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FORMATION AND RETENTION AND BIOLOGICAL ACTIVITY OF N^{10}-PROPARGYL-5,8-DIDEAZAFOLIC ACID (CB3717) POLYGLUTAMATES IN L1210 CELLS *IN VITRO*

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Abstract—The formation, retention and biological activity of the polyglutamate metabolites of the thymidylate synthase (TS) inhibitor N^{10}-propargyl-5,8-dideazafolic acid (CB3717) has been investigated in L1210 murine leukaemia cells grown *in vitro*. CB3717 polyglutamates were measured by HPLC using high specific activity ^{3}H-CB3717. Following the exposure of cells to 50 μM CB3717 for 6, 12 and 24 hr total cellular radioactivity corresponded to 4.5 ± 1.5, 6.8 ± 3.6 and 5.9 ± 3.4 μM drug derived material, respectively. Of this material, >70%, 57 ± 3% and 51 ± 5% was in the form of unchanged CB3717 at 6, 12 and 24 hr respectively. The remaining radioactivity was associated with polyglutamate metabolites of CB3717, predominantly the tetra and pentaglutamate forms. Following the removal of extracellular drug after incubation for 24 hr and resuspension in drug free medium, unchanged CB3717 was lost rapidly from the cells such that after 6 hr it accounted for only 5% of total cellular radioactivity. In contrast, levels of CB3717 tetra and pentaglutamates declined solely due to dilution during cell division. Measurement of the whole cell TS activity by ^{3}H-deoxyuridine incorporation into DNA indicated that, despite the loss of unchanged CB3717 from the cell, enzyme activity remained suppressed (<10% of control) for at least 24 hr after resuspension in drug free medium. The TS inhibitory activity of the polyglutamated metabolites of CB3717 was investigated using enzyme purified from L1210 cells. As inhibitors, the metabolites were 26-, 87-, 119- and 114-fold more potent than CB3717 as the di-, tri-, tetra- and pentaglutamate forms, respectively. However, as inhibitors of dihydrofolate reductase prepared from rat liver, CB3717 polyglutamates were no more than 5-fold more potent than the parent compound. This study has shown that CB3717 can undergo polyglutamation in tumour cells and that the metabolites are preferentially retained giving rise to prolonged TS inhibition. By virtue of their potent TS inhibitory activity these metabolites are, therefore, most probably the intracellular effectors of CB3717 cytotoxicity.

It has been recognised for many years that intracellular folate cofactors exist almost exclusively as gamma-polyglutamate derivatives containing up to seven glutamic acid residues [1]. Like the physiological folate cofactors, classical antifolate drugs such as methotrexate (MTX‡) [2], aminopterin [3], 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin [4] are also metabolised to polyglutamate forms. This metabolic pathway has been characterised for MTX in a variety of cell types, both normal and neoplastic [5–17].

The ability of cells to form polyglutamates has consequences which can influence the cytotoxic action of antifolate drugs. Firstly, polyglutamates are preferentially retained following the removal of free extracellular drug whilst free MTX is readily lost [7, 10, 18–20]. These studies indicate that retention is a direct function of chain length and that the degree of cytotoxicity depends strongly on the ability of the antifolate to undergo conversion to polyglutamate metabolites [7, 10, 19, 20]. Furthermore, it has also been shown that resistance to MTX can be associated with reduced MTX polyglutamate formation [21].

The second important feature of antifolate polyglutamation concerns the increased affnity of the metabolites for certain folate dependent enzymes [5]. For example the addition of glutamate groups increases the binding of MTX to TS (EC 2.1.1.45) [22, 23], 5-aminoimidazole carboxamide ribotide transformylase (EC 2.1.2.3.) [24], glycinamide ribonucleotide transformylase (EC 2.1.2.2) [5] and methylene tetrahydrofolate reductase (EC 1.1.99.15) [5]. In contrast, the polyglutamate derivatives of MTX are no more potent than the parent drug as inhibitors of DHFR (EC 1.5.1.3.) [25] although the polyglutamates of MTX do dissociate less readily from the enzyme [18]. Other classical antifolate polyglutamates are also more potent than the monoglutamate form as inhibitors of TS [26, 27] and it has been suggested that as a result of polyglutamation the intracellular locus of action of the antifolates may be altered [5], although this remains a contentious issue. In addition there is limited evidence which suggests that polyglutamation may be a determinant of the selectivity of antifolates towards tumour as opposed to normal tissues [14, 28, 29], although again further studies are required to clarify this point.

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‡ Abbreviations used: MTX, methotrexate; CB3717, N^{10}-propargyl-5,8-dideazafolic acid; TS, thymidylate synthase; DHFR, dihydrofolate reductase.

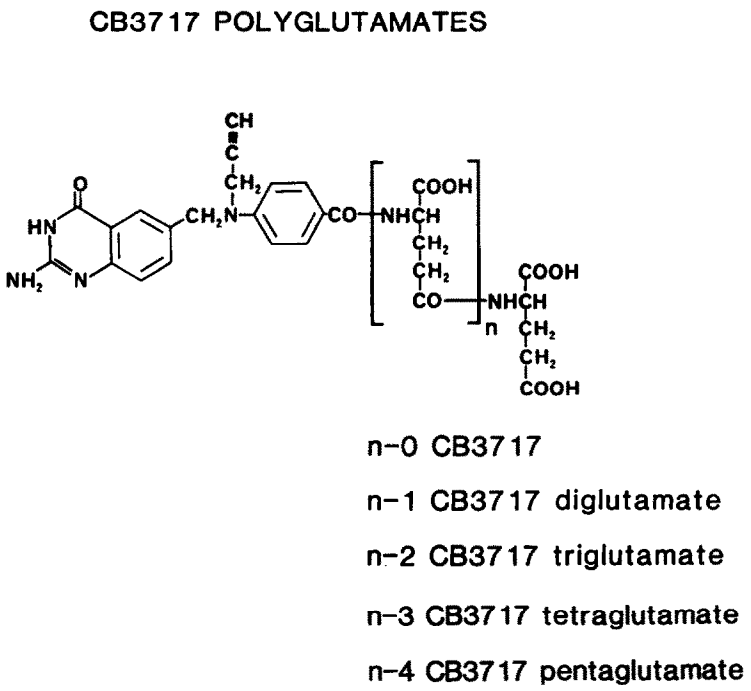

Fig. 1. The structure of CB3717 and its polyglutamate metabolites.

N^{10}-Propargyl-5,8-dideazafolic acid (CB3717, Fig. 1) is a novel antifolate drug which has recently demonstrated activity in clinical trials [30]. Unlike traditional antifolates, the cytotoxicity of CB3717 is not mediated by inhibition of DHFR. CB3717 is a tight-binding inhibitor of TS, which on the basis of currently available evidence, appears to be the sole locus for the cytotoxic action of the drug [31–33]. Recently it has been shown that CB3717 is a substrate for purified mammalian folylpolyglutamate synthetase, the enzyme responsible for the synthesis of both folate and antifolate polyglutamates [34]. Indeed, CB3717 was second only to aminopterin as a substrate amongst the group of compounds studied. Evidence from *in vivo* studies indicates that CB3717 can undergo polyglutamation in normal tissues in mice [35, 36] and also possibly in Ehrlich ascites tumour cells [37]. Studies using either isolated enzyme [38] or a whole cell TS assay [39] have demonstrated that CB3717 polyglutamates are markedly more potent as TS inhibitors than the parent compound. Thus, on the basis of the above evidence it seems highly likely that polyglutamation is an important determinant of the cytotoxicity and possibly selectivity of this new antifolate.

The experiments reported in the present study were performed in order to define in detail the formation and retention of the polyglutamate derivatives of CB3717 in tumour cells. In addition, the biochemical consequences of their formation were investigated by examining the extent of TS inhibition in L1210 cells following the removal of extracellular drug and also by characterising the inhibitory activity of these compounds against purified TS and DHFR. Certain aspects of this study have been previously reported in abstract or preliminary form [40–42].

MATERIALS AND METHODS

The synthesis of the CB3717 polyglutamates used in this study (Fig. 1) is described elsewhere [43], as is the method for the preparation and purification of high specific activity (10.5 Ci/mmole) ^{3}H-CB3717 (specifically labelled in the 2′ position) and the development of the HPLC assay used in this study [44].

CB3717 polyglutamate analysis. CB3717 and its polyglutamated derivatives containing two to five glutamate residues (Fig. 1) were separated on a 10 × 0.46 cm Polygosil 5 μm C18 column (Camlab, Cambridge, U.K.). Synthetic standards were dissolved in 0.15 M sodium bicarbonate at 20 μM and 5 μl aliquots analysed. The compounds were separated by linear gradient elution from 5:95 acetonitrile:0.1 M sodium acetate, pH 5, running at 2 ml/min to 16:84 acetonitrile:0.1 M sodium acetate, pH 5, over 15 min starting at the time of injection of the sample onto the column. An example of the separation of synthetic CB3717 polyglutamates is given in Fig. 2. Synthetic standards were detected by their absorbance at 254 and 280 nm and metabolites present in extracts from tumour cells by fraction collection (0.5 ml) followed by scintillation counting in 10 ml Cocktail T scintillant (BDH Ltd, Poole, U.K.). Quenching was constant throughout the HPLC run and was corrected for by the use of ^{3}H-hexadecane (Amersham International plc, Amersham, U.K.). HPLC separations were performed on Waters Associates chromatograph (Waters Associates, Harrow, U.K.) and scintillation counting on an SL30 Intertechnique liquid scintillation counter (Kontron Ltd., St Albans, Herts, U.K.).

CB3717 polyglutamate formation was investigated

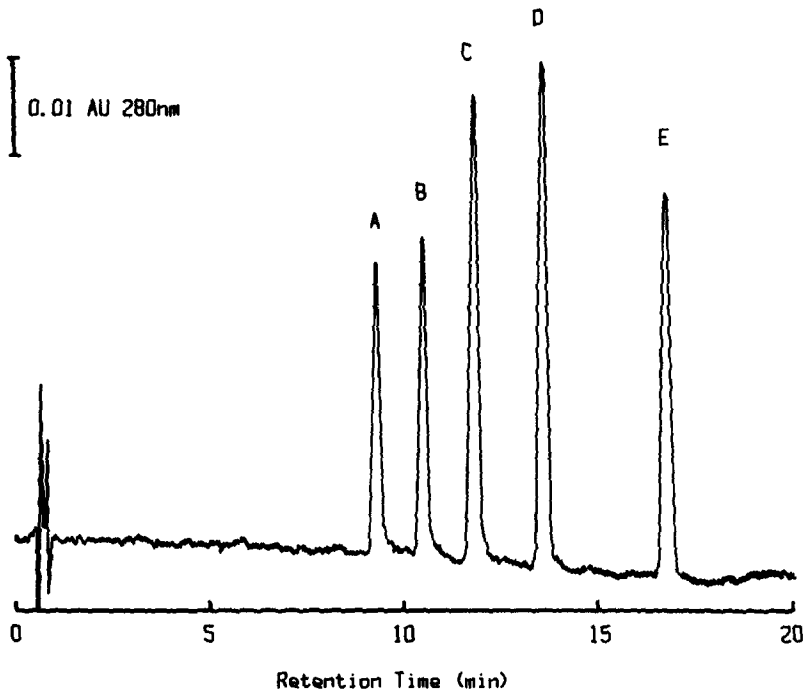

Fig. 2. HPLC separation of synthetic CB3717 polyglutamates. Peaks A, B, C and D are the penta, tetra, tri and di glutamates of CB3717 and peak E is CB3717 (0.1 μmole of each compound, HPLC conditions as described in the text).

in exponentially growing murine L1210 leukaemia cells. Cells were cultured in 9:1 RPMI1640 (containing 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid):donor horse serum (Flow Laboratories, Irvine, Scotland, U.K.) with L-glutamine added to give a final concentration of 2 mM. Cell cultures (200 ml) were exposed to 50 μM ^{3}H-CB3717, diluted to a final specific activity of 0.15 Ci/mmole, in the presence of 10 μM thymidine. This concentration of CB3717 is 10 times that required to inhibit cell growth by 50% [31] and so the thymidine was added to prevent cell death during the period of the experiment. The cell concentration at the beginning of the experiment was 2×10^5/ml and cell counts were performed to confirm exponential growth during the experiment.

After 6, 12 or 24 hr cells were harvested by centrifugation (1000 *g*) at 4° for 5 min and then washed twice with 50 ml phosphate buffered saline at 4°. The resultant cell pellet was resuspended in 1 ml 0.01 M Tris–HCl buffer pH 10 and the cells disrupted by sonication for 30 sec at 0°. The sonicate was boiled for 10 min and stored frozen at −20° for 18–24 hr. The boiled sonicate was thawed and refrozen, thawed again and the precipitated protein removed by centrifugation at 1000 *g* for 15 min at 4°. The supernatant was removed and the pellet resuspended in a further 0.5 ml 0.01 M Tris–HCl buffer pH 10, mixed thoroughly and then centrifuged. The pooled supernatants were lyophilized and the residue redissolved in 0.5 ml water. Aliquots (100 μl) of the extract were analysed by HPLC as described above. For each experiment an additional HPLC chromatogram was always performed, where the synthetic standards were mixed with a sample of cell extract in order that polyglutamate retention times could be calculated. These varied slightly from the retention times (shown in Fig. 2) of the standards dissolved in 0.15 M sodium bicarbonate.

In experiments designed to study the retention of CB3717 polyglutamates following the removal of extracellular drug, L1210 cells were incubated for 24 hr as described above with 50 μM ^{3}H-CB3717 in the presence of 10 μM thymidine. At the end of this period cells were harvested by centrifugation for 5 min at 37° at 1000 *g* and then washed in complete medium, centrifuged again and then resuspended in complete fresh medium, containing 10 μM thymidine, at 2×10^5 cells/ml. After a further 6 or 24 hr incubation the cells were harvested and analysed for CB3717 polyglutamates as described above.

To calculate the cellular levels of radioactivity following incubation of cells with ^{3}H-CB3717 an aliquot of the cell sonicate (25 μl), corresponding to a known number of cells, was digested in 0.5 ml NaOH at 40° overnight, neutralised and counted. The total cellular drug derived material concentration was then determined using a cellular water value of 0.6 ml/10^9 cells [46]. The concentrations of CB3717 and CB3717 polyglutamates were calculated from this value and the fraction of the applied radioactivity associated with each peak in the HPLC radiochromatogram. Recovery of radioactivity was >70% for all stages of the analysis [44].

Enzymology. Thymidylate synthase was prepared from a CB3717-resistant L1210 cell line that overproduces TS 45-fold due to amplification of the TS gene [49, 50]. The cells were centrifuged and resus-

pended (10^7/ml) in 0.05 M potassium phosphate buffer pH 7.4 containing 3 mM dithiothreitol. After sonication and preparation of a 50,000 *g* supernatant, a 30–70% $(NH_4)SO_4$ fraction was made, resuspended in half the original volume and dialysed overnight against the same buffer but containing 0.1 mM dUMP. The dialysate was made 20% with respect to sucrose and stored at −20°. The TS assay was performed as previously described [49] except that the stored enzyme was diluted approximately 1:50 (in 0.125 M potassium phosphate buffer pH 7.4 containing 3 mM dithiothreitol) prior to use and 0.2 ml added to the 0.5 ml reaction mixture. The $Ki_{apparents}$ were determined using the Goldstein equation applicable to tight-binding inhibitors (zone B inhibition). This equation was fitted to the data by a non-linear least squares regression [51]. Dihydrofolate reductase was partially purified from rat liver and assayed as previously described [52]. $Ki_{apparents}$ were determined as above.

6-[^{3}H] *Deoxyuridine incorporation studies.* L1210 cells were incubated with 50 μM CB3717 in the presence of 10 μM thymidine for 24 hr before resuspension and incubation in drug-free medium for 2, 3, 7 and 24 hr. At these times 6-[^{3}H] deoxyuridine (10–15 Ci/mmol, Amersham, Bucks, U.K.) was added and the incorporation into acid-precipitable material was measured after 20 min as previously described [49].

RESULTS

Figure 3 shows a representative chromatogram depicting the radiochemical analysis of an extract of L1210 cells exposed to 50 μM ^{3}H-CB3717 for 24 hr. In addition to the parent compound two radioactive metabolites were detected. These metabolites co-chromatographed with synthetic CB3717 tetra- and pentaglutamates (in parallel cell extract—see Materials and Methods) and were further identified by their ability to inhibit TS, as previously described [44]. The total cellular levels of radioactivity and CB3717 polyglutamates are shown in Fig. 4 for cells exposed to ^{3}H-CB3717 for 6, 12 and 24 hr. In none of the extracts was a peak with a retention volume equivalent to the diglutamate metabolite of CB3717 detected (<1% cellular radioactivity). Furthermore, the levels of the triglutamate metabolite did not rise to above 5% of the cellular radioactivity. However,

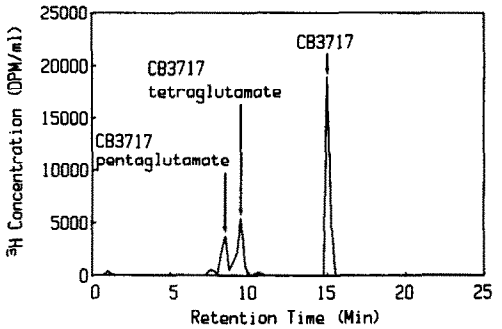

Fig. 3. Radiochemical HPLC analysis of an extract of L1210 cells exposed to 50 μM ^{3}H-CB3717 for 24 hr (extraction methods and quantitation as described in the text).

the tetra and pentaglutamates of CB3717 were readily detected, each accounting for approximately 20% of the cellular drug derived material at 12 and 24 hr. In contrast, at 6 hr the levels of the parent compound greatly exceeded those of the polyglutamates. Although synthetic CB3717 polyglutamates with greater than five glutamate residues were not available for these studies, radioactivity eluting in the region predicted for such compounds (Figs 2 and 3) accounted for no more than 5% of the total cellular radioactivity at any time point. Thus CB3717 underwent polyglutamation in L1210 tumour cells although, over the time period studied, the levels of these metabolites did not exceed those of the parent drug and accumulated only gradually.

The ability of L1210 cells to retain CB3717 and its polyglutamate metabolites was investigated by incubating cells with 50 μM ^{3}H-CB3717 for 24 hr and then culturing the cells for a further 24 hr in drug free medium. As shown in Fig. 5, 6 hr after resuspension in drug free medium levels of unmetabolized CB3717 had dropped substantially such that they represented only 5% of the total cellular radioactivity, as opposed to 50% following 24 hr exposure to the drug. After 24 hr incubation of the cells in drug free medium the levels of free CB3717 had dropped even further such that they represented only 2% of the cellular radioactivity. In contrast to the decline in the levels of CB3717, the levels of the tetra- and pentaglutamate metabolites declined only as a consequence of dilution as the cells replicated. At no point in the efflux experiments were the tri- or diglutamate metabolites of CB3717 detected (<1% cellular radioactivity). Thus CB3717 tetra- and penta-polyglutamates are preferentially retained within L1210 cells following the removal of free extracellular drug. Conversely, unchanged CB3717 associated with the cell is lost following resuspension in drug free medium, a process that is essentially complete after 6 hr.

We measured the incorporation of 6-[^{3}H] deoxyuridine into the acid-precipitable material of L1210 cells which had been incubated for 24 hr with CB3717 (50 μM) plus thymidine and then resuspended in drug-free medium. Deoxyuridine incorporation was suppressed (<10% of control) both immediately after the incubation and even after the 24-hr period in drug-free medium. Cells were resuspended either in drug-free medium containing thymidine to permit growth or medium without additional thymidine. In the latter case the cells did not grow and in neither case was there any recovery of deoxyuridine incorporation.

The synthetic CB3717 polyglutamates were tested against isolated TS and DHFR. Inhibition of L1210 TS was markedly increased (26- and 87-fold) with the addition of one and two additional glutamates. However, further polyglutamation led to little extra increase in TS inhibition. The tightness of the inhibition of the polyglutamates made determination of the mode of inhibition very difficult, and therefore Table 1 gives the $Ki_{apparents}$ only. However, if it is assumed that the inhibition remains competitive upon polyglutamation then the *Ki* values for tetra- and pentaglutamates are 0.032 and 0.034 nM respectively (CB3717 in the same experiment was 3.86 nM).

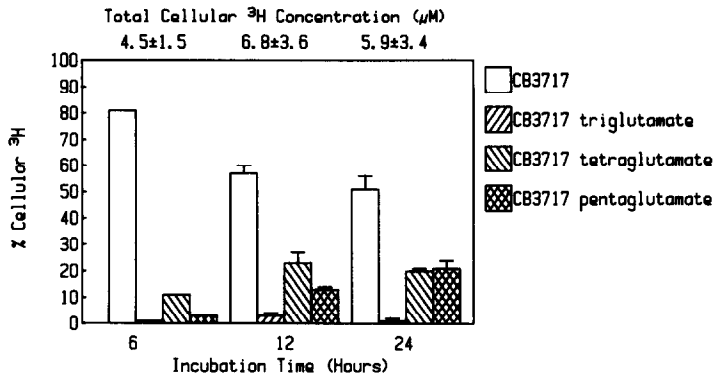

Fig. 4. Total drug derived material, CB3717 and CB3717 polyglutamate levels in L1210 cells following incubation with 50 μM ^{3}H-CB3717 for 6, 12 and 24 hr (values are the mean of duplicate experiments (6 hr) or the mean and standard deviation of 3–6 experiments (12 and 24 hr)).

The polyglutamates of CB3717 were also tested against rat liver DHFR and although a small increase in inhibition was apparent (3- to 5-fold, Table 1) when compared with CB3717 itself the length of the polyglutamate chain was not a determinant of inhibition. The mode of inhibition was determined for CB3717 and its pentaglutamate form and found to be competitive with respect to FH_2.

DISCUSSION

The work described in this paper represents the first detailed study of the formation of the polyglutamate metabolites of the TS inhibitor CB3717 in tumour cells. As shown herein, CB3717 does indeed undergo conversion to polyglutamate metabolites in tumour cells. After 24 hr exposure to 50 μM CB3717 these metabolites comprised approximately 50% of the total cellular radioactivity (Fig. 4). The formation of the polyglutamate metabolites was not rapid in that levels following incubation with the drug for 6 hr did not exceed 10% of the total cellular radioactivity (Fig. 4). Subsequent to 6 hr, the levels of metabolites rose to 50% of the drug derived material at 12 hr and did not increase further despite an additional 12 hr of incubation.

With respect to the kinetics of polyglutamation, CB3717 does appear to differ from other classical antifolates. Thus with MTX, aminopterin and the 10-deazaaminopterins, polyglutamates account for over 50% of the cellular drug derived material within 4 hr of exposure in most of the cell lines studied [3, 6, 9–11] including L1210 cells [10, 12]. However, the analysis of free cellular drug levels must be interpreted with caution since it is possible that a fraction of the unmetabolized drug is associated with the cell membrane and cannot strictly be considered to be intracellular drug. In the present study the washing procedure employed was sufficient to exclude contamination with residual medium; however, the possibility that drug was bound to the cell membrane cannot be excluded. Subcellular distribution studies would be required to answer this question.

For classical antifolates it is now well established that polyglutamation results in the formation of species which do not efflux from the cell as readily as the parent compound [3, 6, 7, 10, 18, 19]. Indeed, polyglutamate derivatives, particularly those with more than three glutamate residues, may not efflux at all [3, 6, 7, 10, 18, 19] although this property does depend upon the cell line studied. As a consequence

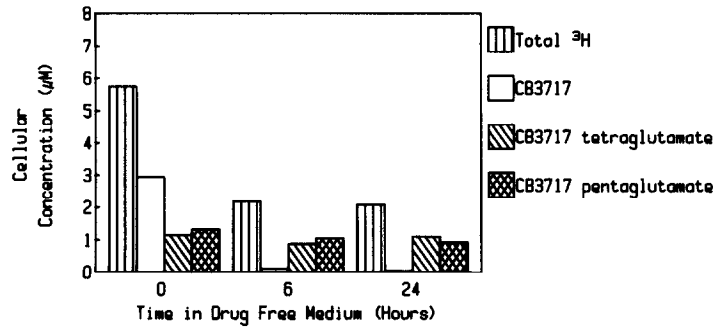

Fig. 5. Total drug derived material, CB3717 and CB3717 polyglutamates in L1210 cells following incubation with 50 μM ^{3}H-CB3717 for 24 hr and then resuspension in drug free medium for either 6 or 24 hr (values are the mean of duplicate experiments and are corrected for cell replication during the period of the experiment).

Table 1. Inhibition of thymidylate synthase and dihydrofolate reductase by CB3717 and its polyglutamates

	L1210* TS Ki_{app} ± SE (nM)	Fold increase in TS inhibition	Rat liver DHFR Ki† ± SE (nM)	Fold increase in DHFR inhibition
CB3717	26.22 ± 2.23‡	—	74.5 ± 10	—
CB3717 diglu	1.00 ± 0.06	26	21.5 ± 2.3	3.5
CB3717 triglu	0.30 ± 0.02	87	26.5 ± 3.5	2.8
CB3717 tetraglu	0.22 ± 0.01	119	14.6 ± 2.9	5.1
CB3717 pentaglu	0.23 ± 0.01§	114	21.2 ± 1.6	3.5

* [±5,10-CH_2FH_4] = 200 μM. Incubation = 1 hr at 37°. Model for calculation of Ki_{app} is the Goldstein equation for Zone B inhibition.

† Ki calculated using the competitive inhibition equation where $Ki = Ki_{app}/1 + (S/K_m)$.

‡ Ki = 3.86 nM (competitive inhibition).

§ Ki = 0.034 nM (assuming competitive inhibition).

of their prolonged retention, antifolate polyglutamates can give rise to continued inhibition of folate dependent enzyme even in the absence of extracellular drug [7–9, 20]. In the present study similar results were obtained for CB3717 in that free drug was readily lost from the cell on resuspension in drug free medium whilst the polyglutamate metabolites were retained (Fig. 5). It is difficult to comment on the efflux or retention of the di- and triglutamates of CB3717 as these metabolites were not detected in significant amounts at any of the time points studied.

With regard to the biochemical consequences of CB3717 polyglutamation, during the 24 hr period following extracellular drug removal, cellular TS activity remained suppressed. Taken together with the data from the efflux experiment (Fig. 5), these two results strongly suggest that the inhibition of TS during the period of culture in drug free medium is solely due to the polyglutamate metabolites. To what exent this is also the case during culture in the presence of drug was not a question specifically addressed in this study. However, the considerable potency of CB3717 polyglutamates as inhibitors of TS (Table 1) [38] suggests that once these metabolites are formed they would act as the species primarily responsible for TS inhibition.

Although the studies reported herein were not designed to study the transport of CB3717, it is noteworthy that despite 24 hr culture in the presence of 50 μM CB3717 (albeit in a growing cell population due to the presence of thymidine) total cellular drug levels did not exceed 10 μM in any of the experiments performed. Furthermore, maximum total drug levels had apparently been reached after incubation for 6 hr. The mechanism of CB3717 transport is not known although it is not thought to be via the active transport mechanisms described for both the natural folates and MTX. For example CB3717 is active against cell lines resistant to MTX due to reduced MTX transport [45] and is also active against a cell line unable to transport either folate or reduced folate into the cell (R. C. Jackson, personal communication).

The greatly increased affinities of the polyglutamates of CB3717 for TS together with the very much smaller increase in the affinity for DHFR is in agreement with the results of Cheng *et al.* [38]. There should therefore be an increase in the specificity of the inhibition of TS as a result of the formation of CB3717 polyglutamates. Experimental data suggests that CB3717 cytotoxicity appears to mediate slowly through TS and that CB3717 polyglutamation does not result in a change in its locus of action. For example the effects are reversed by thymidine alone without the necessity for the addition of a purine [33], CB3717 is active against DHFR overproducing cell lines [31, 45], and L1210 cells with acquired resistance to CB3717 have an amplified TS gene [49, 50]. In contrast, MTX polyglutamation results in a marked decrease in the specificity for DHFR inhibition, due to the activity of MTX polyglutamates against TS, glycinamide ribotide transformylase and 5-aminoimidazole carboximide ribotide transformylase [5, 22–24].

In summary the study reported herein has shown that CB3717 is metabolised to polyglutamate forms in L1210 cells *in vitro*. The substantial increase in the potency of these metabolites as inhibitors of isolated TS suggests that they contribute significantly to the *in vitro* cytotoxicity of CB3717. Furthermore the continued inhibition of TS in the absence of extracellular drug is accounted for by the retention of CB3717 polyglutamates intracellularly.

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