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# Bis(pivaloyloxymethyl) thymidine 5′-phosphate is a cell membrane-permeable precursor of thymidine 5′-phosphate in thymidine kinase deficient CCRF CEM cells

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**Abstract**

Bis(pivaloyloxymethyl) thymidine 5-phosphate ($POM_2$-dTMP) has been investigated as a membrane-permeable prodrugs of dTMP. The growth inhibitory activity of $POM_2$-TMP has been compared with thymidine (TdR) in wild type CCRF CEM cells (CEM) and a strain that lacks TdR kinase (CEM tk−). After 72 h incubation at 37 °C, TdR showed significant antiproliferative activity ($IC_{50}$ = 27 μM) against CEM cells but was weakly effective ($IC_{50}$ = 730 μM) against the mutant cell line. By comparison, bis(pivaloyloxymethyl) thymidine 5′-monophosphate ($POM_2$-dTMP) was equally inhibitory ($IC_{50}$ = 5 μM) to both cell lines. The growth inhibitory effects were reversed by deoxycytidine. Cellular [methyl-$^3$H]dTTP pools increased linearly over 2 h during incubation of CEM or CEM tk− with 5 μM $POM_2$-[methyl-$^3$H]dTMP. The incorporation of [methyl-$^3$H]TdR into $HClO_4$-insoluble cell residue by CEM tk− was <0.1% that of CEM and did not increase over 1 h. In contrast, CEM tk− incorporated radioactivity from $POM_2$-dTMP into acid insoluble residue at a rate 59% that of CEM. These results demonstrate that $POM_2$-dTMP can penetrate into cells and serve as a source of dTMP.
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*Keywords:* Thymidine 5′-phosphate; Prodrugs; Antitumor; Membrane-permeable

## 1. Introduction

Resistance to therapeutic nucleoside analogs is sometimes due to the loss or the reduced activity of primary activating kinases, enzymes that convert the administered compounds to the corresponding 5′-monophosphates [1,2]. This resistance mechanism cannot be overcome by directly administering nucleoside 5′-monophosphates because such compounds are unable to efficiently penetrate cells [3,4] and are usually rapidly dephosphorylated to the parent nucleosides by extracellular phosphatases [5,6]. To overcome this problem, we reported a general method to introduce nucleoside 5′-monophosphates into cells [7]. Our strategy (Scheme 1) was to convert the nucleoside 5′-monophosphates into neutral lipophilic phosphotriesters (**1**) using pivaloyloxymethyl (POM) phosphate-masking groups, which could then penetrate into cells by passive diffusion. Cleavage of one of the POM groups by nonspecific cellular carboxylate esterases gives the hydroxymethyl analogue (**2**) which is inherently chemically labile and spontaneously dissociates with elimination of formaldehyde to give the phosphodiester (**3**). Cleavage of the second POM group by cellular phosphodiesterases regenerates the parent nucleoside 5′-monophosphate (**4**). We described this prodrug strategy for a number of $POM_2$ nucleotides including those derived from 2′-deoxy-5-fluorouridine 5′-monophosphate [8–10]; 3′-azido-3′-deoxythymidine 5′-monophosphate (AZTMP) [11], and 2′,3′-dideoxyuridine 5′-monophosphate (ddUMP) [12], and showed that $POM_2$-AZTMP and $POM_2$-ddUMP

*Abbreviations:* POM, pivaloyloxymethyl; $POM_2$, bis(pivaloyloxymethyl); TdR, thymidine; ddT, dideoxythymidine; dTMP, thymidine 5′-phosphate; ddTMP, dideoxythymidine 5′-phosphate; dTTP, thymidine 5′-triphosphate; NTP, nucleoside triphosphate; CCRF CEM, human acute leukemia lymphoblastic cells; tk, thymidine kinase; tk-, thymidine kinase deficient; AZTMP, 3′-azido-3′-deoxythymidine 5′-monophosphate; ddUMP, 2′,3′-dideoxyuridine 5′-monophosphate; $POM_2$-dTMP, bis(pivaloyloxymethyl) thymidine 5′-monophosphate; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; $HClO_4$, perchloric acid

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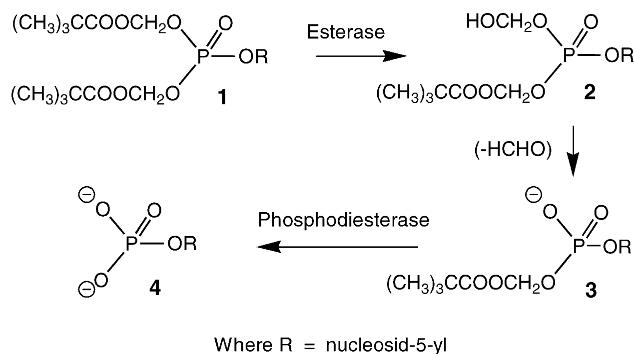


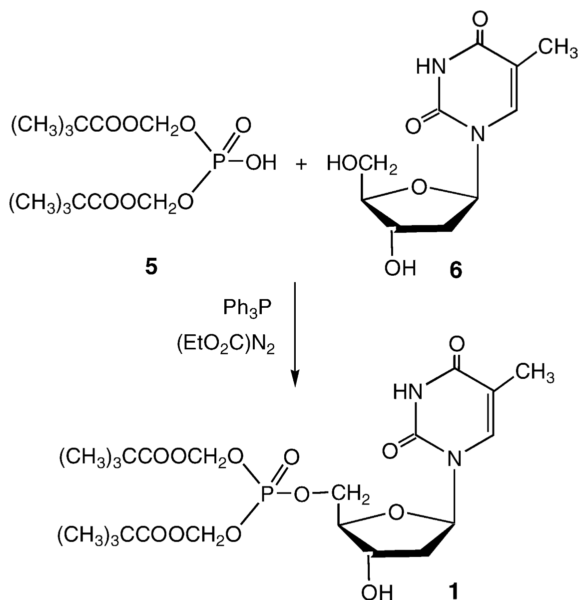

Scheme 1. Biotransformation of $POM_2$ nucleoside 5′-monophosphate prodrugs.

penetrated readily into cells and gave rise to the corresponding mono-, di-, and triphosphates. In conjunction with ongoing studies on the biochemical modulation of anticancer nucleosides, we required evidence that $POM_2$-dTMP could serve as a membrane-permeable prodrug of thymidine 5′-monophosphate (dTMP), and could support DNA synthesis in thymidine kinase (tk) deficient cells. To gain such evidence, we have investigated the biochemical properties and the pharmacologic fate of $POM_2$-dTMP (Scheme 1; R, thymidin-5-yl) in human lymphoblastoid CCRF CEM cells and in tk-deficient CCRF CEM cells (CEM tk−).

## 2. Materials and methods

### 2.1. Analytical methods and prodrug synthesis

Nuclear magnetic resonance spectra ($^1$H NMR, $^{13}$C NMR, and $^{31}$P NMR) were recorded at ambient temperature on an IBM-Bruker Model 300 spectrometer in Fourier transform mode, in $CDCl_3$, MeOD using tetramethylsilane as an internal standard. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN, and where indicated only by symbols of elements were within ± 0.4% of the theoretical values. All reactions were carried out in dry glassware and were protected from atmospheric moisture. Solvents were dried over freshly activated (300 °C/4 h) molecular sieves (type 4A). The homogeneity of the products was determined by ascending thin layer chromatography (TLC) on silica-coated glass plates (silica gel 60 F254, Merck; Bodman Industries, Aston, PA) using mixtures of $CHCl_3$–MeOH (typically 1–10% MeOH) as the eluting solvent. Chromatograms were visualized under a UV lamp (254 nm) or by placing the air-dried plates in a tank of iodine vapor. Compounds containing POM groups were identified by spraying the plates with a 0.25% solution of 4-amino-3-hydrazino-5-mercapto-1,2,4-tetrazole (Purpald) in 0.5 N NaOH solution, then heating them in an oven at 85 °C for 5 min. The liberated formaldehyde reacted with the Purpald reagent to form purple spots against a white background. Preparative separations were performed by flash chromatography on silica gel (230–400 mesh) (Merck; Bodman Industries, Aston, PA) using mixtures of $CHCl_3$/MeOH as eluent. All chemicals and reagents were purchased from Sigma–Aldrich Corporation (St. Louis, MO) and radiolabeled TdR was obtained from Moravek Biochemicals (Brea, CA).




Scheme 2. Synthesis of $POM_2$-dTMP.

### 2.2. Prodrug synthesis

#### 2.2.1. Bis(pivaloyloxymethyl) thymidine 5′-phosphate ($POM_2$-dTMP) (Scheme 2)

A solution of thymidine (484 mg, 2.0 mmol), bis(pivaloyloxymethyl) phosphate (980 mg, 3.0 mmol) (**5**), and triphenylphosphine (786 mg, 3.0 mmol) in dimethylacetamide (5 ml) was stirred magnetically for 10 min. A solution of diethyl azodicarboxylate (0.522 g, 3.0 mmol) in dimethylacetamide (2 ml) was added with stirring, and the reaction mixture was heated at 60 °C for 5 days under a $N_2$ atmosphere. The residue was taken up in the minimum of $CHCl_3$ and chromatographed on a column of silica (70 g) using $CHCl_3$:MeOH (95:5, v/v) as eluent; 10 ml fractions were collected. Fractions containing bis(pivaloyloxymethyl) thymidine 5′-phosphate appeared as a dark quench when viewed under short-wavelength UV light on silica gel 60 F-254 thin layered chromatography plates [Rf = 0.30; $CHCl_3$:MeOH, 95:5) and gave a positive reaction with the Purpald spray reagent. The compound was isolated as colorless oil. Yield: 330 mg (30%). UV ($H_2O$): $\lambda_{max}$ 263 ($\gamma$ 7838). $^1$H NMR ($CDCl_3$): * 7.33 (s, 1H, H-6), 6.30 (dd, 1H, H-1′), 5.66 (dd, 4H, P(O)$OCH_2O$, $J$ = 12 Hz), 5.39 (m, 1H, H-3′), 3.90–4.63 (m, 4H, H-3′, H-4′, H-5′), 2.33 (m, 2H, H-2′), 1.92 (s, 3H, $CH_3$), 1.21 (s, 18H, $C(CH_3)_3$). MS: $m/z$ 551 ($M + H^+$).

#### 2.2.2. Bis(pivaloyloxymethyl) [methyl-$^3$H]thymidine 5′-monophosphate (POM$_2$-[methyl-$^3$H]dTMP)

A solution of [methyl-$^3$H]TdR [1 mCi, 56 Ci/mmol, 1 mCi/ml solution] in EtOH:$H_2O$ (1:1) was added to unlabeled TdR (100 mg, 0.41 mmol). The solution was evaporated, and the residue was dried in vacuo over phosphorus pentoxide for 24 h. It was then taken up in dimethylacetamide (2 ml) and bis(pivaloyloxymethyl) phosphate (148 mg, 0.46 mmol) was added followed by triphenylphosphine (119 mg, 0.46 mmol) and diethyl azodicarboxylate (0.08 ml, 0.45 mmol). The reaction mixture was worked up as described for the unlabeled compound, above. The product was isolated as colorless oil after chromatography of the crude reaction mixture on a column of silica (30 g). It was chromatographically and spectrally identical to the unlabeled preparation. Yield: 45 mg (20%). The specific activity of the compound was 2.06 mCi/mmol.

#### 2.2.3. Bis(pivaloyloxymethyl) 2′,3′-dideoxythymidine 5′-phosphate (POM$_2$-ddTMP)

This compound was prepared similar to POM$_2$-dTMP, above, from 2′,3′-dideoxythymidine (ddT) (100 mg, 0.44 mmol), bis(pivaloyloxymethyl) hydrogen phosphate (360 mg, 1.1 mmol), triphenylphosphine (290 mg, 1.1 mmol), and diethyl azodicarboxylate (0.19 ml, 1.1 mmol) in dimethylacetamide (1.5 ml). The product was isolated as viscous oil. Yield: 115 mg (49%). Rf (silica gel 60) = 0.36 ($CHCl_3$:MeOH, 95:5). $^1$H NMR ($CDCl_3$): 1.23 (s, 18H, $C(CH_3)_3$), 1.95 (s, 3H, $CH_3$), 2.0 (m, 2H, H-2′ and H-2″), 2.18 (m, 2H, H-3′ and H-3″), 4.35 (m, 1H, H-4′), 4.24 (m, 2H, H-5′ and H-5″), 5.68 (dd, 4H, $CH_2$OP(O), $J_{PH}$ = 12 Hz), 6.10 (dd, 1H, H-1′), 7.48 (s, 1H, H-6), 8.50 (s, br, NH). MS: $m/z$ 535 ($M + H^+$).

### 2.3. Cell lines

Human T-lymphoblastoid cells (CEM) were obtained from the American Type Culture Collection (Rockville, MD). A subline deficient in TdR kinase activity, (CEM tk−), was obtained from the AIDS Research and Reference Reagent Program of the National Institute of Allergic and Infectious Diseases, National Institutes of Health. These cell lines were maintained in suspension culture in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with glutamine and 10% heat-inactivated fetal calf serum (GIBCO) at 37 °C.

#### 2.3.1. Growth inhibition of CEM and CEM tk− cells by TdR and POM$_2$-dTMP

CEM and CEM tk-cells ($5 \times 10^5$/well) were incubated for 72 h in 24-well plates in the presence of TdR or POM$_2$-dTMP over the concentration range 2–2000 μM. The viable cells in each culture were determined using a Coulter counter equipped with a model C-1000 particle analyzer (Coulter Electronics, Hialeah, FL).

### 2.4. Deoxycytidine reversal of TdR and POM$_2$-dTMP-induced cell growth inhibition

CEM cells ($5 \times 10^5$/well) in RPMI 1640 media were incubated for 1 h in 24-well plates with TdR (50 μM) or POM$_2$-dTMP (5 μM). Deoxycytidine was added over the concentration range 1–100 μM, and incubation was continued for 3 days. The number of viable cells was determined as before. Similar experiments were conducted with POM$_2$-ddTMP (100 μM).

### 2.5. Cellular metabolism of POM$_2$-[methyl-$^3$H]dTMP in CEM and CEM tk− cells

CEM and CEM tk− cells ($5 \times 10^7$) in exponential growth phase were incubated with 5 μM POM$_2$-[methyl-$^3$H]dTMP for 60 min. At 15 min intervals, $1 \times 10^7$ cells were removed, washed with ice-cold phosphate-buffered saline, and collected by centrifugation. The pellet was extracted overnight with a solution of methanol–water (6:4) (600 μL) at −40°C. After removal of precipitated nucleic acids and cell organelles by centrifugation (1500 rpm), the supernatant was dried under vacuum in an Evapomix volume reduction apparatus (Buchler Instruments, Fort Lee, NJ). The residue was reconstituted in phosphate-buffered saline (500 μL), and analyzed for thymidine mono-, di-, and triphosphates by HPLC (Waters Corporation, Milford, MA). The system was equipped with two model 6000A solvent delivery pumps, a Model 680 gradient solvent programmer, a Partisil 10-SAX anion exchange column (250 mm × 4 mm; Whatman Inc., Clifton, NJ) and was connected to a radioactive flow detector (model A 250; Packard Instrument Co., Meriden, CT). Radioactively labeled nucleotides were separated by using the following mobile phases at a flow rate of 1 ml/min; 0–10 min, 100% buffer A (0.005 M $NH_4$ $H_2PO_4$, pH 2.8); 10–70 min, linear gradient from 100% buffer A to 100% buffer B (0.75 M $NH_4$ $H_2PO_4$, pH 3.7); 70–75 min, 100% buffer B. The eluent was mixed with scintillation fluid (Flo-Scint IV; Packard Instrument Co.) at a ratio of 1:3. The A-200 series Flo-One/data II software version 1.6 (Packard Instrument Co.) was used to analyze the data. The concentration of each metabolite was calculated from the drug specific activity, the number of cell equivalents analyzed, and the mean cell volume of the cells in each sample. External standard quantization was used to determine the concentration of nucleoside triphosphates (NTP), calculated by dividing the NTP amount by the number of cells analyzed and the mean cell volume (13). The identity and concentration of the starting materials and products were confirmed by comparison (retention times and UV-spectra) with authentic standards obtained from Sigma–Aldrich Corporation (St. Louis, MO)

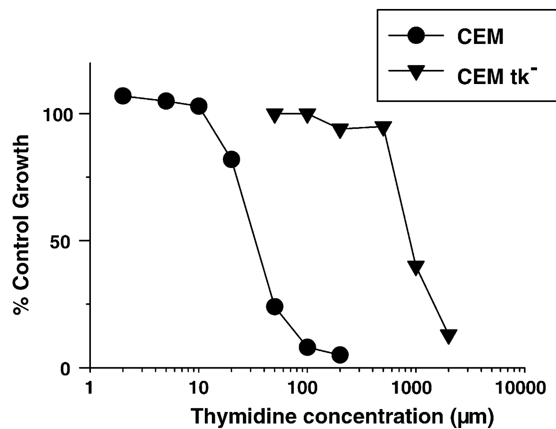



Fig. 1. Growth inhibition of CCRF-CEM and CCRF-CEM tk− cells by TdR. The number of cells in each culture was determined after 72 h.

### 2.6. Accumulation of [methyl-$^3$H]dTTP in CEM and CEM tk− cells exposed to 5: M POM$_2$–dTMP

Exponentially growing CEM and CEM tk− cells ($5 \times 10^7$/ml) were incubated with 5 µM POM$_2$-[methyl-$^3$H]dTMP (specific activity, 2.06 mCi/mmol) for 2 h. At 1 h intervals, $1 \times 10^7$ cells were removed, washed with ice-cold phosphate-buffered saline, and collected by centrifugation. The nucleotides were extracted with 0.4 N $HClO_4$ and neutralized with KOH as described previously [13,14]. The neutralized extracts were analyzed by HPLC (Waters Corporation, Milford, MA) as described above. To confirm that the radioactivity present in CEM tk− cells was associated with deoxyribonucleotides rather than ribonucleotides, the neutralized $HClO_4$ extracts were degraded by periodate oxidation as previously described [15] and reanalyzed by HPLC. The retention times for thymidine mono-, di- and triphosphates were 4, 16, 29.2, and 54 min, respectively.

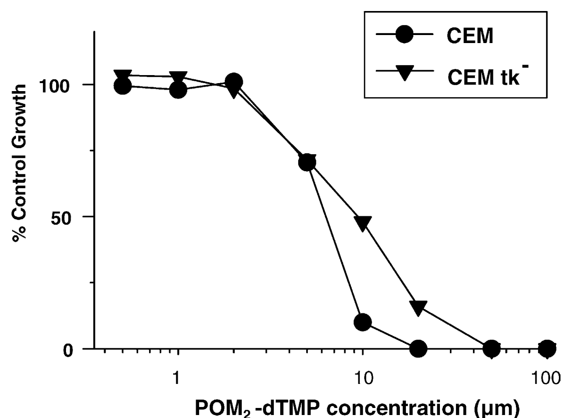



Fig. 2. Growth inhibition of CCRF-CEM and CCRF-CEM tk− cells by POM$_2$-dTMP. Both cell lines were approximately equally sensitive ($IC_{50}$ = 6 µM vs. 9 µM) to the growth inhibitory effects of the compound. The data represent the mean of three determinations.

### 2.7. Incorporation of radioactivity from [methyl-$^3$H]TdR or POM$_2$-[methyl-$^3$H]dTMP into DNA

CEM and CEM tk$^-$ cells ($5 \times 10^7$/ml) in exponential growth phase were treated with 5 µM [methyl-$^3$H]TdR or POM$_2$-[methyl-$^3$H]dTMP for 30 min. The cells were collected on a 25-mm glass fiber disc (Schleicher and Schuell, #34 glass, size 2.5 cm, prewetted with 1% sodium pyrophosphate) by filtration, and washed three times with 4 ml of cold 0.4 N $HClO_4$ and twice with 2 ml of ethanol. Radioactivity retained on the filter disc was determined by liquid scintillation counting.

## 3. Results

### 3.1. Growth inhibition of CCRF-CEM and CCRF-CEM tk− cells by TdR and POM$_2$-dTMP

The $IC_{50}$ of TdR to CEM cells was 27 µM. By comparison, the $IC_{50}$ of TdR to CEM tk− cells was 730 µM, approximately 30-fold greater (Fig. 1). CEM and CEM tk− cells were approximately equally sensitive ($IC_{50}$ = 6 µM versus 9 µM) to the growth inhibitory effects of POM$_2$-dTMP (Fig. 2).

### 3.2. Deoxycytidine reversal of TdR and POM$_2$-dTMP-induced cell growth inhibition

Since it is well established that exogenous deoxycytidine can reverse cell growth inhibition induced by TdR [16], it was of interest to determine whether deoxycytidine could have the same effect on POM$_2$-dTMP-induced growth inhibition. As shown in Fig. 3, equal concentrations

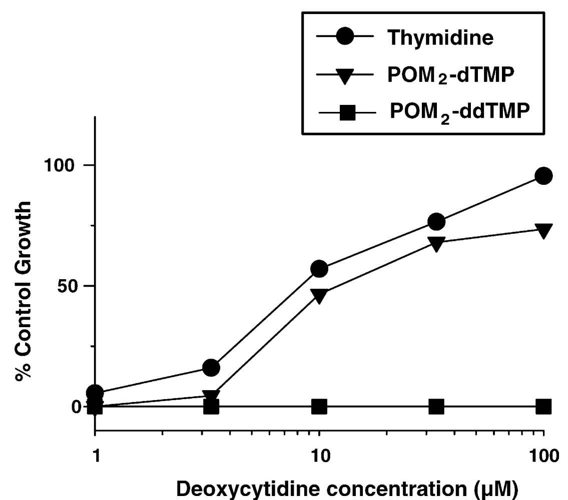



Fig. 3. Deoxycytidine reversal of growth inhibition. Exponentially growing CCRF-CEM cells were incubated with growth inhibitory concentrations of TdR (50 µM), POM$_2$-dTMP (5 µM), or 2′,3′-dideoxythymidine 5′-monophosphate (POM$_2$-ddTMP) (100 µM). Progressively increasing concentrations of deoxycytidine were then added and cell growth monitored. The data represent the mean of three determinations.

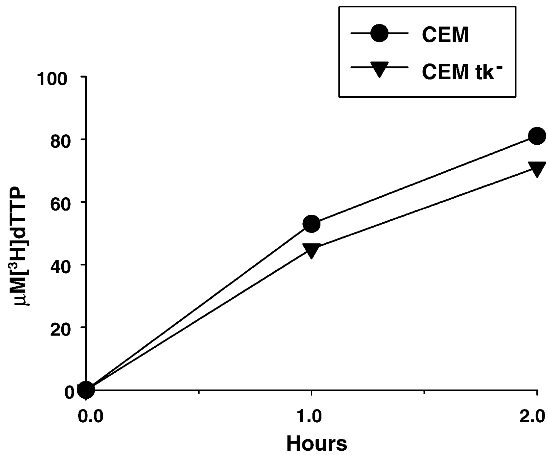



Fig. 4. Accumulation of [methyl-$^3$H]dTTP in CCRF-CEM and CCRF-CEM tk− cells. Exponentially growing CCRF-CEM and CCRF-CEM tk− cells were incubated with 5 μM $POM_2$-[methyl-$^3$H]dTMP, nucleotides were extracted, separated by HPLC, and [5-methyl-$^3$H]dTTP was quantified by flow-through scintillation counting. The data represent the mean of three determinations.

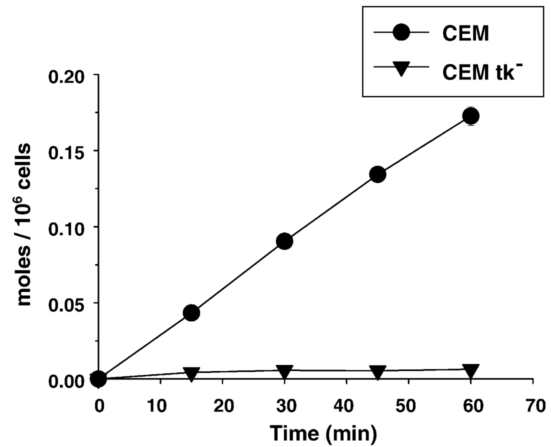



Fig. 5. Incorporation of [methyl-$^3$H]TdR into the DNA of exponentially growing CCRF-CEM and CCRF-CEM tk− cells. The cells were incubated with [methyl-$^3$H]TdR and aliquots of each cell type were withdrawn at intervals and extracted with $HClO_4$. The specific activity of [methyl-$^3$H]dTTP pools of $HClO_4$ soluble extracts and the radioactivity incorporated into $HClO_4$ insoluble material were determined as described in Section 2. The data represent the mean of three determinations.

of deoxycytidine reversed the growth inhibitory effects of TdR and $POM_2$-dTMP to CEM cells to a similar extent. By contrast, the growth inhibitory effects of $POM_2$ 2′,3′-dideoxythymidine 5′-phosphate to CEM cells, could not be reversed by deoxycytidine.

### 3.3. Metabolism of [methyl-$^3$H]TdR and $POM_2$-[methyl-$^3$H]dTMP in CEM and CEM tk− cells

To determine whether $POM_2$-dTMP could act as a precursor of TdR nucleotides, dTTP levels were measured in $HClO_4$ extracts of CEM and CEM tk− cells exposed to 5 μM $POM_2$-dTMP. Both cell lines accumulated dTTP at similar rates (Fig. 4). When the CEM tk− extracts were degraded by periodate oxidation and reanalyzed by HPLC, more than 90% of the radioactivity (in dTTP) was recovered, confirming that the radioactivity was associated with deoxynucleotides rather than ribonucleotides (Table 1).

### 3.4. Incorporation of radioactivity from [methyl-$^3$H]TdR or $POM_2$-[methyl-$^3$H]dTMP into DNA

TdR was progressively incorporated into CEM cells over the 70 min incubation period. However, minimal radioactivity was incorporated into the DNA of CEM tk− cells after [methyl-$^3$H]TdR exposure (Fig. 5). When incubated with 5 μM [methyl-$^3$H]TdR or $POM_2$ [methyl-$^3$H]TdR, CEM cells progressively incorporated radioactivity into DNA (Fig. 5, Table 2). The rate of incorporation of radioactivity from $POM_2$-[methyl-$^3$H]dTMP into the DNA of CEM tk− cells was 59% of that in the wild type cells (Fig. 6).

Table 1
$NaIO_4$-resistance of [methyl-$^3$H]dTTP in CCRF-CEM tk− cells after incubation with $POM_2$-[methyl-$^3$H] dTMP

| | $1.5 \times 10^7$ cells (dpm) | Percentage |
|---|---|---|
| Control | 9603 ± 86 | 100 |
| Mock $NaIO_4$ | 8275 ± 73 | 89 |
| $NaIO_4$ | 8618 ± 81 | 93 |

Table 2
Rate of incorporation of $^3$H into the DNA of cells treated with [methyl-$^3$H]TdR and $POM_2$-[methyl-$^3$H]dTMP

| Compound | Cells | nmol/$10^6$/h | Ratio CEM/CEM tk− |
|---|---|---|---|
| TdR | CEM | 0.172 | |
| | CEM tk− | <0.004 | 48:1 |
| $POM_2$-[methyl-$^3$H]dTMP | CEM | 0.123 | |
| | CEM tk− | 0.072 | 2:1 |

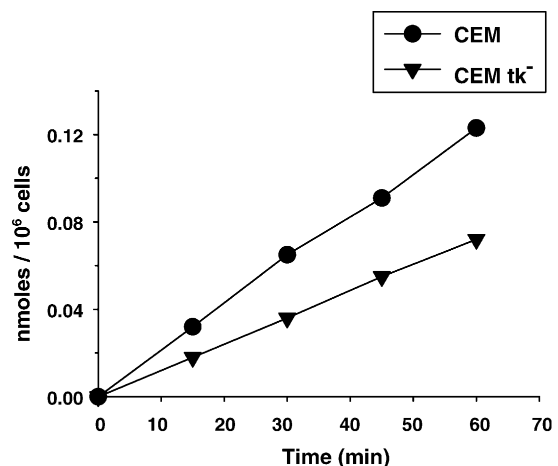



Fig. 6. Incorporation of $POM_2$-[methyl-$^3$H]dTMP into the DNA of exponentially growing CCRF-CEM and CCRF-CEM tk− cells. The experimental design was the same as that described in Fig. 5.

## 4. Discussion

In this study, we wished to determine whether $POM_2$-TMP could serve as a cell-membrane precursor of TMP and support DNA synthesis in cells that were deficient in tk. A well characterized pair of CCRF CEM cells was available to address this question, one wild-type and the other deficient in tk.

The findings are consistent with the conclusion that $POM_2$-dTMP can serve as a cell membrane-permeable precursor of dTMP and can by-pass tk deficiency in the biosynthesis of dTTP. The growth inhibition of CEM cells observed in the presence of TdR is most likely due to high intracellular levels of dTTP. It is well established that dTTP can inhibit ribonucleotide reductase and reduce the biosynthesis of 2′-deoxynucleoside 5′-diphosphates, cellular metabolites that are required for DNA synthesis [17]. The weak growth inhibitory effects of TdR against the CEM tk− cells is consistent with the inability of the cells to convert TdR to TMP, and, subsequently, to dTDP and dTTP. Further support for this interpretation is that $POM_2$-dTMP inhibited the growth of both CEM and CEM tk− cells, findings that are in accord with the ability of the compound to bypass kinase deficiency. Additional evidence in favor of this mechanism is the demonstration that deoxycytidine, at similar concentrations, can reverse the growth inhibitory effects of both TdR- and $POM_2$-dTMP. This is expected because a reduced level of dCDP is the most critical metabolic lesion in dTTP-induced ribonucleotide deficiency. By contrast, deoxycytidine was not able to reverse the growth inhibitory effects of $POM_2$-ddTMP to CEM cells, a compound that closely structurally resembles $POM_2$-dTMP. This observation is consistent with the known locus of action of the ddT, namely anabolism to ddTTP and incorporation into DNA with termination of DNA chain synthesis (Huang et al., 1992). Collectively, these data suggest that TdR and $POM_2$-dTMP exert their growth inhibitory effects by a common mechanism, namely dTTP-induced inhibition of ribonucleotide reductase and depletion of dCDP pools.

The ability of $POM_2$-dTMP to serve as a precursor of dTMP is also supported by the observation that radioactivity was incorporated into the dTTP of CEM tk− cell extracts that had been exposed to radiolabeled $POM_2$-dTMP. The lack of effect of periodate on the radioactivity in the triphosphate pools establishes that the radioactivity was associated with deoxyribonucleotides and not ribonucleotides. As expected, dTR was readily incorporated into the DNA of CEM cells. The low rate of incorporation of radioactivity from TdR into the DNA of CEM tk− cells is consistent with the reduced kinase levels in these cells. $POM_2$-dTMP, on the other hand, was readily incorporated into the DNA of both CEM and CEM tk− cells. The reduced rate of incorporation into the latter is probably due a competing de novo TMP synthesis pathway.

Collectively, these studies demonstrate that $POM_2$ dTMP can serve as a cell membrane- permeable precursor of dTMP. This is consistent with other studies where we have reported that $POM_2$-nucleotides can give rise to the corresponding nucleoside 5′-triphosphates in cells that lack the ability to phosphorylate the free parent nucleosides.

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