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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.

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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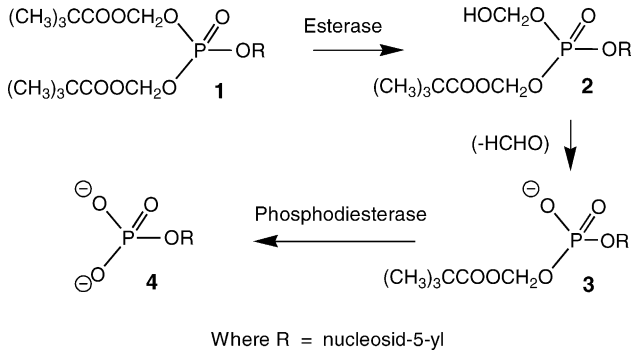


Scheme 1. Biotransformation of POM_2 nucleoside 5′-monophosphate prodrugs.

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

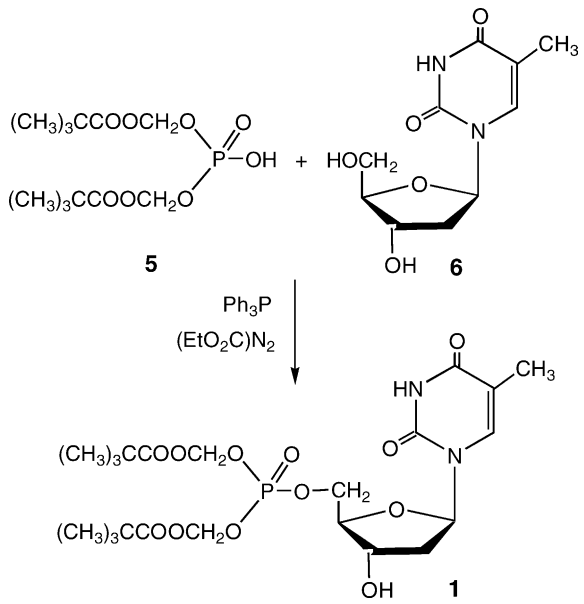
2.1. Analytical methods and prodrug synthesis

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).

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): λ_{max} 263 (γ 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 ($M + H^+$).



Scheme 2. Synthesis of POM_2-dTMP.