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Development of a Whole Cell Assay to Measure Methotrexate-Induced Inhibition of Thymidylate Synthase and *De Novo* Purine Synthesis in Leukaemia Cells

*Matthew J. Barnes, Gordon A. Taylor and David R. Newell**
CANCER RESEARCH UNIT, UNIVERSITY OF NEWCASTLE, NEWCASTLE UPON TYNE NE2 4HH U.K.

ABSTRACT. The cellular pharmacology of methotrexate (MTX) is complex, involving the inhibition of both *de novo* thymidylate and purine biosynthesis. Measurement of MTX-induced inhibition of *de novo* thymidylate and purine biosynthesis may allow optimisation of MTX therapy, and the aim of this study was to develop an assay to measure the activity of both pathways in the same cell sample, and so determine the effects of MTX treatment. *In situ* thymidylate synthase (EC 2.1.1.45) activity was measured by the release of ^3H_2O from [5′-^{3}H]deoxyuridine and *de novo* purine synthesis by the incorporation of [^{14}C]formate into adenine and guanine. Incubation of human leukaemia CCRF-CEM cells for 22 hr with 50 nM MTX resulted in approximately 90% inhibition of *in situ* thymidylate synthase activity, relative to control untreated cells, and after exposure to 1000 nM MTX activity could not be detected. In contrast, *de novo* purine synthesis, measured in the same sample, was not inhibited by exposure to 50 nM MTX, although activity was again completely abolished by exposure to 1000 nM MTX. To demonstrate the utility of the assay, lymphoblasts isolated from a child with acute lymphoblastic leukaemia (ALL) were also incubated for 22 hr with 1000 nM MTX. Both *in situ* thymidylate synthase activity and *de novo* purine synthesis were significantly inhibited, by 70% and 60% respectively, relative to the activity in untreated cells. BIOCHEM PHARMACOL **59**;4:321–328, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. thymidylate synthase; *de novo* purine synthesis; methotrexate; CCRF-CEM; polyglutamation; antifolates

Folic acid and reduced folate cofactors play a critical role in both purine and pyrimidine nucleotide biosynthesis by acting as donors of one-carbon units. The biosynthesis of these DNA precursors can be inhibited by folate analogues such as raltitrexed [1], lometrexol [2], and MTX†, the latter antifolate being particularly important in the treatment of childhood leukaemia [3]. The mechanism of action of MTX was originally thought to be exclusively related to the depletion of intracellular reduced folates following the inhibition of dihydrofolate reductase (EC 1.5.1.3) [4]. However, more recent evidence suggests that MTX, following polyglutamation, can directly inhibit both *de novo* thymidylate [5] and purine synthesis [6]. Cellular MTX uptake [7], levels of cellular dihydrofolate reductase [8], and MTX polyglutamation [9] have all been shown to be potential determinants of the locus of action and cytotoxicity of the drug in childhood leukaemia. However, the measurement of multiple potential determinants of MTX activity is time-consuming and requires a large number of cells. Furthermore, the impact of other, as yet unidentified determinants may be overlooked. As an alternative approach, measuring MTX-induced inhibition of *de novo* thymidylate and purine biosynthesis in whole cell systems should reflect the combined contribution of MTX uptake, polyglutamation, and target enzyme levels on MTX activity, as well as other factors such as intracellular levels of reduced folates. Furthermore, inhibition of these pathways, measured separately, has previously been shown to reflect cellular chemosensitivity to MTX [10, 11].

The combined *de novo* thymidylate/purine biosynthesis assay used here was developed and adapted from those of Masson *et al.* (*de novo* purine biosynthesis) [11] and Taylor *et al.* (thymidylate synthase; EC 2.1.1.45) [12], as adapted from Tomich *et al.* [13] and Rode *et al.* [14]. As the method used by Masson *et al.* for measuring *de novo* purine biosynthesis had been successfully applied to childhood ALL lymphoblast samples, the approach taken here involved adapting the *in situ* TS assay to be compatible with the method for measuring purine biosynthesis. The assay for *de novo* purine biosynthesis is based on the incorporation of [^{14}C]formate into the purine ring structure during *de novo* synthesis via the 10-formyltetrahydrofolate cofactor. Incor-

* Corresponding author: Dr. David R. Newell, Cancer Research Unit, University of Newcastle, Newcastle upon Tyne NE2 4HH, U.K. Tel. +44-191-222.8057; FAX +44-191-222.7556; E-mail: herbie.newell@newcastle.ac.uk

† *Abbreviations:* MTX, methotrexate; ALL, acute lymphoblastic leukaemia; TS, thymidylate synthase; and [^{3}H]dUMP, [^{3}H]deoxyuridine 5′-monophosphate.

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poration occurs at two stages during the *de novo* synthesis of purines in reactions catalysed by glycinamide ribonucleotide transformylase (GARFT; EC 2.1.2.2) and aminoimidazolecarboxamide ribonucleotide transformylase (AICART; EC 2.1.2.3). Activity of these enzymes in the *de novo* pathway is reflected by the presence of ^{14}C-labelled adenine and guanine bases in postincubation hot perchloric acid cellular extracts. The *in situ* TS assay is based on the release of $^{3}H_2O$ in response to incubation with [5′-^{3}H]deoxyuridine. [5′-^{3}H]Deoxyuridine is transported into the cell and phosphorylated by thymidine kinase to [^{3}H]dUMP, in which form it acts as a substrate for TS. [^{3}H]dUMP is methylated in the reaction at the 5′-position by TS, with 5,10-methylenetetrahydrofolate as the methyl-donating cosubstrate, to produce dTMP (thymidylate). The 5′-tritium atom is released from [^{3}H]dUMP during the reaction as $^{3}H_2O$, and extracellular $^{3}H_2O$ is used as a measure of TS activity.

The aims of the studies described here were to combine established methods for measuring *in situ* TS activity and *de novo* purine synthesis, and determine the effects of MTX on the activities of the two pathways. Studies focused on examining the compatibility of the two assays and determining the effects of incubation on *in situ* TS activity and *de novo* purine synthesis. Lastly, the utility of the assay was demonstrated by application to a clinical sample.

MATERIALS AND METHODS

Cell Culture

CCRF-CEM (European Collection of Animal Cell Cultures) human leukaemic cells were grown as a suspension culture in RPMI 1640 medium (GIBCO BRL) supplemented with 2 mM L-glutamine (GIBCO BRL), 12.5 mL of 7.5% (w/v) sodium bicarbonate solution (GIBCO BRL) per 500 mL, and 10% (v/v) charcoal-dialysed foetal bovine serum (Globepharm). The cell line was routinely subcultured twice weekly and maintained in an incubator with 5% CO_2 and a humidified atmosphere at 37°. Regular testing for mycoplasma excluded infection.

Drug Exposure

Five × 10^6 exponentially growing cells were resuspended in 2 mL of dialysed medium, supplemented with 5 μM thymidine (Sigma Chemical Co.) and 10 μM inosine (Aldrich Chemical Co.), to prevent drug-induced cell growth inhibition and toxicity, with or without MTX (Sigma Chemical Co.) at concentrations ranging from 10–1000 nM, and incubated at 37° for 22 hr. At the end of this period, samples were centrifuged (120 *g* for 5 mins), washed and cells resuspended in 2 mL fresh drug-free medium (without added thymidine or inosine) before further incubation for 30 min at 37° prior to the estimation of *de novo* thymidylate and purine biosynthesis. The 30-min incubation in the absence of thymidine and inosine was included to avoid competition between the latter two nucleosides and [5′-^{3}H]deoxyuridine, for transport and/or phosphorylation.

Estimation of De Novo *Thymidylate and Purine Biosynthesis*

Either 5 × 10^6 exponentially growing cells in 2 mL of dialysed medium, or the cell suspensions left after the 30-min re-incubation period following drug exposure as described above, were treated with a mixture of purified [^{3}H]deoxyuridine ([5′-^{3}H]2′-deoxyuridine, Moravek Biochemicals; deoxyuridine, Sigma Chemical Co.) and ^{14}C formic acid (^{14}C formic acid sodium salt, Amersham; sodium formate, Sigma Chemical Co.) so as to achieve final concentrations of 0.3 μM [^{3}H]deoxyuridine (specific activity 227 KBq/mmol) and 0.5 mM formate (specific activity 0.84 KBq/mmol). Samples were then incubated at 37° for a further 2 hr after which they were placed on ice for 5 min and then centrifuged at 120 *g* for 5 mins.

In situ *Thymidylate Synthase Activity*

The supernatant from the above centrifugation step containing the $^{3}H_2O$ released from the *in situ* activity of TS was removed as three 600-μL aliquots and pipetted onto 600 μL of ice-cold 1M perchloric acid (BDH) to precipitate any remaining cellular material. The samples were then placed on ice for 15 min after which time 750 μL of activated charcoal (200 mg/mL suspended in H_2O) (Sigma Chemical Co.) + dextran (10 mg/mL in H_2O) (Sigma Chemical Co.) was added to each triplicate sample at 4° in order to adsorb unutilised [5′-^{3}H]deoxyuridine. The activated charcoal solution was stirred constantly prior to and during the pipetting to ensure that no sedimentation occurred. Samples were left for at least 15 min on ice before centrifugation at 1850 *g* for 10 min at 4°. The supernatant containing the $^{3}H_2O$ released from the [^{3}H]deoxyuridine was removed and 300 μL pipetted into scintillation vials (North Eastern Laboratory Supplies) and 10 mL of scintillant added before scintillation counting. Results were expressed as dpm/10^6 cells/hr or as a percentage of the release of $^{3}H_2O$ in control untreated cells.

Determination of Extracellular [^{3}H]Deoxyuridine Concentration

In situ TS activity was measured in CCRF-CEM cells as described below at various time points during a 240-min incubation. To evaluate deoxyuridine consumption during the assay, 100-μL aliquots of culture medium were removed for the determination of the extracellular [^{3}H]deoxyuridine concentration at time zero, 120 min, and 240 min. [^{3}H]Deoxyuridine was extracted from culture medium by the addition of 100 μL ice-cold 0.2M perchloric acid with brief vortexing. The extracts were then centrifuged at 6700 *g* for 2 min to remove the precipitated protein. The resultant supernatant was decanted directly onto approxi-

mately 200 mg potassium bicarbonate (Aldrich Chemical Co.) and left to stand on ice for 2 min to neutralise the acidic extract. The extract was again centrifuged at 6700 *g* for 2 min to remove any remaining potassium bicarbonate as well as the potassium perchlorate that had formed, and the final supernatant removed and analysed by HPLC. [^{3}H]Deoxyuridine was resolved on a 100 × 4.6 mm Nucleosil 3μ ODS cartridge (Jones Chromatography) using a set of model 510 Waters pumps (Millipore) and a UNICAM PU 4021 multi-channel detector (PYE) to detect standard (Aldrich Chemical Co.) and [^{3}H]deoxyuridine by UV absorbance at 262 and 280 nm. One hundred microlitres of sample and standards (deoxyuridine concentration range 0.03–0.5 μM) was injected onto the column via a 50 × 2 mm pellicular ODS silica precolumn (Whatman) using a Perkin Elmer ISS 200 autosampler (Perkin Elmer), and chromatograms were analysed using the Minicrom chromatography data capture system software (V.G. Data Systems). The mobile phase used to analyse [^{3}H]deoxyuridine concentrations consisted of 0.05 M ammonium acetate (ammonia and glacial acetic acid; BDH) pH 5 + 5% (w/w) methanol (Fisons Chemicals) pumped isocratically at 1 mL/min. Results were expressed as extracellular [^{3}H]deoxyuridine concentration (μM) following quantitation using the standard curve described above.

De Novo *Purine Synthesis*

The cell pellets from the initial centrifugation step described above were resuspended in 2 mL ice-cold fresh drug-free dialysed medium (without added thymidine or inosine) and further centrifuged at 120 *g* for 5 min. The cell pellets were washed a further two times in 2 mL of the same ice-cold medium, and finally resuspended in 500 μL of 1M ice-cold perchloric acid. Following transfer to glass tubes (Baxter Healthcare), samples were vortexed briefly and placed in a dry heating block (Techne) at 100° for 1 hr in order to hydrolyse both polymeric and monomeric purine nucleotides to guanine and adenine free bases. After this hydrolysis, samples were removed from the heating block and allowed to cool for 15 min. The samples were then pipetted into 1.5-mL Eppendorf tubes (Sarstedt) and centrifuged at 6700 *g* for 5 min at 4°. The resultant supernatant was then added to 300 μL of ice-cold 2M tris[hydroxymethyl]aminomethane (Tris-base) (Sigma Chemical Co.) and again centrifuged at 6700 *g* for 5 min at 4°. Finally, the supernatant was removed and the pH verified as neutral before the samples were frozen at −20° prior to analysis by HPLC. HPLC analysis involved the injection of 100-μL samples of the neutralised supernatants via a 50 × 2 mm pellicular ODS silica precolumn with separation of [^{14}C]adenine and [^{14}C]guanine on a 250 × 4.6 mm Spherisorb 5μ ODS2 cartridge (PhaseSep) with UV absorbance of standard (Sigma Chemical Co.) and endogenous adenine and guanine measured at 260 and 280 nm. ^{14}C-Labelled adenine and guanine were detected using an on-line FLO-ONE radiochromatography detector (Packard) with a liquid scintillant cell (flow rate of liquid scintillant 1.5 mL/min). The HPLC mobile phase consisted of 0.1 M sodium acetate (Sigma Chemical Co.) pH 5.5, adjusted using glacial acetic acid (BDH) to achieve the desired pH, run isocratically at 1 mL/min for the first 10 min of the analysis. The isocratic elution was followed by a linear gradient from 0.1 M sodium acetate pH 5.5 to 0.1 M sodium acetate pH 5.5 + 15% (w/w) acetonitrile (Fisons Chemicals) at 1 mL/min over the subsequent 20 min to give a total analysis time of 30 min. A 2-min linear gradient back to the original 0.1 M sodium acetate pH 5.5 was employed, and the column left to re-equilibrate for a further 15 min before the autosampler triggered another injection. Endogenous adenine and guanine were quantitated using linear regression against standard curves containing 0.44–27 and 0.52–33 nmol adenine and guanine, respectively, injected onto the column. Newly formed adenine and guanine were quantitated on the basis of the specific activity of the [^{14}C]formate (0.84 KBq/mmol), and results were expressed as pmol of newly formed bases/nmol of pre-existing (endogenous) bases.

Application of the Whole Cell Assay to an ALL Lymphoblast Sample

Following approval by the Local Ethics Committee, the whole cell assay was applied to a lymphoblast sample taken from a newly diagnosed patient with common, diploid ALL. Briefly, lymphoblasts were isolated from bone marrow aspirates using a Ficoll–Hypaque gradient and washed in Hank's Balanced Salt Solution (GIBCO BRL) before 5 × 10^6 isolated lymphoblasts were incubated in 2 mL of RPMI 1640 + 10% (v/v) dialysed foetal bovine serum supplemented with 5 μM thymidine, 10 μM inosine, and ITS (5 mg/L insulin, 5 mg/L transferrin, and 5 μg/L sodium selenium final concentrations (Sigma Chemical Co.). Drug exposure and estimation of *in situ* thymidylate synthase activity and *de novo* purine synthesis were carried out using the methods described above and CCRF-CEM cells were included in a parallel incubation as a positive control.

RESULTS

Assessment of De Novo *Purine Biosynthesis*

De novo purine synthesis was measured by application of a previously published method [11] based on the incorporation of [^{14}C]formate into the purine bases adenine and guanine. CCRF-CEM cells were incubated in 2 mL dialysed medium plus 0.5 mM [^{14}C]formate for 2 hr, and ^{14}C-labelled adenine and guanine extracted and resolved via HPLC analysis, as shown in Fig. 1. The identity of the ^{14}C-labelled bases was assigned by co-chromatography with standard bases. Investigations into the stability of the free bases during the assay and percentage recovery of radioactivity during the extraction procedure have been published previously [15].

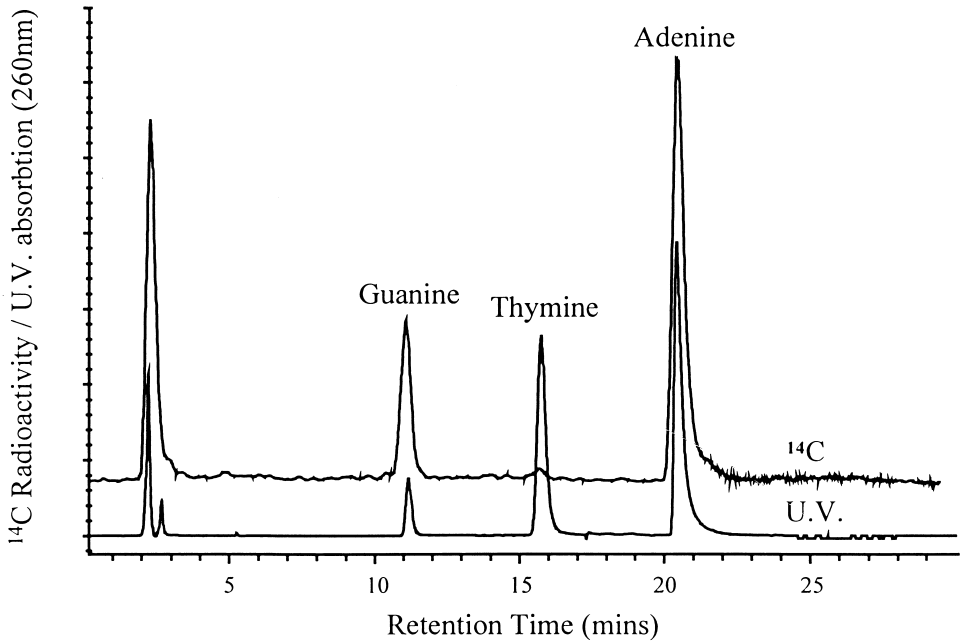

FIG. 1. HPLC chromatogram showing the co-elution of standard purine bases with ^{14}C-labelled compounds from an untreated CCRF-CEM cell extract following incubation with [^{14}C]formate as described in Materials and Methods.

Determination of In Situ *Thymidylate Synthase Activity*

Having reproduced the published technique for the estimation of *de novo* purine synthesis, the next stage in the development of the combined assay was to integrate the method for the estimation of *in situ* TS activity. Initial experiments concentrated on establishing an appropriate incubation period. As shown in Fig. 2, the amount of ^3H_2O released from [^{3}H]deoxyuridine increased linearly with time of incubation up to 240 min ($r^2 = 0.999$, $P < 0.0001$), i.e. double the incubation time used in the method for estimating *de novo* purine biosynthesis. Also shown is the extracellular concentration of [^{3}H]deoxyuridine, which decreased during the incubation but was still greater than 0.15 μM after 240 min. As the rate of ^3H_2O release was still linear at this time, an initial [^{3}H]deoxyuridine concentration of 0.3 μM and a 2-hr incubation period was chosen for the subsequent measurement of TS activity.

Examination of the Compatibility of the Assays for In Situ *Thymidylate Synthase Activity and* De Novo *Purine Synthesis*

As the assay was being developed to measure *in situ* thymidylate synthase activity and *de novo* purine synthesis in the same sample, it was important to demonstrate that the substrate for measuring TS activity did not interfere with the estimation of *de novo* purine synthesis and vice versa. *In situ* TS activity in CCRF-CEM cells was measured in the presence and absence of 0.5 mM [^{14}C]formate, the substrate for the *de novo* purine synthesis assay (Table 1). Following incubation of CCRF-CEM cells with 0.3 μM [^{3}H]deoxyuridine alone, the amount of ^3H_2O released was 173,922 ± 13,618 dpm/10^6 cells/hr (mean ± SD of three separate experiments). When cells were co-incubated with 0.3 μM [^{3}H]deoxyuridine and 0.5 mM [^{14}C]formate, the amount of ^3H_2O released was 173,589 ± 11,397 dpm/10^6

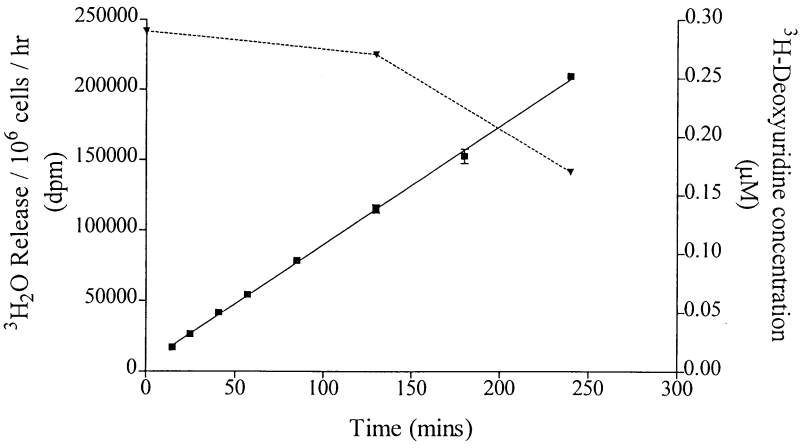

FIG. 2. *In situ* thymidylate synthase activity of CCRF-CEM cells (■) and extracellular [^{3}H]deoxyuridine concentration (▼) during a 240-min incubation. Each TS activity point on the graph represents the mean ± SD of triplicate determinations in a single experiment. Where error bars are not shown they are contained in the symbol. The line was fitted by linear regression analysis. Each [^{3}H]deoxyuridine concentration point on the graph represents a single measurement.

TABLE 1. Summary of *in situ* thymidylate synthase activity and *de novo* purine synthesis in CCRF-CEM cells obtained under different assay conditions

Assay	Conditions	TS activity (dpm/10^6 cells/hr)	*De novo* purine synthesis (pmol/nmol pre-existing base/hr)	
			Guanine	Adenine
Compatibility	+[^{3}H]deoxyuridine	173,922 ± 13,618		
	+[^{14}C]formate	—	6.8 ± 1.55	19.0 ± 2.6
	+[^{3}H]deoxyuridine + [^{14}C]formate	173,589 ± 11,397	6.1 ± 1.0	15.9 ± 2.4
Extracellular thymidine supplement	0 min (dialysed)	229,292 ± 11,110	—	—
	0 min (supplemented)	195,474 ± 2605	—	—
	15 min (supplemented)	213,568 ± 3687	—	—
	30 min (supplemented)	233,745 ± 23,182	—	—
Extracellular thymidine and inosine supplement	2 hr (dialysed)	179,846 ± 23,186	8.3 ± 1.3	17.3 ± 1.8
	24 hr (dialysed)	232,019 ± 27,013	8.2 ± 1.6	21.0 ± 0.9
	24 hr (supplemented)	220,947 ± 40,996	8.1 ± 1.3	21.1 ± 0.5

Values under the heading 'Compatibility' represent the mean ± SD of three separate experiments; 'Extracellular thymidine supplement' represents the mean ± SD of triplicate determinations in a single experiment; 'Extracellular thymidine and inosine supplement' represents the mean ± SD of three separate experiments.

cells/hr (mean ± SD of three separate experiments), demonstrating that 0.5 mM [^{14}C]formate did not alter the *in situ* thymidylate synthase activity of CCRF-CEM cells. The corresponding rate of *de novo* purine synthesis was estimated in the same experiment to assess the effect of 0.3 μM [^{3}H]deoxyuridine on *de novo* purine synthesis determinations. *De novo* purine synthesis activity was also found to be the same in the presence (guanine 6.1 ± 1.0 and adenine 15.9 ± 2.4 pmol/nmol pre-existing base/hr, mean ± SD of three separate experiments) and absence (guanine 6.8 ± 1.55 and adenine 19.0 ± 2.6 pmol/nmol pre-existing base/hr, mean ± SD of three separate experiments) of 0.3 μM [^{3}H]deoxyuridine.

The Effects of Extracellular Thymidine and Inosine on the Estimation of In Situ *Thymidylate Synthase Activity and* De Novo *Purine Synthesis in CCRF-CEM Cells*

Experiments were performed to investigate the effect of extracellular thymidine and inosine, present during the 22-hr MTX incubation period to prevent drug-induced toxicity, on the subsequent estimation of thymidylate and purine biosynthesis. To study the effect of any residual extracellular thymidine on the assay, TS activity was measured in CCRF-CEM cells as described above over a 2-hr period either with or without a 4-hr preincubation in thymidine-supplemented medium. In samples preincubated with thymidine, *in situ* TS activity was measured either immediately after removal of extracellular thymidine, or after washing the cells in dialysed medium followed by 15- or 30-min equilibration periods in dialysed thymidine-free medium prior to the estimation of TS activity. The apparent *in situ* TS activity of cells incubated in thymidine-supplemented medium, when measured immediately (195,474 ± 2,605 dpm/10^6 cells/hr mean ± SD of triplicate determinations in a single experiment), was approximately 85% of, and significantly different ($P < 0.0001$) from, the activity of cells incubated in dialysed non-supplemented medium (229,292 ± 11,110 dpm/10^6 cells/hr mean ± SD of triplicate determinations in a single experiment). After 15 min re-equilibration in dialysed non-supplemented medium, TS activity had begun to return to control levels (213,568 ± 3,687 dpm/10^6 cells/hr, mean ± SD of triplicate determinations in a single experiment), but was still significantly lower ($P = 0.08$) than the activity of cells incubated throughout in dialysed non-supplemented medium. However, after 30 min re-equilibration, TS activity was no longer significantly different ($P = 0.76$) and had returned to control levels (233,745 ± 23,182 dpm/10^6 cells/hr, mean ± SD of triplicate determinations in a single experiment). On the basis of these data, a 30-min re-equilibration period procedure was included in the final assay methodology, following the 22-hr MTX exposure.

Experiments were next performed to confirm that *in situ* TS activity in untreated cells remained constant over the 24-hr period required for the combined assay and to investigate the effect of including the thymidine and inosine supplement. CCRF-CEM cells were incubated in dialysed medium with or without the thymidine and inosine supplement, followed by the washing and re-equilibration steps described above. *In situ* TS activity was not significantly effected by the inclusion of thymidine or inosine (dialysed 232,019 ± 27,013 dpm/10^6 cells/hr versus supplemented 220,947 ± 40,996 dpm/10^6 cells/hr, mean ± SD of three separate experiments) and was not different when measured over 0–2 hr (179846 ± 23,186 dpm/10^6 cells/hr mean ± SD of three separate experiments) or 22–24 hours (232,019 ± 27,013 dpm/10^6 cells/hr) (one-way ANOVA, $P = 0.18$).

As no marked difference in *in situ* TS activity was observed after incubations in dialysed or thymidine- and inosine-supplemented dialysed medium, possible differences in the corresponding rates of *de novo* purine synthesis were examined. Again, there were no significant differences in the mean levels of *de novo* guanine synthesis after incubations in dialysed medium for 2 hr (8.3 ± 1.3 pmol/nmol pre-existing base/hr, mean ± SD of three

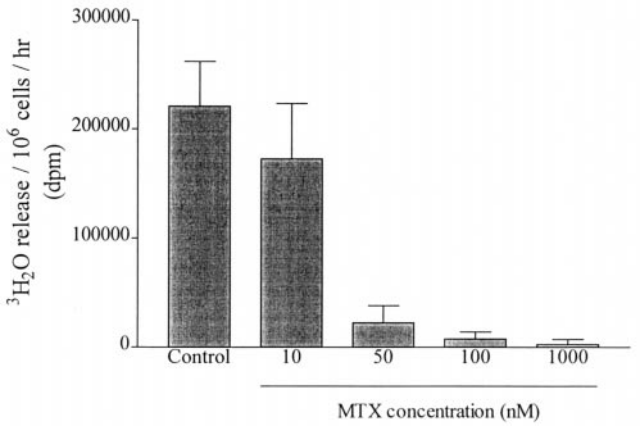

FIG. 3. *In situ* thymidylate synthase activity in CCRF-CEM cells following incubation with MTX for 22 hr at the concentrations shown. Each bar represents the mean ± SD of three separate experiments.

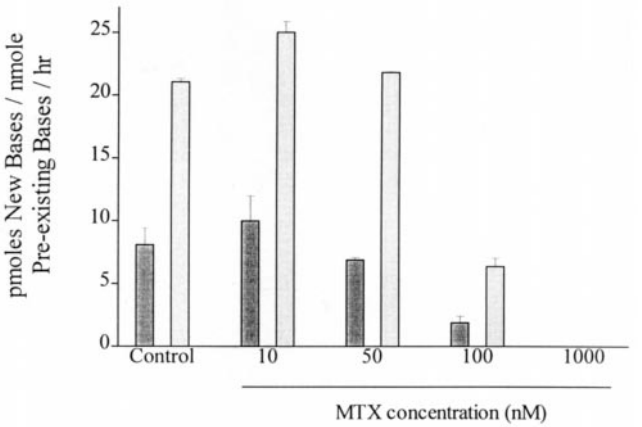

FIG. 4. *De novo* purine synthesis as measured by ^{14}C-labelled adenine (□) and guanine (■) in CCRF-CEM cells following incubation with MTX for 22 hr at the concentrations shown. Each bar represents the mean ± SD of three separate experiments.

separate experiments) or 24 hr (8.2 ± 1.6 pmol/nmol pre-existing base/hr, mean ± SD of three separate experiments) or after incubation in thymidine- and inosine-supplemented medium for 24 hr (8.1 ± 1.3 pmol/nmol pre-existing base/hr mean ± SD of three separate experiments) (one-way ANOVA, $P = 0.989$). However, there was a small and marginally significant difference in mean levels of *de novo* adenine synthesis measured after a 2-hr (17.3 ± 1.8 pmol/nmol pre-existing base/hr, mean ± SD of three separate experiments) versus a 24-hr (21.0 ± 0.9 pmol/nmol pre-existing base/hr, mean ± SD of three separate experiments) incubation in dialysed medium ($P = 0.033$) or after incubation in thymidine- and inosine-supplemented medium for 24 hr (21.1 ± 0.5 pmol/nmol pre-existing base/hr mean ± SD of three separate experiments ($P = 0.025$).

MTX-Induced Inhibition of In Situ *Thymidylate Synthase Activity and* De Novo *Purine Synthesis in CCRF-CEM Cells and ALL Lymphoblasts*

CCRF-CEM cells or ALL lymphoblasts were incubated in dialysed medium supplemented with 5 μM thymidine and 10 μM inosine, and treated with 0–1000 nM MTX for 22 hr. The cells were then washed and equilibrated in dialysed medium for 30 min, and *in situ* TS activity and *de novo* purine synthesis subsequently measured, as described above, over a 2-hr period. The mean *in situ* TS activity in CCRF-CEM cells was determined in relation to exposure to increasing concentrations of MTX and, as shown in Fig. 3, *in situ* TS activity was inhibited by approximately 90% following incubation with 50 nM MTX and was undetectable (<0.2% control) after exposure to 1000 nM MTX. The corresponding mean rates of *de novo* purine synthesis in CCRF-CEM cells were also measured in the same cells, again in relation to MTX concentration (Fig. 4). Untreated cells produced guanine at a rate of 8.1 ± 1.3 and adenine at a rate of 21.1 ± 0.5 pmol/nmol pre-existing base/hr giving a 1:2.6 ratio for newly synthesised guanine and adenine. Exposure to 10 nM MTX produced an apparent increase in the mean levels of both guanine (not significant, $P = 0.239$) and adenine (significant, $P = 0.011$), to approximately 120% of control values. However, as the concentration of MTX was increased further, *de novo* purine synthesis was significantly (guanine, $P = 0.002$; adenine, $P < 0.0001$) inhibited to 25–30% of control levels at 100 nM MTX, with the guanine to adenine synthesis ratio increasing slightly to 1:3.4. Increasing the concentration of MTX still further to 1000 nM produced complete inhibition of *de novo* purine synthesis (limit of detection 0.3 pmol new bases/nmol pre-existing bases/hr). *In situ* TS activity and *de novo* purine synthesis in a childhood ALL lymphoblast sample isolated at presentation from a 16-year-old male patient with diploid ALL was also determined in relation to increasing concentrations of MTX, as shown in Fig. 5. Control, untreated TS activity was 7050 dpm/10^6 cells/hr and *de novo* purine synthesis was 1.54 pmol/nmol of pre-existing adenine/hr, rates both considerably lower than in CCRF-CEM cells. Following exposure to 1000 nM MTX for 22 hr, TS activity and *de novo* purine biosynthesis were inhibited by 70% and 60%, respectively. These data on a clinical sample are consistent with the data from CCRF-CEM cells, and confirm the utility of the assay.

DISCUSSION

The aim of this study was to develop an assay that could be used to measure both *de novo* thymidylate and purine biosynthesis simultaneously, in the same sample of cells. The studies performed were mainly concerned with assay validation and reproducibility and, as methodology for estimating the rate of *de novo* purine synthesis had already been published [11], studies focused on integrating the assay for the estimation of *in situ* TS activity. Investigations into *in situ* TS activity with respect to the time of incubation

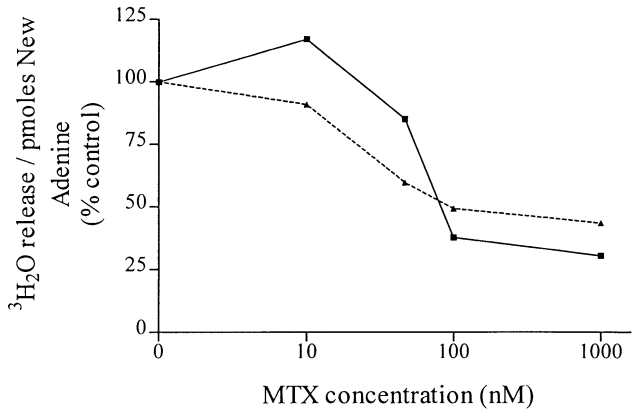

FIG. 5. *In situ* thymidylate synthase activity (—■—) and *de novo* purine synthesis (–▲–) in an ALL lymphoblast sample following incubation with MTX for 22 hr at the concentrations shown. Each point represents either the mean of triplicate (thymidylate synthase) or single (*de novo* purine synthesis) activity determinations in a single experiment expressed as a percentage of control (untreated) activity. The sample was taken from a patient with common diploid ALL at diagnosis.

and [^{3}H]deoxyuridine concentration demonstrated that ^3H_2O release was linear over a 2-hr time period, and that with CCRF-CEM cells at the cell density used, 0.3 μM [^{3}H]deoxyuridine was not limiting (Fig. 2). Comparison of *in situ* thymidylate synthase and *de novo* purine synthesis activities, when measured separately or together, showed there was no difference in the results obtained. Application of the combined assay to study the effects of a 22-hr exposure to MTX on *in situ* thymidylate synthase activity and *de novo* purine synthesis, prior to the 2-hr assay period, necessitated supplementation of the medium during the 22-hr MTX incubation period with thymidine and inosine. Supplementation was necessary in order to prevent the cytotoxic action of the MTX influencing the results, for example, through induction of apoptosis and subsequent cell and enzyme degradation. However, comparison of activities in cells incubated in dialysed medium or dialysed medium supplemented with thymidine and inosine for 22 hr showed no significant differences in control levels of TS ($P = 0.18$) or *de novo* purine synthesis activities ($P = 0.99$).

Having developed the assay, the reproducibility of measuring *in situ* TS and *de novo* purine synthesis activities in both untreated control cells and MTX-treated cells was determined. With respect to *in situ* TS activity in CCRF-CEM cells, the assay was found to be highly reproducible and consistent for both untreated (441,894 ± 81,991 dpm/10^6 cells) and MTX-treated cells over three independent experiments; e.g. 50 nM MTX consistently produced 87–95% inhibition of TS activity (45,128 ± 31,059 dpm/10^6 cells) (Fig. 3). It is difficult to compare the data described here with similar studies involving MTX-induced inhibition of *in situ* TS activity due to differences in cell lines used, concentrations of [^{3}H]deoxyuridine, incubation conditions, and the schedule of exposure to MTX. For example, MTX has been shown to inhibit *in situ* TS activity in H-35 hepatoma cells where, after a 2-hr exposure, cells were maintained in drug-free medium for a further 22 hr prior to measuring TS activity. Exposure to 30 nM MTX under these conditions produced no inhibition of TS activity, whereas exposure to 1000 nM MTX caused 90% inhibition [10]. A similar effect was observed in MCF-7 breast cancer cells treated with 1000 nM MTX, which inhibited TS activity by 75%, after a 3–4 hour MTX exposure [16].

Determination of *de novo* purine synthesis in CCRF-CEM cells also gave reproducible results for untreated cells (guanine − 8.1 ± 1.3 and adenine − 21.1 ± 0.5 pmol new bases/nmol pre-existing bases/hr). Data for MTX-treated cells were also reproducible over three independent experiments, with 70–75% inhibition of *de novo* purine synthesis after exposure to 100 nM MTX (guanine −1.9 ± 0.5 and adenine −6.4 ± 1.2 pmol new bases/nmol pre-existing bases/hr) (Fig. 4). The level of inhibition of *de novo* purine synthesis determined here with CCRF-CEM cells is consistent with observations made with MOLT-4 cells, also a T-lymphoblastic cell line, where complete inhibition of *de novo* purine synthesis, as measured by incorporation of [^{14}C]glycine, was observed after exposure to 200 nM MTX for 24 hr [17]. Similarly, in MCF-7 cells, *de novo* purine synthesis was found to be almost completely inhibited by 1000 nM MTX after 5 hr exposure [16].

Comparison of MTX-induced inhibition of both *de novo* thymidylate and purine biosynthesis demonstrated that the extent of inhibition of the two pathways at a given MTX concentration was different. This difference was most evident following 50 nM MTX, where *in situ* TS activity was inhibited by 90% as opposed to *de novo* purine synthesis, which was essentially uninhibited at this MTX concentration. Previous studies with mouse bone marrow have also shown similar preferential inhibition of TS activity, as compared to *de novo* purine synthesis, following exposure to 10 nM MTX [18]. This difference in pathway inhibition may be explained in terms of the relative levels of MTX polyglutamates produced on exposure to different concentrations of MTX, i.e. higher extracellular MTX concentrations may be required to generate MTX polyglutamate levels (particularly long chain metabolites) capable of directly inhibiting *de novo* purine synthesis [6, 11]. The utility of the assay was demonstrated using lymphoblasts isolated from a child with ALL which were also incubated for 22 hr with MTX and subsequently assayed for *in situ* TS activity and *de novo* purine synthesis. Control, untreated TS activity, and *de novo* purine synthesis were distinctly lower than in CCRF-CEM cells, although the overall pattern of MTX-induced inhibition was similar (Fig. 5).

In summary, the results presented in this paper describe the development and initial application of a whole cell assay for simultaneously measuring MTX-induced inhibition of thymidylate synthase and *de novo* purine synthesis. Potential applications of this assay include further investigations into the cellular pharmacology of MTX and other

antifolates in both cell lines and lymphoblast samples from patients with ALL. In addition, the assay could be used to investigate the impact of various well-defined resistance mechanisms on the cellular pharmacology of such compounds. Lastly, application of the assay to samples from individual leukaemia patients, treated *ex vivo* with a range of antifolate drugs, may allow the selection of the most appropriate agent for each patient.

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References

1. Jackman A, Taylor G, Gibson W, Kimbell R, Brown M, Calvert A, Judson I and Hughes L, ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumour cell growth *in vitro* and *in vivo*: A new agent for clinical study. *Cancer Res* **51:** 5579–5586, 1991.
2. Pizzorno G, Moroson B, Casmore A and Beardsley G, (6*R*)-5,10-Dideaza-5,6,7,8-tetrahydrofolic acid effects on nucleotide metabolism in CCRF-CEM human T-lymphoblast leukemia cells. *Cancer Res* **51:** 2291–2295, 1991.
3. Jonsson O and Kamen B, Methotrexate and childhood leukaemia. *Cancer Invest* **9:** 53–60, 1991.
4. Osborn M, Freeman M and Huennekens F, Inhibition of dihydrofolate reductase by aminopterin and amethopterin. *Proc Soc Exp Biol Med* **97:** 429–431, 1958.
5. Allegra C, Chabner B, Drake J, Lutz R, Rodbard D and Jolivet J, Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. *J Biol Chem* **260:** 9720–9726, 1985.
6. Allegra C, Drake J, Jolivet J and Chabner B, Inhibition of phosphoribosylaminoimadazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc Natl Acad Sci U S A* **82:** 4881–4885, 1985.
7. Gorlick R, Goker E, Trippett T, Waltham M, Banerjee D and Bertino J, Intrinsic and acquired resistance to methotrexate in acute leukemia. *New Engl J Med* **335:** 1041–1048, 1996.
8. Goker E, Waltham M, Kheradpour A, Trippett T, Mazumdar M, Elisseyeff Y, Schnieders B, Steinherz P, Tan C, Berman E and Bertino J, Amplification of the dihydrofolate reductase gene is a mechanism of acquired resistance to methotrexate in patients with acute lymphoblastic leukemia and is correlated with *p*53 gene mutations. *Blood* **86:** 677–684, 1995.
9. Whitehead V, Rosenblatt D, Vuchich M, Shuster J, Witte A and Beaulieu D, Accumulation of methotrexate and methotrexate polyglutamates in lymphoblasts at diagnosis of childhood acute lymphoblastic leukemia: A pilot prognostic factor analysis. *Blood* **76:** 44–49, 1990.
10. Galivan J, Evidence for the cytotoxic activity of polyglutamate derivatives of methotrexate. *Mol Pharmacol* **17:** 105–110, 1980.
11. Masson E, Relling M, Synold T, Liu Q, Schuetz J, Sandlund J, Pui C and Evans W, Accumulation of methotrexate polyglutamates in lymphoblasts is a determinant of antileukemic effects *in vivo*. A rationale for high-dose methotrexate. *J Clin Invest* **97:** 73–80, 1996.
12. Taylor G, Jackman A, Balmanno K, Hughes L and Calvert A, Estimation of the *in vitro* and *in vivo* inhibitory effects of antifolates upon thymidylate synthase (TS) in whole cells. In: *Purine Metabolism in Man VI* (Eds. Mikanagnagi K, Nifhioka K and Kelley WN), pp. 383–388. Plenum Press, New York & London, 1988.
13. Tomich P, Chiu C, Wovcha M and Greenberg G, Evidence for a complex regulating the *in vivo* activities of early enzymes induced by bacteriophage T4. *J Biol Chem* **249:** 7613–7622, 1974.
14. Rode W, Scanlon K, Moroson B and Bertino J, Regulation of thymidylate synthase in mouse leukemia cells (L1210). *J Biol Chem* **255:** 1305–1311, 1980.
15. Matherly L, Barlowe C, Phillips V and Goldman I, The effects on 4-aminoantifolates on 5-formyltetrahydrofolate metabolism in L1210 cells. *J Biol Chem* **262:** 710–717, 1987.
16. Allegra C, Fine R, Drake J and Chabner B, The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells. *J Biol Chem* **261:** 6478–6485, 1986.
17. Bokkerink J, Bakker M, Hulscher T, De Abreu R and Schretlen E, Purine *de novo* synthesis as the basis of synergism of methotrexate and 6-mercaptopurine in human malignant lymphoblasts of different lineages. *Biochem Pharmacol* **37:** 2321–2327, 1988.
18. Zaharko D, Fung W and Yang K, Relative biochemical aspects of low and high doses of methotrexate in mice. *Cancer Res* **37:** 1602–1607, 1977.