Biochemical Pharmacology, Vol. 54, pp. 635–638, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00259-1

SHORT COMMUNICATION

Measurement of Deoxyuridine Triphosphate and Thymidine Triphosphate in the Extracts of Thymidylate Synthase-Inhibited Cells Using a Modified DNA Polymerase Assay

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ABSTRACT. New inhibitors of the enzyme thymidylate synthase (TS) are now reaching clinical application. Alteration of the dUTP: dTTP ratio may be critical to TS inhibition-induced tumor cell death. The DNA polymerase assay with modification was used to rapidly and sensitively measure dUTP, dTTP, and dUTP:dTTP ratios in cell extracts of HT29 human colon carcinoma cells treated with the specific TS inhibitor ZD1694 [*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid]. These results revealed an increase in the dUTP:dTTP ratio at 2 hr after a 2-hr exposure to ZD1694 at concentrations of 0.05 to 0.2 μM with significant normalization at 16 hr after a 2-hr exposure despite evidence of continued TS inhibition. This assay is highly sensitive and reproducible for levels of dUTP and is less labor intensive than traditional assays. BIOCHEM PHARMACOL **54**;5:635–638, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. deoxynucleotides; thymidylate synthase; DNA polymerase; antifolate; colon cancer

Quinazoline-based TS§ inhibitors are currently being investigated in clinical trials. Inhibition of TS leads to depletion of its end-product, TMP, and ultimately to depletion of dTTP. In addition, the substrate for TS, dUMP, accumulates. Phosphorylation of dUMP leads to the production of dUTP. It has been postulated that this increase in the dUTP:dTTP ratio may be important for the induction of cytotoxicity by TS inhibitors [1, 2].

Several methods are available for the measurement of dUTP:dTTP ratios in cell extracts. Both high-performance liquid chromatography [3, 4] and radioimmunoassay [5] have been used to measure dUTP and dTTP. However, the requirement for column chromatography with both of these methods makes it difficult to run large numbers of samples simultaneously. The DNA polymerase method can conveniently assay large numbers of samples simultaneously, but, as originally published, was neither sensitive nor specific enough for this purpose [6]. Recent modifications of the DNA polymerase assay addressed each of these issues. The sensitivity of this assay has been improved significantly by the use of synthesized oligonucleotides [7]. These oligonucleotides have been further modified, permitting measurement of deoxynucleotides in the picomolar range [8]. Furthermore, the specificity for dUTP was improved through the use of a dUTPase predigestion [9]. We have combined these two modifications to produce an assay for dUTP and dTTP that was both sensitive and specific.

This modified DNA polymerase assay was used to measure dUTP and dTTP in standards, mixtures of dUTP and dTTP, and in the extracts of HT29 human colon adenocarcinoma cells whose TS was specifically inhibited by ZD1694.

MATERIALS AND METHODS

Drugs and Reagents

Human dUTPase was purified as described [10]. ZD1694 was provided by Zeneca Pharmaceuticals (Wilmington, DE). DNA polymerase I, large (Klenow) fragment (exo^-) was obtained from New England Biolabs (Beverly, MA). Microcon-3 microconcentrators were purchased from Amicon (Beverly, MA). Synthesized oligonucleotides were obtained from Genset (La Jolla, CA). dUTP and dTTP were obtained from Pharmacia (Uppsala, Sweden). Deoxyadenosine 5′-triphosphate[2,8-^{3}H] tetraammonium salt (sp. act. 32 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). DE81 anion exchange papers were purchased from Whatman (Hillsboro, OR). Culture plates were ob-

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§ *Abbreviations:* TS, thymidylate synthase; ZD1694, *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid; dUTPase, deoxyuridine triphosphate nucleotidohydrolase; FBS, fetal bovine serum; dFBS, dialyzed fetal bovine serum; DFM, drug-free medium; and dNTP, deoxynucleotide triphosphate.

Received 11 March 1997; accepted 14 May 1997.

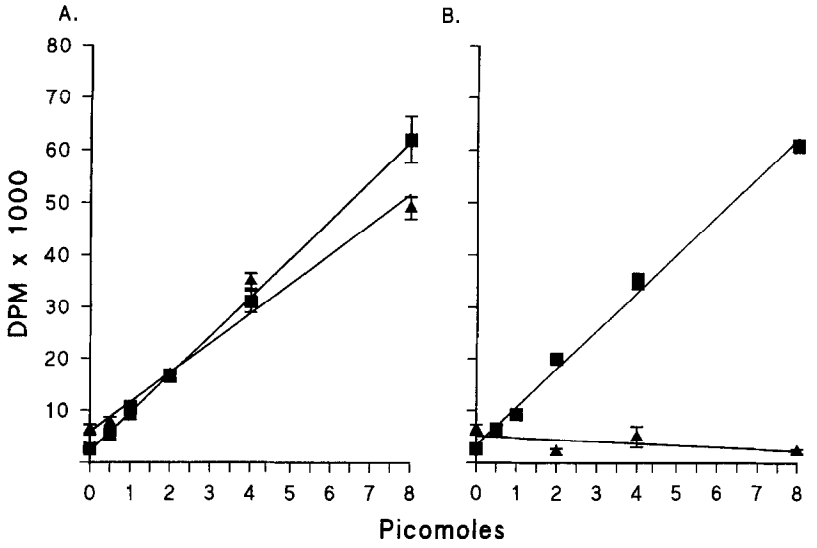

FIG. 1. Standard curves for the modified DNA polymerase assay, demonstrating a linear relationship between radionucleotide incorporation represented by disintegrations per minute (dpm) × 1000 and deoxynucleotide quantity in picomoles. (A) dTTP standard curve from 0.5 to 8 pmol without (■, $r^2 = 0.99$, $P < 0.001$) and with (▲, $r^2 = 0.96$, $P < 0.001$) dUTPase predigestion. (B) dUTP standard curve from 0.5 to 8 pmol without (■, $r^2 = 0.99$, $P < 0.001$) and with (▲) dUTPase predigestion. Points represent the mean of 6 observations with error bars representing the SEM.

tained from Becton Dickinson (Lincoln Park, NJ). Cell culture media and medium supplements were obtained from GIBCO (Grand Island, NY). All other chemicals were from Sigma (St. Louis, MO).

Cell Culture and Extraction

HT29 human adenocarcinoma cells were maintained in RPMI 1640 with 10% FBS and 1% penicillin–streptomycin. Cells were seeded onto six-well tissue culture plates at an approximate density of 1.25×10^5/well. After 72 hr, the medium was changed to folate-free RPMI 1640, 10% dFBS with 80 nM 5-methyltetrahydrofolate, 1% penicillin–streptomycin. The cells were treated with ZD1694 at final concentrations equivalent to 0.05, 0.1, and 0.2 μM for 2 hr. Then the plates were washed with dFBS-containing medium and supplemented with additional dFBS-containing medium. The cells were maintained in DFM for either 2 or 16 hr. Then the medium was poured off, and the cells were extracted with 400 μL of cold 60% methanol for 1 hr at 4°. The extracts were ultracentrifuged in microcon-3 microconcentrators (3000 mol. wt cutoff) to remove the precipitated protein and DNA. Then the extract from each sample was divided into paired aliquots of 50–100 μL and lyophilized to remove the methanol.

dUTPase Predigestion

The dUTPase predigestion was modified from that of Williams *et al.* [9] as follows. To one of each of the paired aliquots was added 20 ng dUTPase in 40 μL of buffer containing 34 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.25 mg/mL BSA. Samples were incubated for 20 min at 37°; 60 μL of 100% methanol was added to produce a final methanol concentration of 60%. Precipitated protein was removed by centrifugation, and the samples were again lyophilized to remove the methanol.

DNA Polymerase Assay

The DNA polymerase assay, modified from that of Sherman and Fyfe [7], was employed. The template sequence employed was:

5′TTT ATT TAT TTA TTT ATT TAG
GCG GTG GAG GCG G-3′

The primer sequence was:

5′-CCG CCT CCA CCG CC-3′

To each sample was added 0.3 U of DNA polymerase I, large (Klenow) fragment (exo^-) in 100 μL of buffer containing 34 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.25 mg/mL BSA, 0.01 μCi/μL deoxyadenosine triphosphate[2,8-^{3}H], and 32 nM oligonucleotide (template + primer). The mixture was incubated for 45 min at 42°. Then 30 μL of each sample was blotted onto a DE81 filter. The filters were washed for 5 min with 1% sodium pyrophosphate/5% trichloroacetic acid six times and dried, and the radioactivity was counted by liquid scintigraphy.

Data Analysis

Standard curves were obtained by assaying serial dilutions of dUTP and dTTP, both with and without dUTPase pretreatment. dUTP controls with dUTPase pretreatment were also included to document complete dUTP digestion. dTTP concentrations were determined by comparing the results of the dUTPase-treated aliquot to a standard curve for dTTP treated with dUTPase. dUTP concentrations were determined by subtracting the counts attributable to dTTP from the results of the aliquot not treated with dUTPase (dTTP + dUTP). Samples for standard curve and experimental samples were assayed in triplicate, and the experiment was performed twice. Mixtures of dTTP and

TABLE 1. dUTP and dTTP concentrations in the extracts of HT29 cells 2 hr after treatment with ZD1694 for 2 hr

ZD1694 (μM)	dNTP	pmol/10^6 cells	dUTP:dTTP (range)
0	dTTP	92 ± 10*	
	dUTP	4.8 ± 3	0.05 (0.02–0.1)
0.05	dTTP	8.8 ± 1	
	dUTP	38 ± 5	4 (3–6)
0.1	dTTP	4.8 ± 0.4	
	dUTP	40 ± 5	8 (7–10)
0.2	dTTP	3.6 ± 0.4	
	dUTP	37 ± 7	10 (8–13)

* Mean ± SEM, N = 6.

dUTP were assayed four times in triplicate. Results are expressed as means ± SEM.

RESULTS AND DISCUSSION

This modified DNA polymerase assay accurately measured dTTP and dUTP in standards. Standard curves for dUTP and dTTP were linear from 0.5 to 8.0 pmol with an r^2 value of >0.95 (Fig. 1). dUTPase predigestion did not result in a significant change in the values for dTTP ($P > 0.05$) (Fig. 1A). dUTPase pretreatment did result in digestion of dUTP to levels at or below background (Fig. 1B). A mixture of dUTP (1 pmol) and dTTP (1 pmol) was found to contain 1.3 ± 0.2 (mean ± SEM) pmol of dUTP and 1.0 ± 0.2 pmol of dTTP with this assay.

For cell extracts, treatment with ZD1694 led to an increase in the dUTP:dTTP ratio at all concentrations and time points (Tables 1 and 2). This increase was more striking at 2 hr after a 2-hr exposure (2 hr + 2 hr DFM) than at 16 hr after a 2-hr exposure (2 hr + 16 hr DFM), although the dUTP:dTTP ratio was still elevated markedly at 16 hr. This drop in the dUTP:dTTP ratio from 2 hr + 2 hr DFM to 2 hr + 16 hr DFM occurred despite continued TS inhibition, based on continued dTTP depletion. Of note, a significant difference in baseline dTTP levels was observed between the untreated controls from the 2 hr + 2 hr DFM and 2 hr + 16 hr DFM samples ($P = 0.02$). The reason for this difference is unclear, but may have been due to a difference in cell cycle kinetics at the time of harvest.

TABLE 2. dUTP and dTTP concentrations in the extracts of HT29 cells 16 hr after treatment with ZD1694 for 2 hr

ZD1694 (μM)	dNTP	pmol/10^6 cells	dUTP:dTTP (range)
0	dTTP	57 ± 4*	
	dUTP	4.0 ± 2	0.07 (0.03–0.1)
0.05	dTTP	17 ± 1	
	dUTP	11 ± 2	0.6 (0.5–0.8)
0.1	dTTP	8.4 ± 0.8	
	dUTP	8.8 ± 1	1 (0.8–1.3)
0.2	dTTP	7.6 ± 1	
	dUTP	6.0 ± 1	0.8 (0.5–1.1)

* Mean ± SEM, N = 6.

The DNA polymerase assay with appropriate modifications has been used to measure dNTPs with a lower limit of detection of about 1 pmol (reviewed in Ref. 11). Improvements to this method included the use of copolymers [11] and, subsequently, synthetic oligonucleotides, which allowed reproducible measurements to 0.2 pmol with sensitivities in cell extracts comparable to HPLC [7]. Measurements of dUTP levels using the DNA polymerase assay are more problematic because of low levels in cell extracts and because of cross-reactivity with dTTP. This problem was partially resolved with the use of dUTPase digestion; however, sensitivities for dUTP were still in the range of about 10 pmol [9]. Combining synthetic oligonucleotides with dUTPase digestion, however, increased the sensitivity to 0.5 or 1 pmol in cell extracts. This is comparable to that achieved with a radioimmunoassay [5], but is less labor intensive, allowing for the assay of large numbers of samples simultaneously.

These results also confirm that TS inhibition with the folate analogue ZD1694 does lead to an increase in the dUTP:dTTP ratio. Although the dUTP:dTTP ratio was increased at all drug concentrations and time points, the increase was greater at 2 hr after a 2-hr ZD1694 exposure than at 16 hr after the same 2-hr drug exposure. This drop in the dUTP:dTTP ratio to or below 1:1 occurred despite continued TS inhibition as evidenced by continued dTTP depletion. Therefore, dUTP levels were not being maintained, possibly due either to decreased activity of the enzymes responsible for production such as deoxycytidylate deaminase, ribonucleotide reductase, and thymidine kinase, or to increased activity of the enzyme responsible for catabolism, dUTPase. We hope to elucidate the mechanism of these changes in the dUTP:dTTP ratio after ZD1694-induced TS inhibition in future studies.

This work was supported, in part, by Cancer Center Support Grant CA13330 from the National Cancer Institute. We thank Dr. Wynne Aherne for helpful discussions.

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