unpredictable toxicity found clinically [25]. However, MTX and CB3717 have different target enzymes and the lack of any antipurine effect of CB3717 [16, 19] may help us to understand how normal tissue toxicity and antitumour activity relate to inhibition of *de novo* thymidylate synthesis in the presence of plasma dThd.

CB3717 is currently in phase I/II clinical study in which antitumour activity has been observed in the absence of myelosuppression or mucositis [34]. Early in the study we failed to show any decrease in plasma dThd following CB3717 (up to 330 mg/m^2) although one patient had an increase in plasma dUrd [23]. Fluctuations in deoxynucleosides may become more apparent at higher doses of drug.

Finally in view of such large differences in plasma deoxynucleosides observed between man and mice perhaps rodent tumours *in vivo* (or human tumour xenografts) are not the most suitable models for testing the antitumour activity and toxicity of inhibitors of *de novo* thymidylate synthesis and data from these should be interpreted with caution.

Acknowledgements—We would like to thank Dr S. E. Barrie for her invaluable criticism of *in vitro* techniques that led to part of this study. This work was supported by grants from The Cancer Research Campaign and The Medical Research Council.

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