Biochemical Pharmacology, Vol. 42, No. 2, pp. 431–437, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INHIBITION OF CELLULAR THYMIDYLATE SYNTHESIS BY CYTOTOXIC PROPENAL DERIVATIVES OF PYRIMIDINE BASES AND DEOXYNUCLEOSIDES

THOMAS I. KALMAN,*† EDMUND R. MARINELLI,‡§ BIN XU,‡‖ A. R. VENUGOPALA REDDY,* FRANCIS JOHNSON‡ and ARTHUR P. GROLLMAN‡

*Departments of Medicinal Chemistry and Biochemical Pharmacology, State University of New York at Buffalo, NY 14260; and ‡Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY 11794, U.S.A.

(*Received* 26 *September* 1990; *accepted* 25 *February* 1991)

Abstract—A series of cytotoxic propenal (3-oxoprop-1-enyl) derivatives of pyrimidine bases and deoxynucleosides was evaluated for their ability to block thymidylate synthesis in intact and permeabilized murine leukemia L1210 cells. Several were potent inhibitors of this process, likely contributing to their cytotoxicity. The IC_{50} values of thymidine-3-propenal, the prototype of this series, in intact and permeabilized L1210, L-M and L-M(TK$^-$) cells were 21, 7.5, and 75 μM and 1.5, 1.7, and 3.5 μM, respectively. The related base analogue, thymine-1-propenal, is a product of bleomycin-induced DNA strand-scission; the results of the present study bear on the mode of action of this antibiotic.

The primary reaction products released during bleomycin-induced DNA strand-scission have been identified as *N*-propenal substituted purines and pyrimidines [1], the thymine derivative predominating. These propenals are chemically reactive and cytotoxic to mammalian cells in culture [2]. A series of structurally related nucleoside derivatives was prepared as potential chemotherapeutic agents and a prototype of this group, thymidine-3-propenal (P-dThd¶), was shown to be cytotoxic to several mammalian cell lines and active against L1210 leukemia *in vivo* [3]. The ability of P-dThd to inhibit selectively DNA synthesis in HeLa cells, as measured by precursor incorporation into cellular macromolecules, correlated with inhibition of dThd kinase [2], demonstrating that P-dThd blocks the salvage pathway of DNA-thymine biosynthesis. However, neither this effect nor the weak DNA polymerase α inhibitory activity [2] accounts adequately for the profound cytotoxicity observed. The cytotoxicity and biological activity of the corresponding propenal derivative of dUrd [3] contrasts with the greatly reduced activities of the propenal derivatives of thymine arabinoside and riboside, suggesting that dTMP synthase (EC 2.1.1.45), a key enzyme of *de novo* DNA-thymine biosynthesis, may be a target for P-dThd and its analogues.

The evaluation of the propenal derivatives as inhibitors of dTMP synthase is complicated by their reactivity toward thiols [3]. Purified dTMP synthase cannot be assayed reliably without exposure to 0.02 to 0.1 M 2-mercaptoethanol or other thiols. Our recently reported assay for dTMP synthase [4] permits cellular activity of this enzyme to be measured in the absence of added thiols, thus providing an experimental system for monitoring *in situ* inhibition of dTMP synthesis by propenal derivatives. In this system, effects on cellular uptake and phosphorylation of precursor nucleosides cannot be separated from effects on *de novo* dTMP synthesis. The additional use of permeabilized cells [5, 6] and thymidine kinase-less mutant strains can overcome this limitation.

In this paper we describe the effects of P-dThd and related propenal derivatives on thymidylate synthesis both in intact and permeabilized murine lymphocytic leukemia L1210 cells and in two cell lines L-M and L-M(TK$^-$), derived from mouse connective tissue. P-dThd is a novel nucleoside antimetabolite capable of blocking both the salvage and *de novo* pathways of dTMP biosynthesis. Our studies suggest that dTMP synthase may be a cellular target of P-dThd and related propenal derivatives; inhibition of this enzyme could contribute significantly to their cytotoxicity.

MATERIALS AND METHODS

Materials. [5-^{3}H]dCyd (22 Ci/mmol) and [5-^{3}H]dUrd (25 Ci/mmol) were purchased from ICN; [2-^{3}H]Hyp (22 Ci/mmol) was from Moravek Biochemicals and [5-^{3}H]dUMP (10.9 Ci/mmol) from Amersham. Dextran sulfate 500 (sodium salt) was

† Correspondence: Dr. Thomas I. Kalman, Department of Medicinal Chemistry, State University of New York at Buffalo, 457 Cooke Hall, Buffalo, NY 14260.

§ Present address: The Squibb Institute for Medical Research, 1 Squibb Drive, P.O. Box 191, New Brunswick, NJ 08903.

‖ Present address: Shanghai Institute of Materia Medica, Academia Sinica, 319 Yue-Yang Road, Shanghai 20031, China.

¶ Abbreviations: dCyd, 2′-deoxycytidine; dThd, thymidine; dTMP, thymidylate; dUMP, 2′-deoxyuridine 5′-phosphate (deoxyuridylate); dUrd, 2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; FU, 5-fluorouracil; Hyp, hypoxanthine; IMP, inosine 5′-phosphate; and P-dThd, 3-(3-oxoprop-1-enyl)thymidine.

purchased from Pharmacia. All other chemicals were obtained from the Aldrich or the Sigma Chemical Co. Media and other tissue culture supplies were from GIBCO Laboratories.

Chemistry. Proton NMR spectra were measured on a Varian HFT-80 spectrometer in $CDCl_3$ or Me_2SO-d_6 using Me_4Si as the internal standard. UV spectra were recorded on a Perkin-Elmer Lambda 5 UV/visible spectrophotometer. Mass spectra were obtained using a Kratos MS-30 high resolution instrument. TLC analyses were performed using Analtech 250 μm analytical silica gel GF plates with solvent systems A: CH_2Cl_2/EtOAc, 4/1; B: CH_2Cl_2/EtOAc, 9/1; C: CH_2Cl_2/EtOAc, 1.5/1; D: $CHCl_3$/tetrahydrofuran, 4/1; E: EtOAc/CH_3OH, 95/5; and F: EtOAc/tetrahydrofuran, 1/1. The syntheses of 5-fluoro-3-(3-oxoprop-1-enyl)-2′-deoxyuridine, the 3-propenal derivative of FdUrd; 3-methyl-1-(3-oxoprop-1-enyl)thymine, the 1-propenal derivative of 3-methylthymine; 1-methyl-3-(3-oxoprop-1-enyl)uracil, the 3-propenal derivative of 1-methyluracil; and 3-methyl-1-(3-oxoprop-1-enyl)-uracil, the 1-propenal derivative of 3-methyluracil are described below. Other analogues were prepared as previously described [3]. Drug solutions were prepared freshly before experiments.

3-Methyl-1-(3-oxoprop-1-enyl)thymine. A 25-mL round bottom flask equipped with a septum-capped inlet and magnetic stirrer was charged with freshy prepared sodium ethoxide (1.2 mmol), 2.0 mL dry ethanol, 4.0 mL dry dimethylformamide (DMF) and 0.280 g (2.0 mmol) 3-methylthymine [7]. The mixture was stirred for 1 hr at room temperature, and then 1.0 mL dry ethanol and 2.0 mL DMF were added. The flask was cooled to −45° and 215 mg (4.0 mmol) propargylaldehyde was added. The mixture was stirred for 1 hr at −45°, then allowed to warm to 25°, and the reaction was quenched with excess aqueous NH_4Cl solution. Volatile components were removed *in vacuo* and the residue was partitioned between water and ethyl acetate. The aqueous layer was extracted twice with ethyl acetate and the organic layers were combined and dried over $MgSO_4$. Evaporation yielded 140 mg (36%) of crude white solid. This material was purified by silica gel flash chromatography using a CH_2Cl_2/EtOAc mixture as the eluant to yield 50 mg (12.8 %) of the desired product as a white solid: m.p. 173–175°; TLC, homogenous in solvent systems C and D; ^{1}H-NMR ($CDCl_3$) δ 2.04 (d, J = 0.8 Hz, 3 H, 5-CH_3), 3.41 (s, 3 H, N-CH_3), 6.13 (dd, J_1 = 14.4 Hz, J_2 = 7.5 Hz, 1 H, CHCHO), 7.32 (br s, 1 H, 6-H), 8.19 (d, J = 14.4 Hz, 1 H, CH=CHCHO), 9.61 (d, J = 7.5 Hz, 1 H, CHO); UV (1% DMF), λ_{max} 286, ε_{max} 2.7 × 10^4. Calcd. for $C_8H_{10}N_2O_3$: m/e 194.0691. Found: m/e 194.0698 [M^+].

3-Methyl-1-(3-oxoprop-1-enyl)uracil. This compound was prepared by the method described above. Yield 8.7%; m.p. 181–183°; TLC, homogenous in solvent systems A and D; ^{1}H-NMR (Me_2SO-d_6) δ 3.22 (s, 1 H, CH_3), 5.99 (d, J = 8.2 Hz, 1 H, 5-H), 6.54 (dd, J_1 = 14.6 Hz, J_2 = 7.5 Hz, 1 H, CHCHO), 8.15 (d, J = 14.5 Hz, 1 H, CHCHO, overlaps with 6-H), 8.25 (d, J = 7.5 Hz, 1 H, 6-H, overlaps with CH=CHCHO), 9.61 (d, J = 7.5 Hz, 1 H, CHO); UV (1% DMF) λ_{max} 295, ε_{max} 2.8 × 10^4. Calcd for $C_8H_8N_2O_3$: m/e 180.0535. Found: m/e 180.0544 [M^+].

1-Methyl-3-(3-oxoprop-1-enyl)uracil. This compound was also prepared by the method described above. Yield 17.2%; m.p. 145.5–147.0°; TLC, homogenous in solvent systems B and D; ^{1}H-NMR ($CDCl_3$) δ 3.45 (s, 3 H, CH_3), 5.79 (d, J = 8.0 Hz, 1 H, H-5), 7.20 (d, J = 8.0 Hz, 1 H, H-6), 7.30 (dd, J_1 = 14 Hz, J_1 = 14 Hz, J_2 = 7.7 Hz, 1 H, CHCHO), 8.14 (d, J = 14.9 Hz, 1 H, CH=CHCHO), 9.60 (d, J = 7.7 Hz, 1 H, CHO); UV (1% DMF) λ_{max} 268, ε_{max} 1.7 × 10^4. Calcd for $C_8H_8N_2O_3$ − CHO: m/e 151.0508. Found: m/e 151.0494 [M^+ − CHO].

5-Fluoro-3-(3-oxoprop-1-enyl)-2′-deoxyuridine. This analogue was prepared essentially as described for P-dThd [3]: yield 9.8%; pale yellow glass; TLC, homogenous in solvent systems E and F; ^{1}H-NMR (Me_2SO-d_6) δ 2.20 (t, J = 6.0 Hz, 2 H, CH_2-2′), 3.66 (m, 2 H, CH_2-5′), 3.87 (q, J = 6 Hz, 1 H, H-4′), 4.27 (m, 1 H, H-3′), 5.20 (m, 2 H, OH-3′ and OH-5′), 6.17 (dt, J_1 = 5.0 Hz, J_2 = 1.6 Hz, 1 H, H-1′), 7.05 (dd, J_1 = 14.4 Hz, J_2 = 7.0 Hz, 1 H, CHCHO), 8.09 (d, J = 14.6 Hz, 1 H, CH=CHCHO), 8.45 (d, $J_{H\text{-}F}$ = 6.9 Hz, 1 H, H-6), 9.63 (d, J = 7.7 Hz, 1 H, CHO).

Transplantation, maintenance and harvest of L1210 cells. The murine leukemia L1210 cell line was maintained by weekly passage of 10^5 cells into 6- to 9-week old DBA/2HA mice. On day 6 following transplantation, three or more recipient mice were killed by cervical dislocation an the cells were recovered by peritoneal lavage using ice-cold isotonic buffer. Following centrifugation, the cell pellet was resuspended in hypotonic buffer to lyse erythrocytes, after which 10 mL of Eagle's BME culture medium containing streptomycin and penicillin was added. Cells were washed twice by centrifugation and the density was adjusted to 2.5 × 10^7 cells/mL. Cell viability was measured by dye-exclusion.

Cell culture techniques. Leukema L1210 cells were cultivated in RPMI 1640 medium supplemented with 20 mM Hepes and 10% heat-inactivated fetal calf bovine serum. Mouse L-M and L-M(TK$^-$) cell lines were obtained from the American Type Culture Collection. The L-M(TK$^-$) strain is a mutant made resistant to 5-bromo-2′-deoxyuridine and lacking dThd kinase activity. Both cell lines were maintained in suspension in minimal essential medium supplemented with 10% fetal bovine serum, and harvested during log phase.

Cellular enzyme assays. The activity of cellular dTMP synthase was determined *in situ* as previously described [4]. Briefly, 200-μL aliquots of cell suspension (2–5 × 10^7 cells/mL) were placed in tubes and incubated for 40 min in a shaking bath at 37° with or without the test compounds. Tritium-labeled nucleosides, [5-^{3}H]dCyd or [5-^{3}H]dUrd, were added and the incubation was continued for 15 min. The reaction was terminated by transferring 100-μL aliquots of the reaction mixture into centrifuge tubes containing 100 μL of a 15% mixture of activated charcoal suspension in 4% aqueous perchloric acid. After mixing, the tubes were centrifuged in a Beckman microfuge. Aliquots (40–100 μL) of the supernatant were removed and the radioactivity of

Table 1. Inhibition of dTMP synthase activity in intact and permeabilized L1210 cells

Compound	Substitutents on uracil			Inhibition of tritium release*, IC_{50} (μM)		
				Intact		Permeabilized
	N-1	C-5	N-3	[5-^{3}H]dCyd	[5-^{3}H]dUrd	[5-^{3}H]dUMP
Uracil-1-propenal	CH=CHCHO	H	H	90	80	4.2
3-Methyluracil-1-propenal	CH=CHCHO	H	CH_3	70	20	9.0
Thymine-1-propenal	CH=CHCHO	CH_3	H	160	150	6.1
3-Methylthymine-1-propenal	CH=CHCHO	CH_3	CH_3	90	80	18
5-Fluorouracil-1-propenal	CH=CHCHO	F	H	90	25	ND†
Thymine-1-propenal	CH=CHCH$_2$OH	CH_3	H	>1000	>1000	>1000
1-Methyluracil-3-propenal	CH_3	H	CH=CHCHO	120	20	6.0
Deoxyuridine-3-propenal	2-Deoxyribosyl	H	CH=CHCHO	6.6	3.7	0.36
Thymidine-3-propenal (P-dThd)	2-Deoxyribosyl	CH_3	CH=CHCHO	21	2.1	1.5
5-Fluorodeoxyuridine-3-propenal	2-Deoxyribosyl	F	CH=CHCHO	0.009	0.008	ND
Adenine-9-propenal				80	20	34

* Measured as described in Materials and Methods. Exposure time was 40 min for intact cells and 45 min for permeabilized cells followed by incubation for 15 and 30 min, respectively. Results are the means of two or more determinations which varied by less than 20%.

† Not determined.

tritiated water was measured by liquid scintillation procedures. Counting efficiency for tritium was 35–40%. The amount of tritium released into water was calculated as the percentage of the total amount of radioactivity added. Inhibition of dTMP synthase activity was expressed as the fraction of tritium released relative to control experiments. IC_{50} values were determined from semi-log plots of the concentration–response curve. Results represent the average of two to five separate determinations.

Release of tritium from [2-^{3}H]Hyp, a measure of intracellular IMP dehydrogenase activity and its inhibition [8], was determined in the presence of 5 μM allopurinol by a procedure identical to that described above, with the exception that [2-^{3}H]Hyp was incubated with the cells in place of [5-^{3}H]dCyd or [5-^{3}H]dUrd for 60 min instead of 15 min.

dTMP synthase activity was measured in suspensions of permeabilized cells [5]. Partial permeabilization [6] was achieved by incubating 10^8 cells/mL at 4° for 20 min, in 50 mM Tris-Cl, pH 7.4, buffer containing 93 mM NaCl, 2 mM $MgCl_2$ and 400 μg/mL dextran sulfate, followed by centrifugation, washing and resuspension of the pellet. After incubation of a 100-μL cell suspension (2–5 × 10^7 cells/mL) in the presence or absence of 25 μL drug solution for 45 min, 100 μL of dTMP synthase assay mixture (0.4 mM *d,l*-L-tetrahydrofolate, 7.5 mM formaldehyde, 200 mM 2-mercaptoethanol, 40 mM $MgCl_2$, 0.6 mM EDTA, 100 mM NaF and 80 mM Tris-acetate buffer, pH 7.4) and 25 μL of [5-^{3}H]dUMP (final concentration = 1 μM) were added. Following incubation at 37° for 30 min, the reaction was terminated and the amount of tritium released, representing the remaining dTMP synthase activity, was determined as described for the intact cell assay [4]. If 2-mercaptoethanol was present when the compounds were added, no inhibition could be observed, presumably due to destruction of the propenal derivatives by the thiol [3, 9].

RESULTS

Inhibition of cellular thymidylate synthesis by various propenal derivatives. All derivatives bearing the propenal side chain at the 1- or 3-position of the pyrimidine ring or at the 9-position of adenine inhibited dTMP synthesis in L1210 cells; the IC_{50} values obtained are shown in Table 1. Nucleosides were more potent than base derivatives. Chemical reduction of the aldehyde moiety abolished inhibitory activity. The relative potency of certain analogues depended on the radioactive substrate used as precursor.

With the exception of adenine-9-propenal, compounds tested were up to 25-fold more inhibitory in the permeabilized cellular enzyme assay using [5-^{3}H]dUMP as substrate. Inhibitory effects of 1- and 3-propenal derivatives of 5-fluorouracil and 5-fluoro-2′-deoxyuridine did not differ significantly from the corresponding unsubstituted fluoropyrimidines (data not shown).

Inhibition of cellular thymidylate synthesis of P-dThd. P-dThd was selected for study to gain further insight into the mechanism by which propenal derivatives inhibit cellular dTMP synthesis. Concentration–response curves for inhibition of dTMP synthesis *in situ* by P-dThd are shown in Fig. 1. P-dThd inhibited release of tritium more effectively from [5-^{3}H]dUrd than from [5-^{3}H]dCyd. In both cases, complete inhibition of tritium release occurred at concentrations of P-dThd approximately 50-fold higher than the respective IC_{50} values. Inhibition of the release of tritium from [2-^{3}H]Hyp, a measure of interference with the IMP dehydrogenase catalyzed reaction [8], was less pronounced reaching 31% at 100 μM P-dThd. Mycophenolic acid, a specific IMP dehydrogenase inhibitor [10] at a 10 μM concentration gave 90% inhibition of tritium release from [2-^{3}H]Hyp, but was without any effect on tritium release from [5-^{3}H]dCyd (data not shown).

In permeabilized L1210 cells, dTMP synthesis

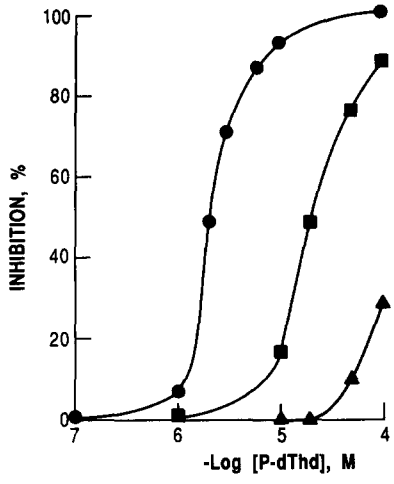

Fig. 1. Concentration-dependent inhibition, by P-dThd, of the release of tritium from [5-^{3}H]dUrd (●), [5-^{3}H]dCyd (■) or [2-^{3}H]Hyp (▲). L1210 leukemia cells were incubated for 40 min at 37° in the absence or presence of various concentrations of P-dThd, followed by further incubation for 15 min in the presence of [5-^{3}H]dUrd or [5-^{3}H]dCyd, or for 60 min in the presence of [2-^{3}H]Hyp. The amount of tritium released into water was quantitated as described in Materials and Methods.

from dUMP was inhibited by P-dThd with an IC_{50} value of 1.5 μM (Table 1). The extent of inhibition increased with exposure time. The ability of dTMP to protect the enzyme against inactivation by P-dThd was investigated (data not shown). Complete protection was achieved with 1 mM dTMP at concentrations of P-Thd which caused up to 25% inhibition of enzyme activity. At higher levels of inhibition, the protective effect of dTMP was diminished; dTMP did not protect when enzyme inhibition by P-dThd was greater than 50%.

Reversibility of inhibitory effects. The chemical reactivity of P-dThd toward nucleophiles [3] and the established susceptibility of the sulfhydryl group at the active site of dTMP synthase to inactivation by a variety of electrophilic reagents [11] suggest that a covalent bond forms between this analogue and the enzyme. The recovery of enzyme activity in the intact cell system, after the removal of extracellular P-dThd, was measured. This approach was employed previously to study the interaction of cellular dTMP synthase with inhibitors known to form covalent bonds at the active site of the enzyme [12].

L1210 cells were treated with various concentrations on P-dThd (0.05 to 1.0 mM) for 40 min at 37°. Cellular dTMP synthesis was blocked. During subsequent incubation of the cells at 37° for 20 min (Fig. 2A) or 60 min (Fig. 2B) in the absence of P-dThd, dTMP synthesis resumed. The extent and rate of this reversible effect depended on the initial extracellular concentration of P-dThd. The data in Fig. 3 show that the rate of regeneration of enzyme activity followed pseudo-first-order kinetics only after exposure to the lowest concentration of P-dThd (0.05 mM) used in the experiment. At 1.0 mM P-dThd, inhibition was irreversible over the time period studied. The data indicate multiple modes of interaction between P-dThd and the enzyme.

Inhibition of thymidylate synthesis in L1210, L-M and L-M(TK$^-$) cells by P-dThd. Following cellular uptake, most cytotoxic nucleoside analogues undergo enzymatic phosphorylation. Although dThd kinase is inhibited by P-dThd [2], finite substrate activity of the analogue is not precluded. Thus, we examined the role that phosphorylation by dThd kinase may play in the dTMP synthase inhibitory effects of P-dThd. In Table 2, the inhibitory effects of P-dThd are compared using three different mouse cell lines; one, L-M(TK$^-$), is a strain lacking dThd kinase activity. Inhibition of dTMP synthesis in the three cell lines was similar as revealed by the permeabilized cell assay, and did *not* require the addition of ATP in the two strains with dThd kinase activity. In the intact cell assay, the mutant strain was 10-fold less sensitive to inhibition by P-dThd than the parent cell line, and approximately 4-fold less sensitive than L1210.

DISCUSSION

The observation that the propenal derivatives inhibit cellular dTMP synthesis in L1210 cells suggests that dTMP synthase may be a target for the cytotoxic action of these compounds. The results obtained agree with the structure–activity relationships previously established [3, 9] with respect to cytotoxicity and inhibition of macromolecular synthesis.

Both the 1- and 3-propenal-substituted pyrimidines were active inhibitors; nucleoside derivatives substituted at the 3-position were superior to the base analogues in this respect. Inhibition of dTMP synthesis in permeabilized L1210 cells by dUrd-3-propenal, an analogue of the natural substrate, dUMP, was two orders of magnitude higher than that by adenine-9-propenal, a purine base derivative. Chemical reduction of the propenal moiety to the propenol abolished enzyme inhibitory activity, demonstrating that the conjugated aldehyde group is required for inhibition. Structural variations in the sugar moiety of nucleosides alter activity [3]. Replacement of the 3′-OH groups of P-dThd with an azido group (3′-azido-3′-deoxythymidine-3-propenal) [13] resulted in an 18-fold decrease in the inhibition of dTMP synthesis in permeabilized L-M cells (data not shown), indicating a decreased interaction of this derivative with the target enzyme.

Propenal derivatives of FU and FdUrd are unstable in aqueous solutions. Their susceptibility to hydrolysis is likely due to the presence of the electron withdrawing 5-fluoro substituent. Loss of the side-chain to form the parent 5-fluoropyrimidines could explain the apparent high potency of the propenal derivatives.

Results obtained in intact L1210 cells using P-dThd revealed that complete inhibition of dTMP synthesis can be achieved *in situ*. The inhibitory effect of P-dThd on release of tritium is greater from [5-^{3}H]dUrd than from [5-^{3}H]dCyd, suggesting interference with dThd kinase necessary for the phosphorylation of [5-^{3}H]dUrd [4, 14]. This effect may result from direct inhibition of dThd kinase and

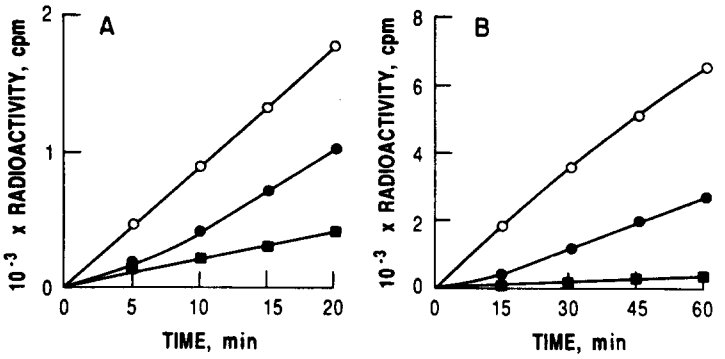

Fig. 2. Recovery of cellular dTMP synthase activity from inhibition by P-dThd during a 20-min (A) and 60-min (B) period. Leukemia L1210 cells grown in culture were incubated at 37° in the absence (○) or presence of various concentrations of P-dThd: 0.05 mM (A, ●), 0.1 mM (B, ●), 0.2 mM (A, ■), and 1.0 mM (B, ■). After 40 min, the cells were resuspended in fresh medium without P-dThd, [5-^{3}H]dCyd was added, and the cell suspensions were further incubated at 37° for 20 min (A) or 60 min (B). At the time intervals indicated, the amount of tritium released into water was determined as described in Materials and Methods.

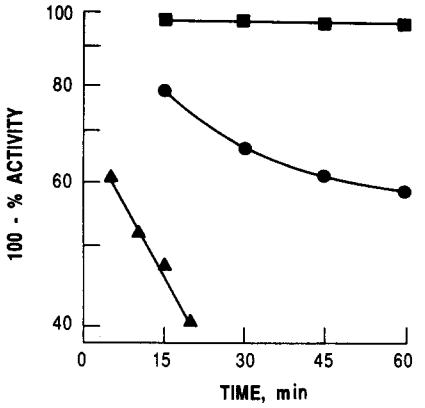

Fig. 3. Recovery of dTMP synthase activity after incubation of L1210 cells for 40 min at 37° in the presence of 0.05 mM (▲), 0.1 mM (●) and 1.0 mM (■) P-dThd (see legend for Fig. 2 for experimental details).

Table 2. Comparison of the dTMP synthase inhibitory effects of P-dThd in L1210, L-M and L-M(TK$^-$) cells

	Inhibition of dTMP synthase*, IC_{50} (μM)		
Cells	L1210	L-M	L-M(TK$^-$)
Intact	21 ± 7.1†	7.5 ± 2.4	75 ± 29
Permeabilized	1.5 ± 0.8	1.7 ± 0.5	3.5 ± 0.4

* Measured by the release of tritium into water from [5-^{3}H]dCyd for intact cells and from [5-^{3}H]dUMP for permeabilized cells as described in Materials and Methods and in the legend of Table 1.

† Means ± SD of two to three separate experiments, each run in duplicate.

from competition with [5-^{3}H]dUrd for dThd kinase by dThd liberated during reaction of P-dThd with nucleophiles [3, 9]. The inability of P-dThd to inhibit significantly IMP dehydrogenase *in situ*, measured by release of tritium from [2-^{3}H]Hyp, is consistent with its DNA-selective effects on macromolecular synthesis [2].

In permeabilized cells, potent inhibition by P-dThd (IC_{50} = 1.5 to 3.5 μM) of the dTMP synthase catalyzed reaction was observed in all three cell lines tested. The enzyme was only partially protected from inactivation by P-dThd in L1210 cells by a high concentration of dTMP*, indicating that P-dThd may interact with more than one site on the enzyme, in agreement with the conclusions drawn from the reversal experiments.

The first step of the reaction catalyzed by dTMP synthase involves nucleophilic attack by the active site cysteine sulfhydryl group at position 6 of dUMP, resulting in an enzyme-linked covalent thioether intermediate [15–19]. Analogous nucleophilic attack may occur at the reactive C-1 position of the side chain of P-dThd [3, 9], similar to that taking place during inactivation of the enzyme by the maleimide derivative showdomycin and its 5′-phosphate [20]. Since the propenal side chain is bifunctional, irreversible cross-linking may also occur by Schiff-base formation between the aldehyde and an amino group of the enzyme.

ATP was *not* required for dTMP synthase inhibitory activity of P-dThd in permeabilized cells suggesting that kinase-mediated phosphorylation is not involved. The 10-fold difference in inhibitory potency of P-dThd between the L-M and L-M(TK$^-$) cells is also inconsistent with a requirement for enzymic phosphorylation [21]. The differential

*dTMP is a competitive inhibitor (K_i = 10 μM) with respect to dUMP (K_m = 2 μM) in this system (Kalman TI and Hsiao MC, unpublished results).

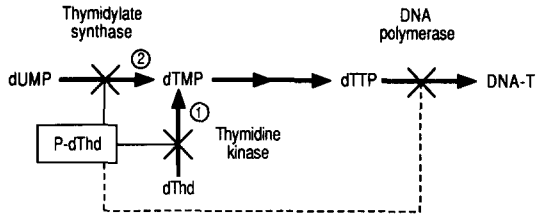

Fig. 4. Postulated mode of action of P-dThd involving the inhibition of salvage (1) and *de novo* (2) pathways of DNA-thymine biosynthesis.

activity observed could be due to a coexisting defect in the nucleoside transport system [22] of the mutant cells, which were selected by continuous exposure to 5-bromo-dUrd, a powerful mutagen [21, 23].

We considered the possibility that P-dThd inhibits dTMP synthase directly without prior phosphorylation, causing thymine-less death of the cell [24]. Inhibition of DNA polymerase α [2], while insufficient by itself to account for the cytotoxicity of P-dThd, may enhance this effect. The demonstrated inhibition of dThd kinase by P-dThd [2] prevents rescue of the cell by preformed dThd, also a product of the reaction of P-dThd with nucleophiles [2, 3]. Indeed, it was noted previously [2] that dThd fails to reverse inhibition by P-dThd of the growth of L1210 cells in culture. We conclude from this analysis that P-dThd is capable of blocking both the salvage and *de novo* pathways of DNA-thymine biosynthesis (Fig. 4) without a demonstrated requirement for activation by kinase-mediated phosphorylation. P-dThd, a chemically reactive bifunctional electrophile, may interact with other cellular sites which may contribute to its observed cytotoxicity. Interactions of P-dThd and other propenal derivatives with those cellular targets remain to be elucidated.

The biological activities of P-dThd have implications for its potential cancer chemotherapeutic applications. In contrast to most dThd analogues, loss of dThd kinase activity cannot result in resistance against P-dThd, since this enzyme is not required for the action of the analogue. However, resistance may arise by alteration of the membrane transport properties of the cell [22] or by increased levels of dTMP synthase arising by gene amplification [25].

As postulated earlier [2, 9, 26, 27], base propenals generated during bleomycin-induced DNA strand-scission may account for some of the cytotoxic and toxic effects of this antibiotic. Thymine-1-propenal, the most cytotoxic of the four base propenals formed from bleomycin-treated DNA, is also the most abundant [1]. Inhibition of macromolecular syntheses in HeLa cells by bleomycin [28] is qualitatively and quantitatively similar to effects observed in cells treated with base propenals [2].

Our results suggest that thymine-1-propenal is a potent inhibitor (only 4-fold less potent than P-dThd; Table 1) of dTMP synthase. Although this enzyme is induced during the S-phase of the cell cycle [29, 30], cycling cells are most sensitive to bleomycin during the G_2 and M phases [31], when basal dTMP synthase activity is very low. However, it has also been observed that bleomycin is more lethal to mutant mouse FM3A cells containing reduced levels of dTMP synthase than to the parent cell line [32]. The available data do not permit a rational explanation of all these observations. Further work is required to establish the significance of the effects of thymine-1-propenal on dTMP synthesis with respect to the mode of action of bleomycin.

Acknowledgements—The authors thank Martha C. Hsiao for her able technical assistance, Dr. Tai-Shun Lin for samples of 3′-azido-3′-deoxythymidine (AZT) and its 3-propenal derivative, and Dr. Alexander Bloch for his comments on the manuscript. This work was supported in part by Grants CA17395 (A.P.G.) and CA35212 (T.I.K.) from the National Cancer Institute, NIH, DHHS, and CH-240 (A.P.G.) from the American Cancer Society.

REFERENCES

1. Giloni L, Takeshita M, Johnson R, Iden C and Grollman AP, Bleomycin-induced strand-scission of DNA: Mechanism of deoxyribose cleavage. *J Biol Chem* **256**: 8608–8615, 1981.
2. Grollman AP, Takeshita M, Pillai KMR and Johnson F, Origin and cytotoxic properties of base propenals derived from DNA. *Cancer Res* **45**: 1127–1131, 1985.
3. Johnson F, Pillai KMR, Grollman AP, Tseng L and Takeshita M, Synthesis and biological activity of a new class of cytotoxic agents: *N*-(3-oxoprop-1-enyl)-substituted pyrimidines and purines. *J Med Chem* **27**: 954–958, 1984.
4. Yalowich JC and Kalman TI, Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 leukemia cells *in vitro*. *Biochem Pharmacol* **34**: 2319–2324, 1985.
5. Kalman TI and Hsiao MC, The dependence of the thymidylate synthase cycle on the extent of polyglutamylation of folate cofactors in permeabilized L1210 leukemia cells. *Proc Am Assoc Cancer Res* **23**: 235, 1985.
6. Kucera R and Paulus H, Studies on ribonucleoside-diphosphate reductase in permeable animal cells. 1. Reversible permeabilization of mouse L cells with dextran sulfate. *Arch Biochem Biophys* **214**: 102–113, 1982.
7. Ledvina M and Farkas J, Synthesis of methyl 3-(*N*′-alkylureido)-2-methyl-2-propenoates and their cyclization to 3-alkyl-5-methyluracils. *Collect Czech Chem Commun* **47**: 676–688, 1982.
8. Kalman TI and Hsiao MC, Rapid measurement of IMP dehydrogenase and its inhibition in intact L1210 leukemia cells. *Proc Am Assoc Cancer Res* **28**: 16, 1987.
9. Johnson F and Grollman AP, Cytotoxic base propenals from DNA. In: *Experimental and Clinical Progress in Cancer Chemotherapy* (Ed. Muggia FM), pp. 1–12. Martinus Nijhoff, Boston, 1985.
10. Franklin TJ and Cook JM, The inhibition of nucleic acid synthesis by mycophenolic acid. *Biochem J* **113**: 515–524, 1969.
11. Plese PC and Dunlap RB, Sulfhydryl group modification of thymidylate synthetase and its effect on activity and ternary complex formation. *J Biol Chem* **252**: 6139–6144, 1977.
12. Kalman TI and Yalowich JC, Survival and recovery: The reversibility of covalent drug–enzyme interactions. In: *Drug Action and Design: Mechanism-Based Enzyme Inhibitors* (Ed. Kalman TI), pp. 75–91. Elsevier North Holland, New York, 1979.
13. Lin TS, Guo JY, Schinazi RF, Chu CK, Xiang JN and Prusoff WH, Synthesis and antiviral activity of various 3′-azido analogs of pyrimidine deoxyribonucleosides

against human immunodeficiency virus (HIV-1, HTLV-III/LAV). *J Med Chem* **31**: 336–340, 1988.
14. Balzarini J, De Clercq E, Shugar D and Torrence PF, 5-Substituted 2′-deoxyuridines: Correlation between inhibition of tumor cells growth and inhibition of thymidine kinase and thymidylate synthetase. *Biochem Pharmacol* **31**: 3673–3682, 1982.
15. Kalman TI, On the mechanism of the reaction catalyzed by thymidylate synthetase. *Ann NY Acad Sci* **186**: 166–167, 1971.
16. Pogolotti AL and Santi DV, On the catalytic mechanisms of thymidylate synthetase. In: *Bioorganic Chemistry* (Ed. van Tamelen EE), Vol. 1, pp. 277–311. Academic Press, New York, 1977.
17. Danenberg PV, Thymidylate synthetase – A target enzyme in cancer chemotherapy. *Biochim Biophys Acta* **473**: 73–92, 1977.
18. Cisneros RJ, Silks LA and Dunlap RB, Mechanistic aspects of thymidylate synthase: Molecular basis for drug design. *Drugs Future* **13**: 859–881, 1988.
19. Montfort WR, Perry KM, Fauman EB, Finer-Moore JS, Maley GF, Hardy L, Maley R and Stroud RM, Structure, multiple site binding, and segmental accommodation in thymidylate synthase on binding dUMP and an anti-folate. *Biochemistry* **29**: 6964–6977, 1990.
20. Kalman TI, Inhibition of thymidylate synthetase by showdomycin and its 5′-phosphate. *Biochem Biophys Res Commun* **49**: 1007–1013, 1972.
21. Kit S, Dubbs DR, Piekarski LJ and Hsu TS, Deletion of thymidine kinase activity from L cells resistant to bromodeoxyuridine. *Exp Cell Res* **31**: 297–312, 1963.
22. Aronow B and Ullman B, Thymidine incorporation in nucleoside transport-deficient lymphoma cells. *J Biol Chem* **260**: 16274–16278, 1985.
23. Hsu TC and Somers CE, Effect of 5-bromodeoxyuridine on mammalian chromosomes. *Proc Natl Acad Sci USA* **47**: 396–403, 1961.
24. Cohen SS, On the nature of thymineless death. *Ann NY Acad Sci* **186**: 292–301, 1971.
25. Berger SH, Jenh CH, Johnson LF and Berger FG, Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol Pharmacol* **28**: 461–467, 1985.
26. Grollman AP, Base propenals and the toxicity of bleomycin. In: *Organ Directed Toxicities of Anticancer Drugs* (Eds. Hacker MP, Lazo JS and Tritton TR), pp. 79–90. Martinus Nijhoff, Boston, 1988.
27. Takeshita M and Grollman AP, A molecular basis for the interaction of bleomycin with DNA. In: *Bleomycin: Chemical, Biochemical and Biological Aspects* (Ed. Hecht SM), pp. 207–221. Springer, New York, 1979.
28. Takeshita M, Horwitz SB and Grollman AP, Bleomycin, an inhibitor of vaccinia virus replication. *Virology* **60**: 455–465, 1974.
29. Navalgund LG, Rossana C, Muench AJ and Johnson LF, Cell cycle regulation of thymidylate synthetase gene expression in cultured mouse fibroblasts. *J Biol Chem* **255**: 7386–7390, 1980.
30. Rode W, Scanlon KJ, Moroson BA and Bertino JR, Regulation of thymidylate synthetase in mouse leukemia cells (L1210). *J Biol Chem* **255**: 1305–1311, 1980.
31. Baranco SC, A review of the survival and cell-kinetics effects of bleomycin, In: *Bleomycin: Current Status and New Developments* (Eds. Carter SK, Crooke ST and Umezawa H), pp. 81–90. Academic Press, New York, 1978.
32. Ayusawa D, Iwata K and Seno T, Unusual sensitivity to bleomycin and joint resistance to 9-β-D-arabinofuranosyladenine of mouse FM3A cell mutants with altered ribonucleotide reductase and thymidylate synthase. *Cancer Res* **43**: 814–818, 1983.