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POTENTIATION OF QUINAZOLINE ANTIFOLATE (CB3717) TOXICITY BY DIPYRIDAMOLE IN HUMAN LUNG CARCINOMA, A549, CELLS

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Abstract—A potent quinazoline antifolate inhibitor of thymidylate synthase, CB3717, inhibits the growth of A549 human lung carcinoma cells: ID_{50} 2.74 ± 0.53 μM. The toxic effects of thymidylate synthase inhibition may be prevented by salvage of exogenous thymidine. The nucleoside transport inhibitor, dipyridamole, at the non-toxic concentration of 1 μM, inhibited [^{3}H]thymidine uptake/incorporation by more than 95% and significantly reduced the ID_{50} of CB3717 to 0.98 ± 0.28 μM. Elimination of salvagable thymidine by the use of dialysed serum also enhanced CB3717 toxicity. Since dipyridamole was equally effective in the presence or absence of dialysed serum and was more effective than dialysed serum alone, inhibition of nucleoside efflux may be an important aspect of its potentiation. Efflux of [5-^{3}H]deoxyuridine was inhibited by 89% and [^{3}H]thymidine efflux by 61% in the presence of 1 μM dipyridamole. Inhibition of thymidylate synthase increases the deoxyuridine nucleotide/thymidine nucleotide pool ratio. Dipyridamole could exacerbate the nucleotide pool imbalance caused by CB3717, thereby potentiating its toxicity.

Thymidylate synthase is a key enzyme of DNA synthesis, and inhibitors of this enzyme are used extensively as cancer chemotherapeutic agents. Clinically used drugs of this type, such as 5-fluorouracil, 5-fluorodeoxyuridine and methotrexate, also have other sites of action. A specific inhibitor of thymidylate synthase, CB3717 (*N*-(4-(*N*-((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynylamino)-benzoyl)-L-glutamic acid), acts as a competitor of N^5N^{10}-methylene tetrahydrofolate, the methyl donor for the formation of dTMP from dUMP; K_i 1–5 nM [1, 2].

Cells can be both protected and rescued from CB3717 toxicity by exogenous thymidine [2] and the toxicity of CB3717 *in vitro* is not expressed until the concentration of thymidine in the medium is less than 1×10^{-7} M [3]. The levels of thymidine in human plasma in normal and cancer patients are in the range 4×10^{-8} to 6×10^{-7} M [4, 5] so circulating thymidine may protect tumour cells *in vivo* from CB3717 toxicity. Furthermore, tumours often have increased salvage capacity compared to their normal counterparts [6] and in hepatomas, at least, pyrimidine salvage increases in both a transformation-linked and progression-linked way [7]. The prevention of salvage would therefore be likely to increase the cytotoxicity of these antimetabolites.

Paterson *et al.* [8] suggested that an inhibitor of nucleoside transport might be used to enhance the effect of antimetabolites which block *de novo* nucleotide synthesis. Nucleoside transport is carrier-mediated and is thought to be the rate-limiting step in the salvage of exogenous nucleosides [9]. Dipyridamole, a compound in frequent clinical use, is an inhibitor of nucleoside transport; K_i for deoxynucleoside transport is 1–2 μM [9]. Dipyridamole increases the cytoxocity of agents that can be circumvented by the salvage of nucleosides, e.g. methotrexate [10–12], acivicin [13, 14] and *N*-phosphonacetyl-L-aspartate [15]. Augmentation of antimetabolite toxicity by dipyridamole has also been demonstrated *in vivo* [15–17]. None of the drugs used in those studies are specifically thymidylate synthase inhibitors and many act on more than one pathway. The combination of CB3717 and dipyridamole permits the study of the role of nucleoside transport inhibition during thymidylate synthase inhibition alone.

MATERIALS AND METHODS

Materials. CB3717 solution (10 mg/ml) was obtained from ICI Pharmaceuticals (Macclesfield, Cheshire, U.K.). Thymidine, deoxyuridine and dipyridamole were purchased from Sigma (Fancy Road, Poole, Dorset, U.K.). Dipyridamole was dissolved in 1 N HCl, the pH adjusted to 3 with NaOH (limit of solubility), filter sterilized and stored at 0–4° in the dark. All routine chemicals were of AnalR grade and purchased from BDH (Poole, Dorset, U.K.), [methyl-^{3}H]thymidine (44 Ci/mmol; 1 Ci/ml) and [6-^{3}H]deoxyuridine (21 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.) and [5-^{3}H]deoxyuridine (23 Ci/mmol; 1 Ci/ml) was obtained from ICN Radiochemicals (Eschwege, F.R.G.). Cell culture media, trypsin and foetal bovine serum were obtained from either Gibco (Paisley, Scotland) or Northumbria Biologicals (Cramlington, Northumberland, U.K.). Dulbecco's modified phosphate buffered saline without magnesium and calcium (DulA), Titertek cell harvester and filters came from Flow Laboratories (Irvine, Scotland) and plastic culture dishes were supplied by Nunc (Gibco: 6-well plates) or Falcon (Becton-

Dickinson, Oxford: flat bottomed 96 well plates). PEI cellulose TLC plates (Merck) were purchased from BDH (Blaydon, Tyne & Wear, U.K.).

Cell culture and growth inhibition assays. A549 lung carcinoma cells [18, 19] were grown in RPMI + 10% foetal bovine serum (FBS: heat inactivated 56°, 30 min) with 500 IU/ml penicillin and 500 μg/ml streptomycin at 37° in an atmosphere of 5% CO_2 in air. For cell growth assays, cells were seeded into 6-well plates at a density of 2×10^4 cells/well in 2 ml RPMI + 10% FBS. Twenty-four to 48 hr later, depending on cell growth, the medium was replaced by fresh control or experimental medium containing CB3717 and/or dipyridamole. Cells were trypsinised and counted on a Coulter counter (Coulter Electronics, Luton, Beds., U.K.) 72 hr later. To eliminate salvagable thymidine (estimated at 0.5 μM in calf serum [20]), cells were grown in the presence of dialysed serum. Cells growing in medium supplemented with dialysed serum grew at a reduced rate (doubling time in dialysed serum approx. 30 hr, in undialysed serum 20–24 hr), and in order to have a similar number of cell doublings, the incubation period was extended to 96 hr.

[^{3}H]*Nucleoside incorporation.* Cells were seeded at 2–2.5 $\times 10^4$ cells/well in 0.125 ml RPMI + 10% FBS in 96-well trays. The following day the medium was removed, the cells washed with DulA and then given 80 μl serum-free medium supplemented with CB3717 and/or dipyridamole where indicated for various pre-incubation periods. Then 20 μl 500 nM [^{3}H]thymidine or [6-^{3}H]deoxyuridine (final concentration = 100 nM) was added for the stated incubation period, after which the medium was rapidly aspirated, the cells washed twice with ice-cold DulA and solubilised with 0.5 M NaOH and transferred to glass fibre filters using the Titertek apparatus. (Blanks were: no cells, no label or 2 mM cold thymidine or deoxyuridine.) The filters were dried and counted in 5 ml scintillant (Optiphase-safe: LKB, South Croydon, Surrey) in a LKB1217 liquid scintillation counter.

Nucleoside efflux. Subconfluent monolayers of A549 cells in 6-well plates were preincubated for 2 hr with 1 ml serum-free medium or 1 ml 30 μM CB3717 in serum-free medium. Then 200 μl were withdrawn from each well and 200 μl 500 nM [^{3}H]thymidine added. Two hours later, cells were washed three times in ice-cold DulA and 1 ml serum-free medium containing 0, 1 or 10 μM dipyridamole was added. Blanks were incubated in the presence of 1 mM cold thymidine. Aliquots of 25 μl were withdrawn immediately from each well and dispensed into scintillation vials and at regular intervals thereafter. At the end of the period of efflux measurement, the cells were again washed three times with ice-cold DulA and the cells removed from the dish by trypsinisation. Half of the cell suspension was counted to give an estimate of cell number, the other half was pelleted, the supernatant discarded and 0.5 ml ice-cold 0.4 M PCA added to the pellet and mixed thoroughly. After 30 min on ice, the acid insoluble material was pelleted at 3000 rpm for 20 min at 4° in a MSE Europa 24M (Crawley, Sussex). The supernatants (acid soluble, nucleotide pool) were dispensed into scintillation vials and the pellets (acid insoluble material DNA and RNA) were washed twice with 0.2 M PCA and solubilised in 0.5 ml 0.3 M KOH prior to transfer to scintillation vials. Scintillant was added and the samples were counted.

For the measurement of deoxyuridine efflux, cells were preincubated with 1 ml serum-free medium or that containing 30 μM CB3717 for 2 hr as above, followed by a 1 hr incubation with 100 nM [5-^{3}H]deoxyuridine. (Blanks were incubated in the presence of 2 mM cold deoxyuridine). After washing three times in ice-cold DulA, the cells were reincubated with 1 ml serum-free medium containing 0, 1 or 10 μM dipyridamole. Aliquots (100 μl) from each well were transferred to scintillation vials immediately and at regular intervals thereafter, and at the final time point all the remaining medium was transferred to scintillation vials. Cells were trypsinised and PCA-treated as above. All samples were taken to dryness to eliminate ^3H_2O (resulting from thymidylate synthase activity) and rehydrated with 0.5 ml water prior to counting. Analysis of efflux material by TLC [21] revealed no degradation of [^{3}H]thymidine to [^{3}H]thymine or [5-^{3}H]deoxyuridine to [5-^{3}H]uracil.

To have an estimate of the amount of labelled material inside the cell prior to the efflux incubation (and its distribution between acid soluble and insoluble pools), duplicate wells incubated for 2 hr in serum-free medium +30 μM CB3717 and 2 hr with [^{3}H]thymidine or 1 hr [5-^{3}H]deoxyuridine were washed and trypsinised as above and aliquots taken for PCA treatment and cell counting as above.

RESULTS

Thymidine uptake/incorporation

[^{3}H]thymidine incorporation by A549 cells was linear over a 6 hr incubation period, the rate of incorporation for two experiments being 6.5 and 7.5 pmol/10^6 cells/hr respectively. Incorporation of radioactivity from [6-^{3}H]deoxyuridine was also linear at about 4.1 pmol/10^6 cells/hr.

Effect of CB3717 on the incorporation of [^{3}H]thymidine and [6-^{3}H]deoxyuridine

Cells were preincubated for 0, 2 or 4 hr in serum-free medium or that containing 3 or 30 μM CB3717. Incorporation of label was measured after a further 2 hr incubation with either 100 nM [^{3}H]thymidine or 100 nM [6-^{3}H]deoxyuridine in the same medium. CB3717 causes a depletion of endogenous thymidylate and consequently dTTP pools [2]. Thus [^{3}H]thymidine incorporation increased with increasing concentrations of CB3717 and the length of time cells were exposed to it (Fig. 1A). Similarly, inhibition of thymidylate synthase and consequent expansion of the endogenous deoxyuridine nucleotide pools, resulting from exposure to CB3717, led to a decreased incorporation of radiolabel from [6-^{3}H]deoxyuridine in a dose and time-related way (Fig. 1B).

Effect of dipyridamole on [^{3}H]thymidine incorporation

Increasing concentrations of dipyridamole resulted in progressively decreased incorporation of

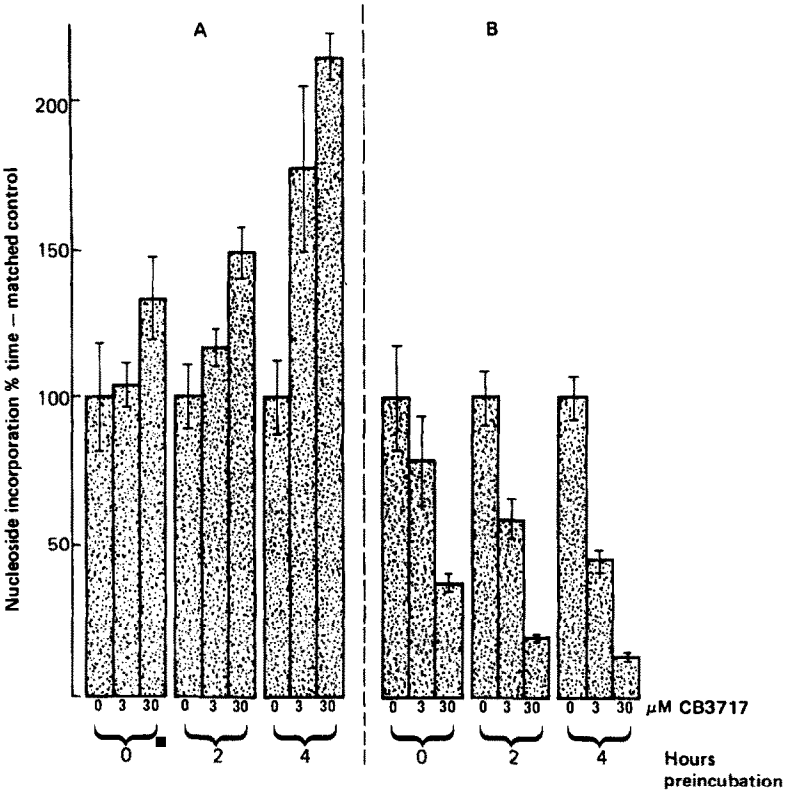

Fig. 1. Incorporation of label from [^{3}H]thymidine (A) and [6-^{3}H]deoxyuridine following CB3717 treatment (B). Incorporation measured after 2 hr incubation with 100 μM labelled nucleoside following preincubation with 0, 3 or 30 μM CB3717 for 0, 2 and 4 hr. Figures are mean, expressed as % time matched drug-free control, error bars represent standard deviation.

[^{3}H]thymidine both in control cells and those that had been preincubated with 30 μM CB3717 (Fig. 2). The degree of inhibition of incorporation of [^{3}H]thymidine caused by various concentrations of dipyridamole was the same in control and CB3717 treated cells. 1 μM dipyridamole was sufficient to inhibit the incorporation of [^{3}H]thymidine by more than 95%. At this concentration, dipyridamole does not affect cell growth.

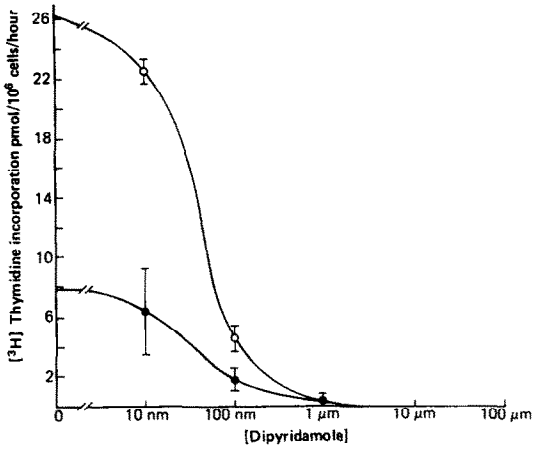

Fig. 2. [^{3}H]Thymidine incorporation during exposure to dipyridamole with or without 30 μM CB3717. Cells were preincubated for 2 hr in serum-free medium with varying concentrations of dipyridamole followed by 2 hr exposure to [^{3}H]thymidine in the same medium (●); or preincubated for 2 hr in serum-free medium + 30 μM CB3717 and varying concentrations of dipyridamole followed by 2 hr exposure to [^{3}H]thymidine in the same medium (○).

Augmentation of the toxicity of CB3717 by dipyridamole

A cell growth assay of cells exposed for 72 hr to varying concentrations of CB3717 and dipyridamole is shown in Fig. 3. From these results, it can be seen that increasing concentrations of dipyridamole gave increasing potentiation of CB3717 toxicity and that 1 μM dipyridamole, without being toxic itself, significantly enhanced the toxicity of CB3717.

Augmentation of CB3717 toxicity by dialysed serum and dipyridamole

If the observed potentiation of CB3717 toxicity by dipyridamole is due to the inhibition of uptake of salvagable thymidine, then a similar degree of potentiation should be seen when cells are exposed to CB3717 in the absence of salvagable thymidine, i.e. using dialysed serum. The results from pooled experiments are shown in Table 1.

Dialysed serum increases the toxicity of CB3717 to A549 cells but because of the variability of ID_{50} measurements in this group, the significance was

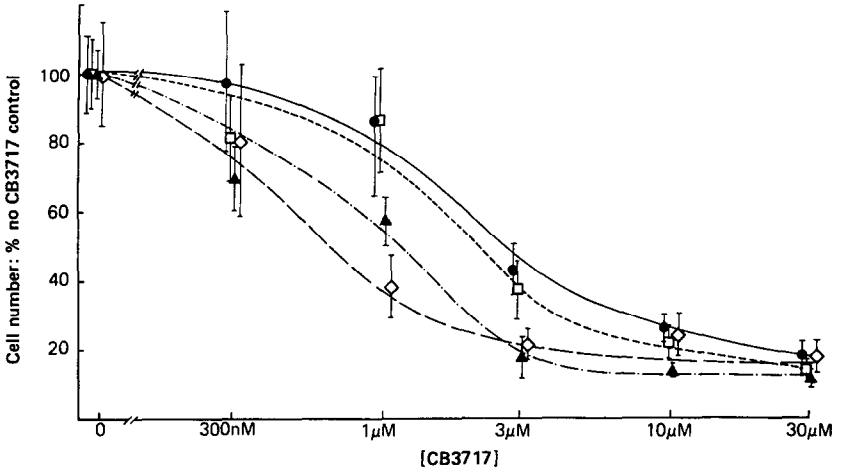

Fig. 3. Cell growth assay of cells exposed to CB3717 and dipyridamole. Cell number as a percent of controls in the absence of CB3717 following a 72 hr exposure to varying concentrations of CB3717 and no dipyridamole (●); 100 nM dipyridamole (□); 1 μM dipyridamole (▲); or 10 μM dipyridamole (◇).

given by $P = 0.07$. Dipyridamole (1 μM) was a more potent enhancer of CB3717 toxicity. Furthermore, dipyridamole in the presence of dialysed serum was significantly more effective in augmenting the toxicity of CB3717 than dialysed serum alone. Dipyridamole enhances the toxicity of CB3717 to approximately the same extent in dialysed as undialysed serum.

Effect of dipyridamole on nucleoside efflux

(a) *Thymidine.* The rate of efflux of [^{3}H]thymidine was inhibited by dipyridamole in a dose related manner in both control and CB3717-treated cells for the first 30–45 min (Fig. 4A, 4B, Table 2). After this time, the amount of [^{3}H]thymidine in the extracellular medium from cells incubated without dipyridamole did not increase, probably because of re-uptake of thymidine by the cells during the 2 hr efflux incubation. The rate of efflux was therefore calculated for the initial linear efflux period (a plateau in [^{3}H]thymidine content in the medium does not occur in dipyridamole-treated cells, due to the inhibition of thymidine re-uptake, Fig. 2). Thus the amount of labelled material in the extracellular medium at the end of the 2 hr efflux incubation does not reflect the inhibition of the rate of efflux by dipyridamole. The retention of labelled material in the acid-soluble pools was correspondingly enhanced by dipyridamole and was approximately doubled by 10 μM dipyridamole (Table 3). Prior to the 2 hr efflux period, about 20% of the labelled material was in the acid-soluble pool. Over half of the [^{3}H]thymidine in the pool was subsequently incorporated into acid insoluble material in cells that had been treated with CB3717. A greater proportion was incorporated into the acid insoluble portion in cells that had been treated with CB3717.

(b) *Deoxyuridine.* In cells that had not been pretreated with CB3717, most of the [5-^{3}H]deoxyuridine was converted to thymidylate and ^3H_2O and consequently counts were low in all fractions (Table 4). However, in cells which had been treated with 30 μM CB3717, the levels of [5-^{3}H]deoxyuridine in the acid-soluble pools were comparable to the levels of [^{3}H]thymidine prior to efflux (Table 4). In the absence of dipyridamole, most of the radioactivity entered the extracellular medium. There was a marked reduction in the rate of efflux by 1 and

Table 1. ID_{50} values* for CB3717 using undialysed or dialysed serum in the presence or absence of 1 μM dipyridamole

Culture medium	Exposure time (hr)	ID_{50} + [CB3717] μM
Control (undialysed serum)	72	2.74 ± 0.3 (8)†
Undialysed serum + 1 μM dipyridamole	72	0.98 ± 0.28 (7) $P < 0.001$‡
Dialysed serum	96	1.48 ± 0.96 (7) $P < 0.01$
Dialysed serum + 1 μM dipyridamole	96	1.03 ± 0.29 (7) $P < 0.001$

* ID_{50}; the concentration of CB3717 that causes a 50% reduction in cell growth.

† Figures are mean ± SD with the number of experiments given in parenthesis.

‡ Significant difference from control (paired Students *t*-test). In addition, there was a significant enhancement of toxicity of CB3717 in dialysed serum + 1 μM dipyridamole compared with dialysed serum alone; $P < 0.05$ (paired *t*-test). There was no significant difference between undialysed serum + 1 μM dipyridamole and dialysed serum + 1 μM dipyridamole.

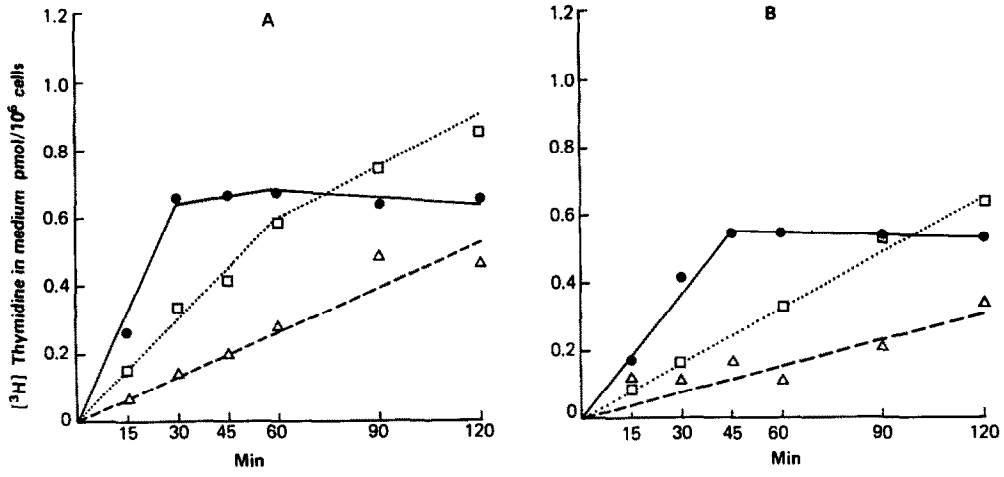

Fig. 4. [^{3}H]Thymidine efflux from cells in serum-free medium (A), or serum-free medium containing 30 μM CB3717 (B). Cells were preincubated with 100 nmoles [^{3}H]thymidine in medium with or without CB3717 as appropriate, washed and the efflux of label into the medium measured in the presence of no dipyridamole (●); 1 μM dipyridamole (□); or 10 μM dipyridamole (△).

10 μM dipyridamole (Fig. 5). As with thymidine, the extracellular medium from cells incubated without dipyridamole showed an initial rapid increase in the amount of radiolabel followed by a decrease, probably representing re-uptake. Again, the rate of efflux was calculated from the initial linear efflux period. Dipyridamole inhibited deoxyuridine efflux more than thymidine efflux (Table 2). This inhibition of efflux by dipyridamole was accompanied by a large increase in radioactive material remaining in the acid-soluble pool (Table 4).

DISCUSSION

The ID_{50} for CB3717 of 2.74 ± 0.53 μM obtained in the cell growth inhibition assays is within the range of plasma concentrations achieved in patients [22] and is similar to that reported for other cell lines [1, 23, 24]. At a concentration that is not toxic by itself (1 μM) and within the range of plasma levels of patients under normal dosing schedules [25], dipyridamole inhibited by more than 95% the uptake/incorporation of [^{3}H]thymidine in both control and CB3717 treated cells. At this concentration, dipyridamole significantly enhanced the toxicity of CB3717. Growth of cells in medium supplemented with dialysed serum also enhanced the toxicity of CB3717 but not as markedly as did dipyridamole. Other authors [26] have reported that dipyridamole (and more recently nitrobenzylthioinosine) [27], but not dialysed serum, enhanced the cytotoxicity of 5-fluorouracil. These authors suggest that dipyridamole may be inhibiting the efflux of fluorodeoxyuridine. Similarly, dipyridamole may enhance the toxicity of other drugs by the inhibition of the efflux of toxic metabolites, e.g. cytosine arabinoside [28] and deoxycofomycin [29] *in vitro* and *in vivo* [28].

CB3717 treatment of human lymphoblastoid cells not only reduces cellular dTTP by 85% but also increases cellular dUMP by 2300% [2]. In our study, treatment with 30 μM CB3717 caused a 4100% increase in the amount of label from [5-^{3}H]deoxyuridine in the acid soluble pool (Table 4). This is because thymidylate synthase is inhibited and [5-^{3}H]dUMP is not converted to dTMP and ^3H_2O.

Table 2. Inhibition of nucleoside efflux by dipyridamole

	% Reduction* in [^{3}H]thymidine efflux		% Reduction in [5-^{3}H] deoxyuridine efflux
Preincubation	No CB3717	30 μM CB3717	30 μM CB3717
Efflux incubation			
1 μM dipyridamole	60.7 ± 5.7 (3)	61 ± 4.7 (3)	89.1 (89.0–89.2)
10 μM dipyridamole	81.7 ± 4.0 (3)	80.3 ± 8.5 (3)	95.5 (95.3–95.7)

Figures are mean ± SD with numbers of experiments given in parenthesis or mean and range in parenthesis.

* Compared to controls incubated in the absence of dipyridamole. The rate of efflux was calculated from the linear portions of efflux measurement (Figs 4A, B and 5) as in control incubations after the initial linear efflux period there followed a plateau or decrease in the radioactivity in the medium due to re-uptake.

Table 3. Distribution of label after incubation with [^{3}H]thymidine

	[^{3}H]thymidine pmol/10^6 cells (% total)		
	Acid-insoluble pool	Acid-soluble pool	Extracellular medium
Control			
Prior to efflux	15.10 (81.2%)	3.500 (18.8%)	0
After 2 hr efflux period			
0 μM dipyridamole	17.54 (94.2%)	0.423 (2.3%)	0.655 (3.5%)
1 μM dipyridamole	16.55 (92.5%)	0.481 (2.7%)	0.876 (4.9%)*
10 μM dipyridamole	19.24 (93.5%)	0.869 (4.2%)	0.469 (2.3%)
30 μM CB3717			
Prior to efflux	24.20 (82.9%)	4.990 (17.1%)	0
After 2 hr efflux period			
0 μM dipyridamole	33.40 (97.4%)	0.386 (1.1%)	0.535 (1.6%)
1 μM dipyridamole	27.42 (95.4%)	0.559 (1.9%)	0.767 (2.7%)*
10 μM dipyridamole	29.20 (96.6%)	0.676 (2.2%)	0.346 (1.1%)

* The total efflux of [^{3}H]thymidine in the presence of 1 μM dipyridamole is greater than in the absence of dipyridamole due to the inhibition of re-uptake by the cells (Fig. 4A).
Figures are mean of two estimations.

Elevated dUMP may lead to increased levels of dUTP which in turn may lead to uracil misincorporation into DNA because DNA polymerase can utilise dUTP and dTTP with equal efficiency [30, 31]. Uracil is rapidly excised from the DNA leaving an AP site which is a target for endonuclease attack, thus causing DNA strand breakage. The role of uracil misincorporation into DNA as a mechanism for thymineless death is well documented in bacteria [32–34]. In mammalian cells, the misincorporation of uracil has been demonstrated in the presence of methotrexate [35] and metoprine, another antifolate, causing DNA strand breaks [36]. In cells treated with methotrexate without allowing the intracellular dTTP pool to diminish [37], elevated dUTP pools led to DNA fragmentation and cell death.

Following treatment with methotrexate, cellular deoxyuridine nucleotides increased and deoxyuridine was released to the medium [38]. Similarly, CB3717 treatment of tumour-bearing mice causes a doubling of plasma deoxyuridine concentration [3]. The inhibition of the efflux of deoxyuridine may help to maintain high deoxyuridine nucleotide pools, increase uracil misincorporation and thereby enhance the toxicity of CB3717. Dipyridamole (1 μM) reduced the efflux of deoxyuridine by nearly 90% and this is greater than the inhibition of thymidine efflux (around 60%). This may exacerbate further the nucleotide pool imbalance, particularly as thymidine uptake is greatly reduced (>95%) by 1 μM dipyridamole. Grem and Fisher [39] demonstrated inhibition of deoxyuridine efflux by dipyridamole with expansion of the dUMP pool. Thus dipyridamole may have a dual mechanism of the potentiation of CB3717 toxicity.

Uracil misincorporation into DNA may not be the

Table 4. Distribution of label after incubation with [5-^{3}H]deoxyuridine

	[5-^{3}H]deoxyuridine pmol/10^6 cells (% total)		
	Acid-insoluble pool	Acid-soluble pool	Extracellular medium
Control			
Prior to efflux	0.041 (34.3%)	0.079 (65.7%)	0
After 1 hr efflux period			
0 μM dipyridamole	0.032 (41.6%)	0.044 (58.4%)	0
1 μM dipyridamole	0.031 (39.2%)	0.048 (60.8%)	0
10 μM dipyridamole	0.032 (37.7%)	0.053 (62.3%)	0
30 μM CB3717			
Prior to efflux	0.020 (0.6%)	3.253 (99.4%)	0
After 1 hr efflux period			
0 μM dipyridamole	0.013 (0.2%)	0.040 (0.8%)	4.84 (98.9%)
1 μM dipyridamole	0.015 (0.4%)	1.501 (39.0%)	2.328 (60.6%)
10 μM dipyridamole	0.025 (0.6%)	3.328 (77.5%)	0.94 (21.9%)

Figures are means of duplicate estimations.

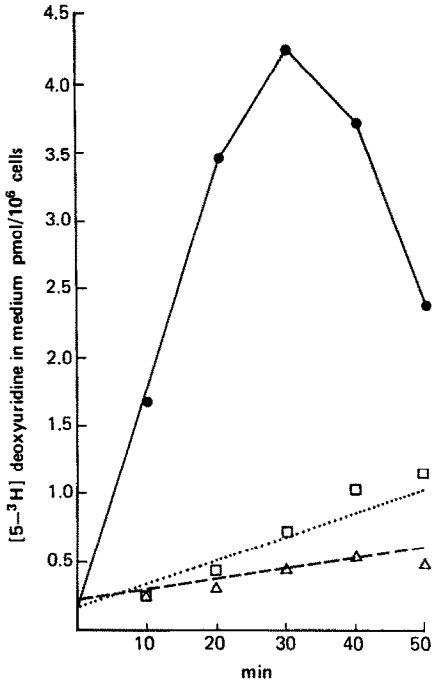

Fig. 5. [5-^{3}H]Deoxyuridine efflux from cells pretreated with 30 μM CB3717. Cells were preincubated with 100 nmol [5-^{3}H]deoxyuridine and 30 μM CB3717, washed and the efflux of labelled nucleoside into the medium measured in the presence of no dipyridamole (●); 1 μM dipyridamole (□); or 10 μM dipyridamole (△).

mechanism of thymineless death in all cells. Ayusawa *et al.* [40] failed to demonstrate significant incorporation of uracil in a thymidylate synthase-deficient mutant mouse cell during thymineless death. More recently, Fraser and Pearson [41] have also been unable to detect uracil incorporation into DNA of both HeLa and human lymphoblastoid cells exposed to methotrexate. Moreover, in lymphoblastoid cells, deoxyuridine did not potentiate CB3717 toxicity and inhibitors of uridylate and deoxyuridylate accumulation did not strongly antagonise CB3717 as would be expected if uracil misincorporation into DNA was the primary lethal event [2].

We have shown that dipyridamole, at concentrations that are non-toxic and within the levels achievable in patients, enhances the toxicity of a pure thymidylate synthase inhibitor, CB3717. Although dipyridamole is extensively bound to the serum component α_1-acid glycoprotein [42], there may be a reversible equilibrium between the fraction bound to α_1-acid glycoprotein and that interacting with the nucleoside transporter [43]. Under normal dipyridamole dosing schedules, the inhibition of ^{14}C adenosine uptake by platelets in whole blood was up to 92% [25]. Thus, the efficiency may not be substantially reduced but clinical studies and *in vivo* measurements will be necessary to evaluate the therapeutic value of dipyridamole. The enhancement of CB3717 toxicity by dipyridamole was greater than that achieved by elimination of salvageable thymidine (dialysed serum) and a significant enhancement of CB3717 toxicity was seen in the presence of dialysed serum +1 μM dipyridamole compared with dialysed serum alone. This suggests that dipyridamole enhances the cytotoxicity due to thymidylate synthase inhibition by a second mechanism, namely inhibition of deoxyuridine efflux which may further expand deoxyuridine nucleotide pools and increase uracil misincorporation into DNA. We are currently investigating the mechanism of thymineless death by the measurement of intracellular dUTP levels following treatment with CB3717 with and without dipyridamole in relation to DNA damage and cytotoxicity.

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