Biochemical Pharmacology, Vol. 51, pp. 1293–1301, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00035-4

Immunoreactive dUMP and TTP Pools as an Index of Thymidylate Synthase Inhibition; Effect of Tomudex (ZD1694) and a Nonpolyglutamated Quinazoline Antifolate (CB30900) in L1210 Mouse Leukaemia Cells

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ABSTRACT. The inhibition of thymidylate synthase (TS) as a drug development target has received much attention in recent years, and several compounds have reached clinical evaluation. During drug development, the effectiveness of target inhibition can be assessed by determination of the perturbations of deoxythymidine 5-triphosphate (TTP) and deoxyuridine 5′-monophosphate (dUMP) pools in drug-treated cells. Rapid, sensitive, and reproducible radioimmunoassays for TTP pools and immunoreactive dUMP pools have been developed to meet our requirement for the rapid assessment of TS inhibition by quinazoline antifolates. The assays can be carried out on 1–2 million cells, and require minimal sample preparation. The limit of detection for TTP is 1 pmole/10^6 cells and for immunoreactive dUMP ("dUMP"), 3.0 pmole/10^6 cells, both assays being performed on the same cell extract.

TTP and "dUMP" pools have been measured in mouse L1210 leukaemia cells treated with the quinazoline antifolates ZD1694 (N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamine]-2-thenoyl)-L-glutamic acid) and CB30900 (N-[N-[4-[N-[(3,4-dihydro-2,7-dimethyl-4-oxo-6-quinazolinyl)methyl]-N-prop-2-ynylamino]-2-fluorobenzoyl]-L-γ-glutamyl]-D-glutamic acid). Unlike ZD1694, CB30900 is a TS inhibitor that does not rely on polyglutamation for activity. In L1210 cells, both compounds caused a rapid inhibition of TTP pools in a dose- and time-related manner. Greater than 90% TS inhibition was achieved following a 4-hr exposure to each compound at equitoxic doses (up to 100 times the IC_{50} determined by a 48-hr growth inhibition assay). For both compounds, this was accompanied by a 5–10-fold increase in "dUMP" pools. For ZD1694, neither the TTP pool or "dUMP" levels were normalised when cells were resuspended in a drug-free medium for 4 hr and, at the higher doses studied, TS was still inhibited after a 16-hr period in the absence of drug. This is consistent with the formation and intracellular retention of potent polyglutamated forms of ZD1694. In contrast, TS activity as determined by repletion of the TTP pools and normalisation of "dUMP" levels were demonstrated for CB30900. However, at a high dose (50 μM, equivalent to 250 times the IC_{50}), retention of TS inhibition was observed following 4 hr, but not 16 hr in the absence of drug. The radioimmunoassays described will prove useful to further define the extent and time-course of TS inhibition by novel antifolate compounds, and will also provide valuable *in vitro* and *in vivo* pharmacodynamic information on established antimetabolites when used alone or in combination with other drugs and modulators. BIOCHEM PHARMACOL 51;10: 1293–1301, 1996.

KEY WORDS. cellular pharmacology; drug development; antimetabolite

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† *Abbreviations:* RIA, radioimmunoassay; TS, thymidylate synthase; FPGS, folylpolyglutamate synthase; RFC, reduced folate carrier; PCA, perchloric acid; DFM, drug-free medium; dUTP, dUDP, dUMP, 2′-deoxyuridine 5′-tri, di, and monophosphate; dCMP, dCTP, 2′-deoxycytidine-5′-mono and triphosphate; dATP, 2′-deoxyadenosine-5′-triphosphate; FdUMP, fluorodeoxyuridine-5′-triphosphate; ZD1694, N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl)-L-glutamic acid; ICI 198583, 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid; CB30900, N-[N-[4-[N-[(3,4-Dihydro-2,7-dimethyl-4-oxo-6-quinazolinyl)methyl]-[N-prop-2-ynylamino]-2-fluorobenzoyl]-L-γ-glutamyl]-D-glutamic acid; CB3717, (*N*-(4-*N*-((2-amino-4-hydroxy-6-quinazolinyl)methyl)prop-2-ynylamino)benzoyl)-L-glutamic acid.

Received 25 July 1995; accepted 18 December 1995.

TS† catalyses the reductive methylation of dUMP to form TMP, which is the rate-limiting step for the *de novo* synthesis of TTP. The methyl group involved in this reaction is donated by 5,10 methylene tetrahydrofolate (10-CH_2FH_4). Inhibition of TS leads to depletion of the TTP pool and accumulation of dUMP behind the enzyme block [1, 2, 3]. This accumulation of dUMP is due to loss of negative feedback control by TTP on three enzymes, deoxycytidylate deaminase, ribonucleotide reductase, and thymidine kinase [4] and leads to the formation of dUTP. Misincorporation of uracil into DNA and the resultant cycle of excision, repair, and reinsertion of uracil results in

DNA strand breaks and is a possible mechanism of cell death in TTP-depleted cells [5, 6, 7].

Recently, there has been considerable interest in the development of specific antifolate TS inhibitors as antitumour agents [8, 9] and several compounds are currently undergoing preclinical and clinical evaluation. ZD1694 (Tomudex^R) is a more potent and water-soluble analogue of the first specific antifolate TS inhibitor (CB3717) to reach clinical trials [10], is actively transported into cells *via* the methotrexate/reduced folate carrier (RFC), and is an effective substrate for the enzyme folylpolyglutamate synthase (FPGS) [11]. This biochemical profile serves to prolong TS inhibition because the polyglutamated species are retained within cells for long periods of time and are substantially more potent inhibitors of TS than the parent compound, itself. A Phase III trial with Tomudex has recently been completed [12] and it is now available in the U.K. for the treatment of advanced colorectal cancer.

In an ongoing program of development of quinazoline TS inhibitors, compounds that are not subject to polyglutamation but are, nevertheless, potent inhibitors of TS, have been identified. These compounds are dipeptide analogues of 2-desamino 2-methyl N^{10} propargyl-5,8-dideazafolic acid (ICI198583) [13] and, although transported into cells in the same way as ZD1694, their potent TS-inhibitory properties have been shown to be independent of polyglutamation [14, 15]. Such compounds may be active, therefore, in tumours in which FPGS is defective or expressed at low levels. Also, lack of drug retention through polyglutamation suggests that TS activity would recover as soon as drug administration had ceased, thereby permitting greater control over the duration of TS inhibition. The 7-CH_3, 2′F-L-glu-γ-D-glu analogue of ICI 198583 (CB30900) is stable *in vivo* to peptide bond hydrolysis [16], uses the RFC and, although not a substrate for FPGS, is a potent inhibitor of TS (K_i = 0.2 nM for L1210 enzyme). This compound is 5 times less potent than ZD1694 as an inhibitor of L1210 cell growth [17, 18].

In addition to pharmacokinetic considerations, many factors may influence the effectiveness of TS inhibitors as anticancer agents, including the level of expression of the enzyme itself [19, 20], the ability of cells to form TTP through the "salvage" pathway [21], relative rates of drug influx and efflux, and the extent to which they are polyglutamated. As part of an ongoing interest in the development of effective inhibitors of TS, an assay that was suitable for relatively large numbers of samples and could provide an index of enzyme inhibition was required. TS inhibition can be measured in intact cells by measuring the rate of ^3H_2O from 5-^{3}H dUrd [17, 22, 23]. The extent and duration of TS inhibition can also be determined by measuring TTP pool depletion and the increase in deoxyuridylate nucleotides.

Deoxynucleotide pools have proven difficult to measure because of their low intracellular concentrations (pmole/10^6 cells) and to the simultaneous presence of 100–1000-fold levels of the corresponding ribonucleotides. Excellent resolution of these compounds using anion exchange [24] or reversed-phase columns [25] is possible, but UV detection generally offers only limited sensitivity (>10 pmole in cell extracts). Improved sample cleanup procedures can increase sensitivity to 0.5 pmole/10^6 cells [26], thereby reducing the number of cells required for each assay. The DNA polymerase assay [27, 28], although highly sensitive, cannot distinguish between TTP and dUTP. This is important in TS-inhibited cells because dUTP is formed to different extents from dUMP accumulated behind the enzyme block [5, 6, 7].

RIA provides an attractive alternative for the sensitive measurement of intracellular deoxynucleotide pools. Specific sensitive RIA procedures for the measurement of dCTP and dUTP pools in cell extracts have previously been described [29, 30]. The production and characterisation of an antibody to TTP have been described briefly [31]. The antiserum was produced in a sheep to a conjugate of TTP and egg albumin. Cross-reactivity of the antibodies with TDP was 89%, but there was minimal recognition of thymidine, TMP, dUMP, dCTP, dATP, and UTP. Cross-reaction with dUTP (7.8%) was effectively eliminated by affinity purification of the antiserum on an activated CH-Sepharose 4B column to which dUTP-thryoglobulin had been conjugated. In the original method, TTP was separated from TMP and TDP by column chromatography, as described for the RIA of dUTP [30]. This paper describes a RIA for the direct measurement of TTP in cell extracts and an adaptation of the dUTP RIA that exploits the cross-reactivity of the antiserum with dUMP. By using dUMP standards and radiolabel in the assay, sufficient sensitivity to obtain a measure of dUMP pools is possible. The effects of ZD1694 and CB30900 on intracellular TTP and immunoreactive "dUMP" pools in mouse leukaemia cells have been compared.

MATERIALS AND METHODS

ZD1694 was supplied by Zeneca Pharmaceuticals Ltd (Macclesfield, Cheshire, U.K.) and prepared in 0.15 M $NaHCO_3$. CB30900 was synthesised as previously described [14]. TTP, dUTP, and QAE Sephadex were purchased from Pharmacia (St. Albans, Herts., U.K.). KH_2PO_4 (Analar grade) was from British Drug Houses (Poole, Dorset, U.K.), and all other chemicals were from Sigma (St. Louis, MO, U.S.A.). Deoxythymidine 5′-tiphosphate, [methyl-^{3}H] tetra sodium salt (NET-221X Specific Activity 78 Ci/mmole), and Deoxy[5-^{3}H]uridine 5′-monophosphate, ammonium salt (TRK287 15.4 Ci/mmole) were purchased from New England Nuclear (NEN) Research Products and Amersham International plc (Little Chalfont, Bucks., U.K.), respectively. Solutions were made in Milli Q deionised water. The TTP (HP/S/1524) and dUTP (R9) antisera have been described previously [30, 31] and were stored at 4°C in the presence of 0.1% sodium azide. Further supplies of both antisera are available.

Cell Culture and Extraction

The L1210 mouse leukaemia cell line was cultured as described before by suspension culture in RPMI 1640 medium

supplemented with horse serum [11, 17]. 10 mL cells (duplicate flasks) at an approximate density of 2×10^5 cells per mL were exposed to either ZD1694 or CB30900 at final concentrations equivalent to 10, 50, and 100 times the IC_{50} for growth inhibition previously determined over 48 hr. After 1 and 4 hr, the cells were washed twice in medium and resuspended in drug-free medium (DFM) for a further 4 or 16 hr. The total number of cells per estimation was 2–3 $\times 10^6$. Control (untreated) cells were included in each experiment. All cell counts were performed using a model ZM Coulter Counter. Cells were harvested by refrigerated centrifugation at 1000 rpm (300 g) for 10 min, and the medium removed. Ice cold PCA (0.5 mL) was added to each pellet and the tubes vigorously vortex mixed, and left in iced water for 30 min. After centrifugation at 4°C for 20 min at 3000 rpm, the supernatant was transferred to chilled Eppendorf tubes and neutralised with ½ volume of cold 0.73 M KOH in 0.16 M $KHCO_3$. The tubes were allowed to stand for 30–60 min in iced water, centrifuged at 10,000 rpm at 4°C for 10 min, and the supernatant stored at −70°C until assay.

Sodium Periodate Treatment of Cell Extracts

The cell extracts were thawed on ice and then treated with sodium periodate as previously described [32]. At this stage, the samples could be refrozen for later assay or kept at 4°C for immediate RIA.

Chromatography of Extracts

Triphosphates were separated from mono- and di-phosphates using QAE Sephadex chromatography, as previously described [30]. 400 μL of extract was applied to a 10 mL column of QAE Sephadex A25 equilibriated in 0.3 M KH_2PO_4 and 40 mL of the same solution used to elute mono- and di-phosphates. In some experiments, this eluate was collected in 10 4-mL fractions. TTP and dUTP were eluted with 0.7 M KH_2PO_4 and collected in 25 0.8 mL fractions. 400 μL of each fraction was assayed by RIA for TTP and, in selected samples, for dUTP.

Radioimmunoassay

A similar procedure was used for both RIAs, but the assay diluent was 0.7 M KH_2PO_4 pH 4.4 for TTP and 0.3 M KH_2PO_4 pH 4.5 for dUMP. All dilutions and dispensing of standards and samples were made with a Dilutrend^R diluter (Boehringer, Lewes, W. Sussex, U.K.). Throughout the assay procedure, all solutions were kept in iced water. Standard solutions of TTP and dUMP in water (200 μM and 284 nM) were stored aliquoted at −20°C and diluted for each assay to cover the range of the standard curve. For the direct RIA, extracts were assayed at 3 appropriate dilutions to fall on the standard curve. The affinity purified antibodies were diluted in water just prior to use, so that approximately 30% of total radioactivity was bound (B_o) to antibody. This dilution was previously determined from an antiserum dilution curve and was 1/25 for both TTP and dUMP. The radiolabeled TTP and dUMP were diluted with water so that approximately 0.05 pmole and 0.2 pmole per tube (0.1 mL), respectively was added to each assay tube. Diluted standards and samples (0.1 mL) were added in duplicate to numbered LP3 tubes (Luckham) with 0.3 mL assay diluent, 0.1 mL of diluted antiserum, and 0.1 mL diluted label, the tubes vortexed and left to stand in iced water for 2 hr. Each assay included Total Counts and Nonspecific Binding tubes containing only the radiolabel and buffer, and zero binding tubes (B_o) that contained only radiolabel and antiserum. The antibody-bound ligand was separated from unbound ligand by the addition of ice-cold dextran-coated charcoal [2.5% (w/v) activated charcoal (Sigma) coated with 0.25% (w/v) Dextran T-70 (Pharmacia)] to all but the Total Counts tubes for 10 min. Following centrifugation at 2500 rpm (1000 g) for 10 min at 4°C, 500 μL aliquots of supernatant were taken from each assay for scintillation counting in 2.5 mL Hionic-Fluor scintillant (Packard). The dNTP concentrations in the cell extracts were calculated from the standard curve using a data reduction program that utilised a 4-parameter logistic plot (RiaSmart Packard), and the amount of TTP or dUMP per 10^6 cells calculated.

RESULTS

Radioimmunoassay of TTP

The standard curve for TTP ranged from 50–2000 fmoles (in 100 μL) and 50% inhibition of binding occurred with the addition of approximately 350 fmoles TTP. Figure 1 shows the mean standard curve with the associated precision profile, obtained over 12 successive assays. The sensitivity of the curve, as determined by a 2SD fall in binding from the average B_o value was 51 fmoles. A solution of TTP in water equivalent to 3400 fmoles/mL was aliquoted and

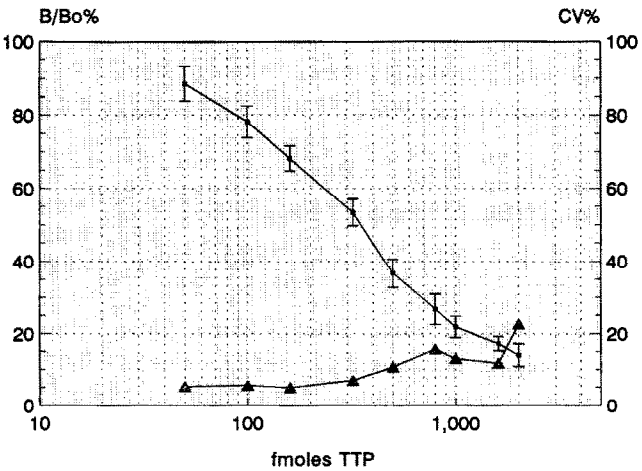

FIG. 1. The standard curve for TTP showing the mean antibody binding ± SD expressed as a percent of that obtained in the absence of TTP (B_o) (■) at each standard point of 12 successive assays. The precision profile (CV% vs dose) is also shown (△).

stored at −20°C and used as the assay control sample. The mean value measured over 17 assays was 3590 fmoles/mL (CV% = 14.5). Within an assay, the mean variation for this sample was 8.0%.

In the assay as originally described, chromatography was used to separate TTP from TMP and TDP. Figure 2 shows the separation of TTP, TDP, and TMP standards by chromatography on a QAE Sephadex column and the immunoreactivity measured in fractions when extracts from both control and TS-inhibited L1210 cells were chromatographed. The elution of TTP from cell extracts occurred in the same fractions as authentic TTP (Fig. 2). No immunoreactivity was found in fractions corresponding to the elution of TMP or TDP with 0.3 M KH_2PO_4. The recovery of TTP from the column was 84.6 ± 12.3%.

To meet our requirements for a rapid assay that could be conveniently used to measure TS inhibition in large numbers of samples, TTP immunoreactivity was measured directly in the sodium periodate-treated cell extracts. Results obtained in this way compared to results obtained following chromatography; the mean value for TTP pools in L1210 cells in logarithmic growth was 22.0 ± 3.1 pmole/10^6 (n = 5) cells when chromatography was included, compared to 22.0 ± 3.6 (n = 11) for direct measurement of extracts by RIA. TTP levels, measured by direct RIA in TS inhibited L1210 cells, also compared to those obtained with the chromatography step prior to RIA (data not shown).

Aliquots of L1210 cells (2.8 × 10^6) were harvested and the pellets spiked with 25 pmole TTP, PCA extracted, and sodium periodate-treated as described. The recovery of the added TTP over that measured in unspiked cell extracts was 72% (67%; 78%). There was no significant difference between results of duplicate samples measured in the same RIA (14.5 ± 9.6 pmoles/10^6 cells and 15.6 ± 10.9 pmoles/10^6 cells) or between results of the same samples assayed on different occasions (15.8 ± 7.9 and 17.6 ± 9.5 pmoles/10^6 cells). The limit of detection for measurement of TTP in cell extracts depends on the number of cells extracted and the dilution of extract used in the RIA. For example, for 2.5 × 10^6 cells extracted as described and diluted 5 times prior to assay, the limit of detection is 1025 fmoles/10^6 cells.

dUMP Radioimmunoassay

The previously reported RIA for dUTP [30] has been modified by substituting dUMP radiolabel and standards for those of dUTP. Because of the low cross-reactivity of the dUTP antiserum with dUMP (3%) this was at the expense of titre, the antiserum needing to be diluted 4 times less than when ^{3}H-dUTP was used as the radiolabel. The elution of dUMP, dUDP, and dUTP standards from the QAE Sephadex column has been shown previously [30]. Figure 3 shows the immunoreactivity measured in fractions of the 0.3 M KH_2PO_4 column eluant following the chromatography of untreated L1210 cells and cells treated for 4 hr with 2 doses of CB30900. The immunoreactivity eluting in the same position as authentic dUMP increased with dose of TS inhibitor. There was no significant difference in results for controls and TS-inhibited samples obtained either with or without chromatography (P = 0.25). No immunoreactivity was detected in fractions corresponding to standard dUDP. Also, in these samples, no dUTP was found in fractions eluted with 0.7 M KH_2PO_4. However, in samples of cells subjected to more prolonged TS inhibition, evidence of immunoreactivity corresponding to dUDP and dUTP was observed. The results reported by direct RIA of cell extracts represent immunoreactive deoxyuridine nucleotide concentrations ("dUMP") and will be subject to interference by the presence of any dUDP and dUTP formed as a result of TS inhibition. Nevertheless, this is a useful measure of total deoxyuridine nucleotide levels accumulating in the cell due to TS enzyme inhibition. As expected, com-

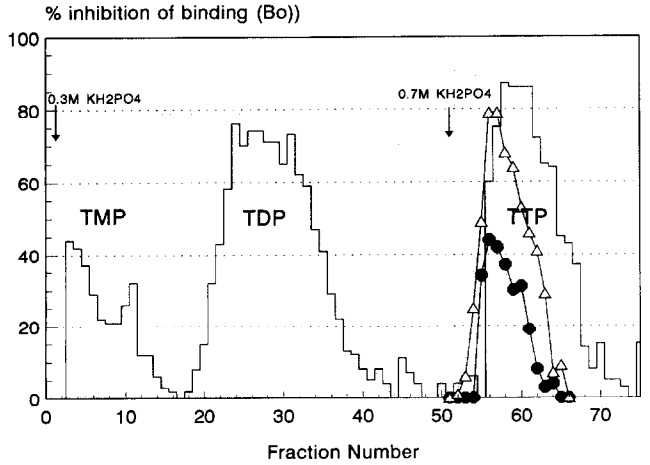

FIG. 2. The elution of TMP (25 µmoles), TDP (28 pmoles), and TTP (25 pmoles) standards from a QAE Sephadex column detected in fractions of 0.3 M or 0.7 M KH_2PO_4 eluate using the RIA. The immunoreactivity detected in fractions following the chromatography of an untreated cell extract (●) and a TS-inhibited cell extract (△) is also shown.

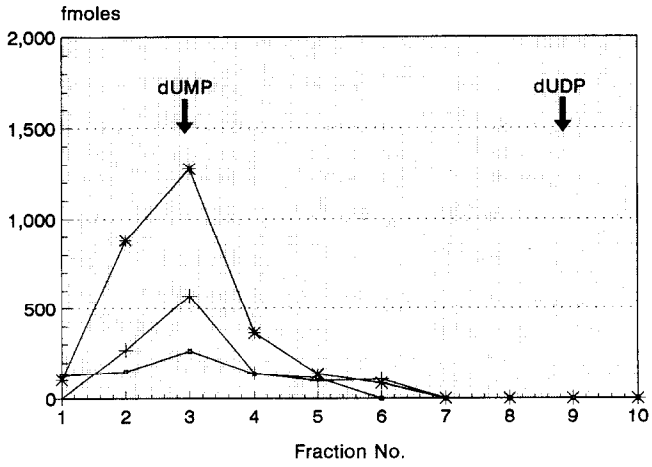

FIG. 3. Immunoreactivity detected in 0.3 M KH_2PO_4 fractions corresponding to the elution of authentic dUMP and dUDP following chromatography of extracts of untreated (□) L1210 cells and those treated with 2 µM (+) and 10 µM (*) CB30900 for 4 hr.

pounds such as TMP, deoxyuridine, thymidine, and FdUMP did not cross-react in the assay (<0.1%).

The dUMP standard curve ranged from 83–14199 fmoles added with 50% inhibition of binding occurring at approximately 1050 ± 84 fmoles added. Figure 4 shows the mean standard curve for dUMP obtained on 12 different occasions, with the associated precision profile. The sensitivity of the standard curve was 100 fmole compared to only 4 fmole for the dUTP standard curve [30]. Using 2 million cells and assaying the extract at a dilution of ⅕ results in a limit of detection of 3 pmoles/10^6 cells. A pooled cell extract included in each assay as a control sample gave a CV% of 12.6% (12.0 ± 1.5 pmoles/mL). The recovery of dUMP throughout the entire sample preparation procedure was 73 ± 8.5%. As with the TTP RIA, there was no significant difference between samples assayed in duplicate within an assay or on separate occasions.

Measurement of TTP and "dUMP" Pools in TS-Inhibited Cells

The RIAs described were used to define the perturbations in TTP and "dUMP" pools in L1210 cells treated with ZD1694 and CB30900. Exposure of L1210 cells to both compounds caused a time- and dose-dependent depletion of TTP pools and a concomitant rise in immunoreactive dUMP indicative of effective inhibition of TS activity.

ZD1694. The effect of time and dose (10, 50, and 100 times the IC_{50}) of ZD1694 on TTP and "dUMP" pools in L1210 cells is shown in Table 1. In the presence of ZD1694, TTP pools were significantly depleted ($P < 0.001$) compared to untreated cells, except at the lowest dose (0.1 μM) studied when TTP pools had been depleted by only 23% at 1 hr ($P = 0.08$). At 4 hr, TTP levels were near to the limit of detection of the assay (<10% of controls). TS inhibition was maintained when the cells were resuspended in DFM for 4 hr. Indeed, at the highest dose (1.0 μM), TTP levels were below the limit of detection in all samples assayed. TTP remained depleted following a further 12 hr in DFM (68% and 80% inhibition compared to control levels) at the two higher doses studied but, at the lowest dose, TTP pools had completely recovered, exceeding those of untreated cells.

Following TS inhibition with ZD1694 "dUMP" pools were significantly increased ($P < 0.01$) and largely mirror those for TTP. At 1 hr, the "dUMP" pool had risen between 4- and 8-fold compared to untreated cells and by 8–10 times at 4 hr. Further incubation in the absence of drug did not allow "dUMP" pools to normalise (4.5–7.4-fold increase over controls at 16 hr). dUTP was specifically measured in selected samples by including the chromatography step prior to RIA. dUTP pools increased to 1–2 pmoles/10^6 cells (controls 0.75 pmoles/10^6 cells), but only following a 4-hr exposure to 50 and 100 times the IC_{50} and subsequent overnight incubation in DFM.

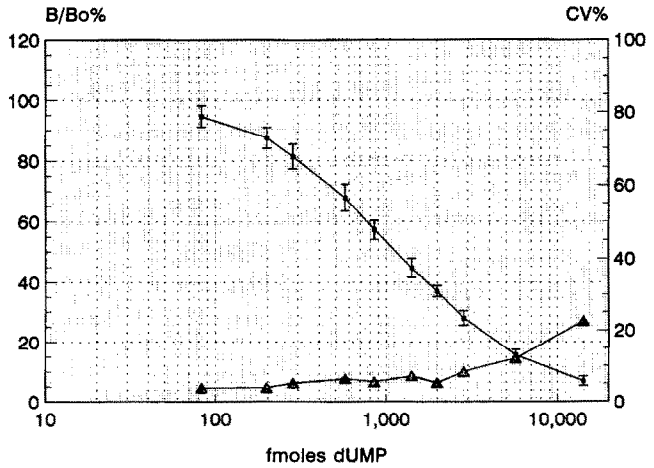

FIG. 4. The standard curve for dUMP showing the mean percent binding ± SD at each standard point of 12 successive assays. The precision profile (CV% vs dose) is also shown (△).

CB30900. Table 2 shows the effect on TTP pools of exposing L1210 cells to 10, 50, and 100 times the IC_{50} (2, 10, and 50 μM) of the nonpolyglutamated compound CB30900. As for ZD1694, there was a time- and dose-dependent depletion of TTP pools but, unlike ZD1694, a 4-hr period in the absence of drug allowed the pools to become largely normalised. TTP pools were 76% of untreated controls ($P = 0.14$) following exposure of L1210 cells to 2 μM CB30900 (10 × IC_{50}) for 1 hr. At the 2 higher doses used, TTP pools had been depleted ($P < 0.001$) 28% and 31% of controls at 1 hr and by 4 hr in drug-containing medium TTP pools, were depleted further at all doses (9%, 10%, and 6%, respectively). As was observed for ZD1694-treated cells, "dUMP" pools were increased 2–4-fold at 1 hr compared to untreated cells ($P = 0.03$) and 5–7-fold 4 hr following exposure to CB30900 ($P < 0.005$) (Table 2). "dUMP" pools remained increased, but at a lower level (4–5-fold higher than untreated controls) following a 4-hr period in drug-free medium.

In untreated L1210 cells, the ratio of dUMP pools to TTP pools was 0.32; the effect of TS inhibitors is to markedly increase this ratio. Figure 5 shows the effect of ZD1694 and CB30900 on the "dUMP"/TTP ratio, which is increased over 100 times during drug exposure. The prolonged inhibition of TTP pools for ZD1694-treated cells, even following resuspension in drug-free medium, is consistent with the formation and intracellular retention of potent polyglutamated species of the drug. This retention of TS inhibitory activity was not observed when the same growth inhibitory doses of CB30900 were used. When L1210 cells were exposed for 4 hr to a higher dose (50 μM) of CB30900 (250 × IC_{50}), inhibition of TTP pools and elevation of "dUMP" pools were similar to those observed at the lower doses during the drug-exposure period (4 hr). However, TTP pools remained depleted (84% inhibition) and "dUMP" pools remained elevated (5-fold) 4 hr after resuspension in DFM (Fig. 6). A further 8 hr in DFM allowed the cells to completely restore the normal pool sizes of "dUMP" and TTP.

TABLE 1. The effect of time and dose of ZD1694 exposure on TTP and "dUMP" pools in L1210 mouse leukaemia cells and the effect of resuspension in drug-free medium

Treatment	0.1 μM ZD1694		0.5 μM ZD1694		1.0 μM ZD1694	
			TTP pools (pmole 10^6 cells)			
1 hr	16.2 ± 6.0	(n = 4)	6.4	(4.2/8.5)	5.2	(3.0/7.3)
4 hr	9.2	(9.1/9.3)	1.8 ± 1.0	(n = 4)	1.2 ± 0.4	(n = 6)
4 hr + 4 hr DFM	6.1 ± 4.0	(n = 3)	1.9 ± 1.6	(n = 3)	1	(n = 4)*
4 hr + 16 hr DFM	32.8	(28.2/37.4)	6.7	(3.9/9.4)	4.1	(2.1/6.1)
			"dUMP" pools (pmoles/10^6 cells):			
1 hr	29.0 ± 6.6	(n = 3)	32.7	(31.8/33.6)	36.5	(39.2/33.8)
4 hr	65	(51.2/78.4)	52.1 ± 27.0	(n = 3)	54.0 ± 19	(n = 5)
4 hr + 4 hr DFM	35.1	(n = 1)	43.2	(53.2/33.2)	44.5 ± 10	(n = 3)
4 hr + 16 hr DFM	30.4	(n = 1)	50.6	(59.0/42.2)	48.0	(52.7/43.2)

Cells were treated with 10, 50, and 100 times the IC_{50} (48 hr growth inhibition assay) for 1 and 4 hr and resuspended in drug-free medium for 4 and 16 hr. The data are the results of 1–6 experiments carried out in duplicate. Cell extracts were prepared as described and assayed directly for TTP and "dUMP" pools. Results below the limit of detection have been assigned a value of 1 pmole/10^6 cells. *All results were below the limit of detection. The mean values for control (untreated) cells during these experiments was 21 ± 3.6 pmole/10^6 cells (n = 11) for TTP and 6.8 ± 7.2 (n = 8) for dUMP pools.

DISCUSSION

Practical problems of measuring the low dNTP concentrations within cells have often precluded extended investigations of pool perturbations following exposure of cell lines and tissues to antimetabolites. The use of RIA has not been widely used, but offers an alternative to HPLC and DNA polymerase methodologies. Highly sensitive RIAs for dCTP [29] and dUTP [30] have exemplified the potential advantages of immunological techniques for studies on the cellular pharmacology of enzyme inhibitors. In this paper, we have described the use of RIAs to determine the extent and duration of TS inhibition by the measurement of the relevant pools either side of the enzyme block (i.e. immunoreactive TTP and dUMP).

Because a rapid assay was required to facilitate our studies during the development of novel TS inhibitors, the RIA has been carried out without chromatography, previously described to separate TTP from TDP and TMP [31]. Intracellular TDP and TMP concentrations have been reported to be approximately 5 and 35 times lower, respectively, than TTP pools [1, 33]. Thus, interference from the low concentrations of TMP would cause negligible interference with the measurement of TTP because of its low cross-reaction with the antibody (<0.2%). Interference by TDP would be more likely to interfere with the measurement of TTP because it cross-reacts with the antiserum by 89%. However, in view of this particular application, where TS inhibition would result in depletion of TDP as well as TTP, the time-consuming chromatography step was omitted. In support of this, the immunoreactivity in fractions obtained following chromatography of extracts from both untreated and drug-treated cells was undetectable in those corresponding to TMP and TDP (Fig. 2). Results obtained by RIA directly on cell extracts were not significantly different from those obtained following chromatography.

The RIA for TTP is sensitive to <1 pmole/10^6 cells (depending on the number of cells harvested), is robust, reproducible, and suitable for the sensitive and rapid measurement of TTP pools in drug-treated cells, allowing the measurement in duplicate of at least 20 samples within an assay. Recovery of TTP from the RIA itself was complete

TABLE 2. The effect of time and dose of CB30900 on TTP and "dUMP" pools in L1210 mouse leukaemia cells and the effect of resuspension in drug-free medium

Treatment	2 μM CB30900		10 μM CB30900		20 μM CB30900	
			TTP pools (pmoles/10^6 cells):			
1 hr	16.0	(10.6/21.3)	5.8	(6.0/5.7)	6.4	(5.8/7.0)
4 hr	1.9 ± 0.6	(n = 4)	2.0 ± 1.0	(n = 4)	1.3 ± 0.4	(n = 5)
4 hr + 4 hr DFM	25.1 ± 8.7	(n = 3)	16.5 ± 2.7	(n = 3)	14.5 ± 4.1	
			"dUMP" pools (pmoles/10^6 cells):			
1 hr	20.5	(17.9/20.5)	14.9	(17.4/29.8)	29.1	(22.6/35.5)
4 hr	46.5 ± 28.0	(n = 3)	46.3 ± 16.4	(n = 3)	35.5 ± 15.7	(n = 4)
4 hr + 4 hr DFM	29.1	(22.6/35.5)	35.5 ± 15.7	(n = 4)	28.5	(43.1/13.8)

L1210 mouse leukaemia cells were treated with CB30900 at 10, 50, and 100 times the IC_{50} (48 hr growth inhibition assay) for 1 hr and 4 hr and resuspended in drug-free medium for 4 hr. The data are the results of 2–6 experiments carried out in duplicate. Cell extracts were prepared as described and assayed directly for TTP and "dUMP" pools. The mean values for control (untreated) cells during these experiments was 21 ± 3.6 pmole/10^6 cells (n = 11) for TTP and 6.8 ± 7.2 (n = 8) for dUMP pools.

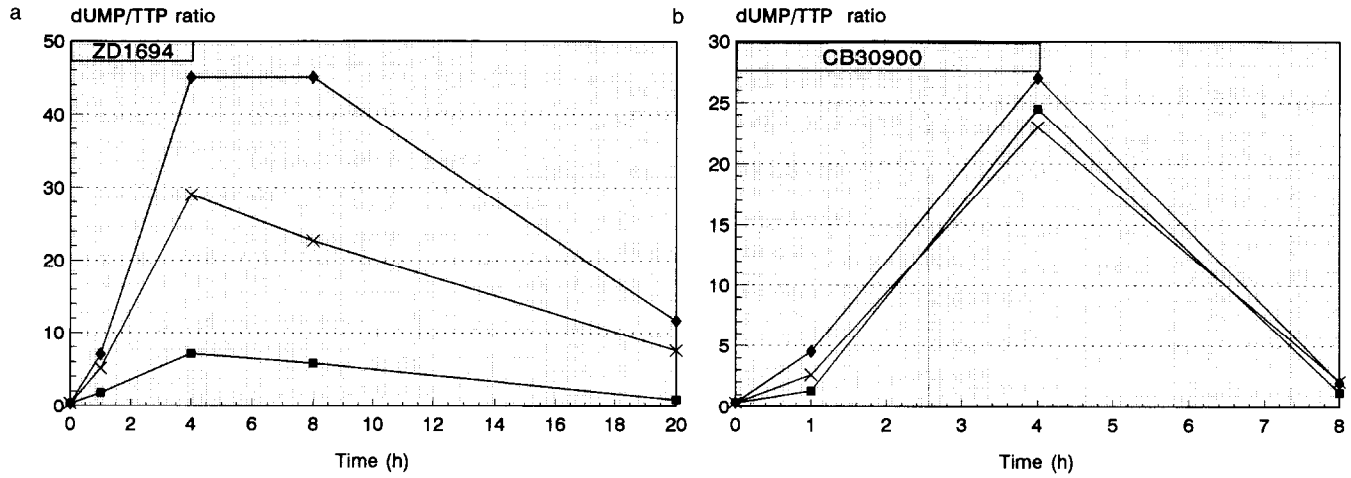

FIG. 5. The effect of dose and time of exposure to (a) ZD1694 and (b) CB30900 on the "dUMP"/TTP ratio in L1210 cells (data from Table 1). Cells were exposed to 0.1 (■), 0.5 (×) and 1.0 μM (◆) ZD1694 equivalent to 10, 50, and 100 times the IC_{50} in a 48-hr growth inhibition assay. Cells were resuspended in drug-free medium at 4 hr.

and reproducible results both between and within assays is less than 12%. Extraction recovery of TTP throughout the whole procedure including PCA extraction, neutralisation, and periodate oxidation was 72%. The precise step at which losses occur has not been determined, but recovery from water rather than a cell pellet was complete (92%). Storage of unextracted cell pellets even at −20°C results in considerable loss of TTP (data not shown), and it is likely that enzymatic degradation occurs prior to PCA extraction. To minimise any loss by enzymatic degradation, the PCA extraction step was always carried out immediately after the cells were harvested. The values obtained for TTP pools in L1210 cells compared to results previously reported and obtained by HPLC (56.7–14.2 pmoles/10^6 cells) and by the polymerase assay (19.0 ± 4 pmoles/10^6 cells) [1, 34].

RIA has previously been utilised to measure dUTP in TS-inhibited cells following chromatography to separate dUMP from dUDP [7]. In A549 lung carcinoma cells,

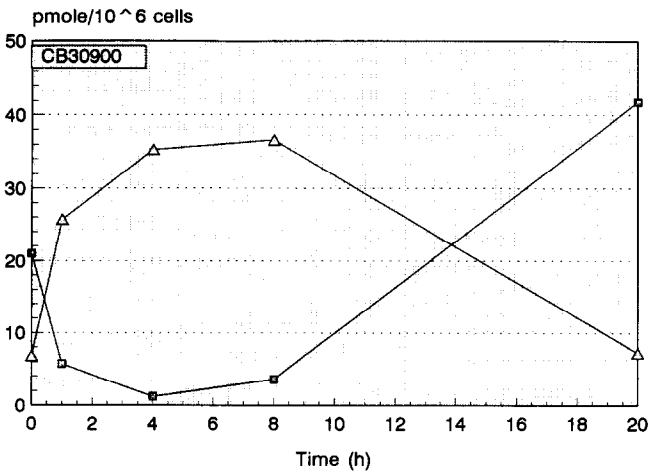

FIG. 6. TTP (□) and "dUMP" (△) pools in L1210 cells treated with 250 × IC_{50} CB30900 for 4 hr and then resuspended in DFM for an additional 16 hr. Results are the mean of at least 2 experiments carried out in duplicate.

dUTP pools were enlarged in a dose- and time-related manner in response to the antifolate CB3717. A 4-hr exposure to CB3717 at its IC_{50} (3 μM) and a 10-fold higher dose, increased dUTP pools to 3.3 and 9.6 pmoles/10^6 cells respectively. In our initial studies with L1210 cells, no marked increase in dUTP could be detected at this relatively short time point with the growth inhibitory doses used. Therefore, the dUTP RIA was adapted to measure total immunoreactivity of deoxyuridine nucleotides so that events behind the enzyme block could be investigated. The dUTP antiserum cross-reacts with dUMP by 3.0%, and this was exploited by replacing dUTP radiolabel with that of dUMP. Results obtained in this way are reported as "immunoreactive" dUMP concentrations, as even small amounts of dUTP and dUDP formed as a result of TS inhibition would interfere markedly. However, in L1210 cells it is only in samples obtained following longer periods of TS inhibition (>4 hr) where significant amounts of dUTP were formed. Comparison of results obtained with and without the use of chromatography showed good correlation for control samples and those obtained up to 4 hr in the presence of inhibitor. "dUMP" pools measured by RIA in this study compared with those previously measured in the human lymphoblastoid W1L2 cell line by HPLC [1].

The "dUMP" measurement can also give an indication of dUTP production. At later time points, when "dUMP" pools have not normalised in the presence of restored or normalising TTP pools, it is likely that dUTP pools are present and can be specifically measured on the same extract if required by utilising the dUTP RIA on the relevant chromatography reactions. The length of time and extent of dUMP pool elevation required before significant quantities of dUTP are formed probably vary with each cell line and will depend on several factors (e.g. deoxyuridine efflux [21, 35] and the activity of deoxyuridine triphosphatase [36]). When efflux of deoxyuridine was inhibited with the nucleoside transport inhibitor, dipyridamole, dUTP pools were significantly increased [7] in cells treated with

CB3717 compared to those treated with the TS inhibitor alone.

Perturbations in TTP and "dUMP" pools as a result of cell exposure to both ZD1694 and CB30900 have been described. Following treatment with ZD1694, there is a rapid depletion of TTP pools as TS is inhibited. Complete inhibition (>90%) was achieved by 4 hr. This is accompanied by a concomitant increase in "dUMP" pools behind the enzyme block caused primarily by loss of negative feedback on deoxycytidylate deaminase [4]. The fold increase in "dUMP" was similar to that observed in W1L2 human lymphoblastoid cells with CB3717 [1] and in MGH-U1 human bladder carcinoma cells treated with ZD1694 [3] although, in the latter case, control levels were 100-fold higher. Effective TS inhibition was also achieved with CB30900 by 4 hr at all dose levels used with an increase in "dUMP" pools similar to those caused by ZD1694. Both ZD1694 and CB3090 appeared to deplete TTP at a similar rate, which may reflect the fact that the two compounds are both transported into cells by the RFC.

Marked differences were, however, observed between the 2 compounds following resuspension in DFM that are consistent with their biochemical profile. Delayed recovery of TTP pools was observed for ZD1694, especially at the higher dose levels. Previous studies have shown that ZD1694 is retained within L1210 cells largely in the form of polyanionic polyglutamates and that, following resuspension, only the parent compound and lower polyglutamates are lost over a period of time [6]. Recent studies have confirmed that this pattern of retention also occurs *in vivo* [37]. As would be expected from a compound that has been designed not to be a substrate for FPGS, TS inhibition by CB30900 was readily reversed by resuspension of drug-treated cells in DFM. However, at the highest dose used in this study, some retention of TS activity was observed. The reason for this is not clear but may be due to slow efflux or by tight binding of inhibitor to the enzyme or another intracellular component.

In summary, we have described the use of two RIAs, one for TTP and one for dUMP immunoreactivity, that are suitable for evaluating and comparing the TS inhibitory properties of novel antifolates currently in development. TS inhibition as determined by RIA of TTP and "dUMP" pools was qualitatively similar to that obtained using the whole cell TS activity assay [38]. However, it is apparent that the whole cell activity assay can underestimate TS activity when dUMP pools are high. Elevated endogenous pools of dUMP will reduce the specific activity of radiolabelled dUMP formed during the assay, thereby giving an apparently low estimate of TS activity [17]. The measurement of TTP pools as described here, on the other hand, may not give an accurate assessment of TS inhibition in the presence of salvageable TTP. TS inhibition in various human tumour cell lines in response to quinazoline antifolates with different biochemical properties is currently being investigated using the RIAs for TTP and "dUMP". These assays have the potential to be widely applied to *in vitro* and *in vivo* pharmacodynamic studies of established drugs, combinations of drugs, and biochemical modulators, as well as during the development of novel TS inhibitors.

This work was supported by the Cancer Research Campaign and Zeneca Pharmaceuticals.

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