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SYNTHESIS AND BIOLOGIC STUDIES OF ARABINOSYLCYTOSINE 5′-METHYLPHOSPHONATE

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Abstract—Arabinosylcytosine (ara-C), a clinically useful antitumor agent, is ineffective against cells that have deleted deoxycytidine kinase, the enzyme necessary for conversion of ara-C to its active nucleotide form. To circumvent this resistance, arabinosylcytosine-5′-methylphosphonate (ara-CMeP) was synthesized as an analogue of ara-CMP that would be membrane-permeable, resistant to serum phosphatase attack, and resistant to nucleoside deaminase inactivation. Ara-CMP was inhibitory to leukemia P388 *in vitro* but required concentrations 90-fold greater than that of ara-C for comparable cell inhibition. Both ara-CMeP and ara-CMP were competitive inhibitors of dCMP kinase from leukemia L1210 with K_i values of 4.0×10^{-3} and 4.4×10^{-3} M respectively. However, ara-CMP is a substrate for dCMP kinase, whereas ara-CMeP was not. Thus, the inability of ara-CMeP to be phosphorylated precludes its usefulness as a functional analogue of ara-CMP.

Since the introduction of arabinosylcytosine (ara-C) in the early 1960s, the drug has proven to be clinically useful in the treatment of leukemias [1]. To be effective, ara-C must be phosphorylated to the triphosphate (ara-CTP), which not only inhibits DNA polymerase but is also possibly incorporated into DNA as a false nucleotide [2, 3]. The usefulness of ara-C has been limited by the rapid development of tumor resistance. In several resistant mouse leukemias, the mechanism of resistance has been cellular deletion of CdR kinase, the initial phosphorylating enzyme for ara-C [4, 5].

The ideal solution to resistance caused by CdR kinase deletion would be to bypass the enzyme and provide the cell with the enzyme product, i.e. the nucleotide, arabinosylcytosine monophosphate (ara-CMP). Unfortunately nucleotides penetrate cell membranes poorly and are highly susceptible to serum phosphatase attack. Phosphatase removal of the phosphate group converts ara-CMP to the parent compound (ara-C), which is ineffective in resistant cells [6]. The phosphate group on ara-CMP is double ionized at physiologic pH, and the resulting negative charges may be a major factor excluding it from cells [7].

A recent report by Wigler and Lozzio [8] on 5-bromo-2′-deoxyuridine-5′-methylphosphonate suggests that substituting a methyl group for an oxygen on the nucleotide phosphate group reduces phosphate ionization and increases membrane penetration. In addition, the methylphosphonate group is resistant to phosphatase attack. Based on these observations, synthesis of the corresponding analogue of ara-CMP has been suggested [9]. The present paper describes the synthesis and biologic properties of ara-C-5′-methylphosphonate (ara-CMeP, Fig. 1).

MATERIALS AND METHODS

Phosphocreatine and creatine phosphokinase were purchased from CalBiochem; NDP kinase and ATP were from Sigma; ara-CMP was from Terra-Marine; and dCMP was from PL-Biochemicals. The [γ-^{32}P]ATP and [8-^{14}C]ATP were from New England Nuclear. The [2-^{14}C]dCMP was from Schwarz-Mann. Radioactive samples were counted in a Beckman LS-230 scintillation counter in 10 ml of scintillation fluid [6 g 2,5-diphenyloxazole (PPO) and 200 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene (POPOP) in 1400 ml toluene and 600 ml methanol]. The DEAE-cellulose plates used for chromatography were made by Analtech Inc. Whatman DE-81 discs were used in the enzyme assay. Tritylchloride and

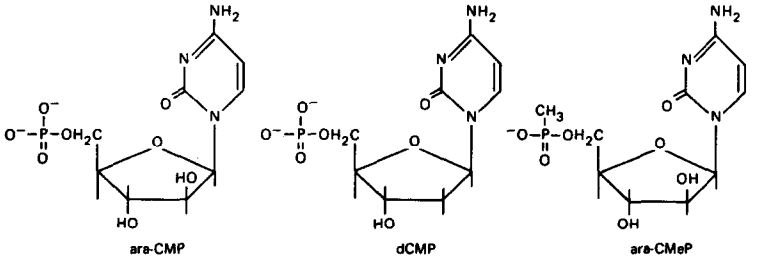

Fig. 1. Chemical structures of ara-C, dCMP and ara-C-5′-methylphosphonate.

p-nitrobenzyl chloride were purchased from Eastman Organic Chemicals.

Synthesis of ammonium arabinosylcytosine 5'-methylphosphonate

5'-O-*tritylarabinosylcytosine* (*ara-C-T*) [10]. Ara-C (10 g, 35.6 m-moles) and tritylchloride (18 g, 62.4 m-moles) in dry pyridine (100 ml) were heated at 80–90° for 5 hr. The reaction mixture was poured into 1.5 liters water and the precipitate was collected by filtration, dried, and triturated with 3 × 100 ml of hot heptane. The product was recrystallized from EtOAc/MeOH to give 6 g (35 per cent), m.p. 228–230° (lit. m.p. 227.5 to 228°).

5'-O-*Trityl*-N^4, 2',3'-O-*tri*-p-*nitrobenzoyl-arabinosylcytosine* (*ara-C-T-Bz*) [10]. Ara-C-T (6 g, 12.4 m-moles) and *p*-nitrobenzylchloride (8.6 g, 49.8 m-moles) in dry pyridine (50 ml) were stirred overnight at room temperature, then heated at 50–60° for 4 hr. The reaction mixture was poured into 200 ml of ice water and saturated $NaHCO_3$ was added. The solid was collected by filtration and dissolved in 100 ml CH_2Cl_2. The CH_2Cl_2 solution was extracted successively with 2 × 50 ml of saturated $NaHCO_3$, 2 × 50 ml H_2O, and 2 × 50 ml of saturated NaCl. The CH_2Cl_2 solution was dried ($MgSO_4$), decolorized (Norit), and filtered, and the solvent evaporated. The product was recrystallized from EtOAc/MeOH to yield 9.4 g (81 per cent), m.p. 144–147°.

N^4,2',3' - O - *tri* - p - *nitrobenzoyl* - *arabinosylcytosine* (*ara-C-Bz*) [10]. Ara-C-T-Bz (9.4 g) was dissolved in $CHCl_3$ (100 ml), and 30% HBr/HOAC (12 ml) was added dropwise. An amorphous precipitate formed immediately. The solvent was decanted; the precipitate was washed with $CHCl_3$ and triturated with ether. The product was obtained by filtration and was crystallized from ETOAc/MeOH by the addition of heptane. The yield was 6.0 g (86 per cent), m.p. 227–231° (dec).

Ammonium arabinosylcytosine 5'-methylphosphonate (*ara-CMeP*) [8]. Ara-C-Bz (2.8 g, 4 m-moles) and methylphosphonic acid (1.2 g, 12 m-moles) were dissolved in 50 ml of dry pyridine and the solvent was removed under vacuum. This procedure was repeated to provide a dry reaction mixture. The residue was dissolved in 75 ml of dry pyridine, and dicyclohexylcarbodiimide (8.2 g, 40 m-moles) was added. After standing at room temperature for 4 days, the reaction mixture was poured into 100 ml of cold water and stirred for 2 hr. The mixture was filtered and the solvent removed under vacuum. The residue was dissolved in 25 ml MeOH, to which solution 25 ml of concentrated NH_4OH was added, and the reaction mixture was stirred at room temperature overnight. The solution was filtered and the solvent removed under vacuum. The residue, after recrystallization from MeOH/ether, was dissolved in water and chromatographed on Sephadex G-10 (3 × 15 cm) with water as eluant. The yield of very hygroscopic product was less than 10 per cent.

The u.v. spectrum of the product ($\lambda_{max}^{H_2O}$ 273.5 nm, λ_{max}^{MeOH} 276 nm) is consistent with results reported for ara-C phosphates. Thin-layer chromatography indicated only one compound (Silica gel, MeOH, $R_f = 0.66$; MeOH–$CHCl_3$ 3:1, $R_f = 0.59$).

Cytotoxicity studies

Cell inhibition experiments *in vitro* were performed with leukemia P388 cells maintained in static culture in RPMI No. 1630 medium with 10% fetal calf serum. A stock culture was diluted to 10^5 cells/ml in a total volume of 5 ml/bottle. Solutions of ara-C-MeP and ara-C were passed through a 0.45 μm Millipore filter, and a volume of 0.1 was added to each bottle. Duplicate bottles were employed at each concentration. The cells were counted with a Coulter counter after 24 and 48 hr of incubation at 37°. Values are the average of two determinations.

dCMP kinase preparation

L1210 leukemic cells were harvested from the peritoneal cavity of CDF_1 mice inoculated 8 days prior with 5 × 10^5 cells. The cells were twice washed in phosphate-buffered saline, with centrifugation at 500 *g* for 10 min. The erythrocytes present were lysed in 3 vol. of distilled water for 30 sec and then 1 vol. of 3.6% NaCl was added. The cell preparation was centrifuged and the lysis repeated. The erythrocyte-free L1210 cells were collected by centrifugation and stored at −70°. The dCMP kinase was obtained by thawing an aliquot of the L1210 cells, adding 1.5 ml of 0.05 M Tris (pH 7.5) containing 3 mM mercaptoethanol, and lysing the cells by freeze-thawing twice. After centrifugation at 40,000 *g* for 30 min, the supernatant was removed, dialyzed against 0.05 M Tris (pH 7.5) containing 3 mM mercaptoethanol, and used for the enzyme reactions.

Kinetic studies

The enzyme reaction mixture contained 0.435 μmole ATP, 0.22 μmole $MgCl_2$, 0.135 μmole creatine phosphate, 3.0 μmoles Tris (pH 7.9), 0.6 mg bovine serum albumin, 0.27 units creatine kinase, 0.05 units NDP kinase, 10 μl of enzyme preparation, [2-^{14}C]dCMP (1.0 to 100 nmoles), and ara-CMP or ara-CMeP (6.5 μmoles) altogether in 60 μl. NDP kinase was added to enhance conversion of diphosphate nucleotides to triphosphates to increase the sensitivity of the disc assay. Incubation was for 10 min at 37°, conditions under which the reaction rate was linear. Reactions were stopped by placing the incubation mixture in boiling water for 1 min. To each tube was added 50 μl water, the contents were mixed, and 50-μl samples of each tube were analyzed for product formation by the DEAE disc method described by Cheng and Prusoff [11]. The 50-μl samples were placed on DE81 discs and the discs air dried. The discs were gently washed three times (15 min each wash) in 4 N formic acid containing 1 mM ammonium formate. The discs were air dried and placed in scintillation vials, 10 ml of scintillation fluid was added, and the discs were counted.

Phosphorylation studies

The reaction mixture contained 1.0 μmole $MgCl_2$, 5.0 μmoles Tris (pH 7.9), 0.42 μmole of either [γ-^{32}P]- or [8-^{14}C]-labeled ATP (approximately 2 × 10^5 dis./min), 1.0 mg bovine serum albumin, 25 μl of the crude enzyme preparation, and ara-CMP, dCMP or ara-CMeP (0.62 μmole) in 120 μl. Unlike the kinetic studies, no NDP kinase was added. The control reac-

tions contained no added substrate. After incubation at 37°, the reaction was stopped by placing the reaction mixture in boiling water for 1 min. Denatured protein was removed by centrifugation and 4 μl of the supernatant was spotted on DEAE-cellulose thin-layer chromatography plates. The plates were developed in 4 N formic acid containing 1 mM ammonium formate. In this system, the nucleoside triphosphate remains at the origin (R_f = 0.03), the diphosphate has an R_f of 0.38, and the monophosphate moves with the solvent front (R_f = 0.96). The diphosphate and triphosphate spots were defined by both autoradiography and fluorescence under 254 nm u.v. light. The spots were scraped, placed in 18 ml of scintillation fluid, and counted.

RESULTS

Cytotoxicity in vitro

The cell inhibition of ara-CMeP was compared to that of ara-C against P388 cells *in vitro*. A 24-hr exposure of P388 cells to ara-CMeP at concentrations as high as 64 μg/ml of culture media produced no inhibition of growth. After 48 hr, inhibition was 37 per cent at an ara-CMeP concentration of 64 μg/ml. Comparable inhibition with ara-C was obtained at concentrations of 0.7 μg/ml. Table 1 shows the inhibition data obtained at 48 hr. From these studies, ara-C appears to be nearly ninety times as inhibitory as ara-CMeP.

Kinetic studies

Both ara-CMeP and ara-CMP inhibit the phosphorylation of dCMP by leukemia L1210 dCMP kinase. The results are presented in Fig. 2 as a Lineweaver–Burk plot. [2-^{14}C]dCMP was used as a substrate of dCMP kinase with ara-CMP or ara-CMeP as inhibitor. The concentration of ara-CMP or ara-CMeP was 5 mM in the reaction mixture. The K_m for dCMP was 2.1 × 10^{-3} M, which is similar to that previously reported for rat liver and calf thymus [12, 13]. Ara-CMP was a competitive inhibitor with a K_i of 4.4 × 10^{-3} M. Ara-CMeP was also a competitive inhibitor with a K_i of 4.0 × 10^{-3} M.

Phosphorylation studies

[γ-^{32}P]ATP was used to measure the rate of phosphorylation of ara-CMeP, dCMP and ara-CMP. The use of ^{32}P label in conjunction with a formic acid-DEAE cellulose chromatography system (see Materials and Methods) allowed the specific examination of ATP-mediated phosphate transfers to nucleotides. The control for these reactions was a mixture containing only enzyme while the reactions of interest contained, in addition, 5.2 mM ara-CMP, dCMP, or ara-CMeP. After chromatography, the nucleoside diphosphate spots were removed, counted, and then corrected by subtracting the radioactivity contained in the same area of the chromatogram of the control reaction. Initially, a 10-min incubation period was used to study the phosphorylation of all three substrates, and ara-CMeP was found not to exceed that of control. The net formation of [^{32}P]-labeled nucleoside diphosphates in the 10-min incubation was 21 μmoles with dCMP, 42 μmoles with ara-CMP, and 0.0 μmole with ara-CMeP. The more rapid rate of phosphorylation of ara-CMP compared to dCMP has been noted in the literature [13]. A 20-min incubation, later extended to 40 min, confirmed that ara-CMeP was not phosphorylated.

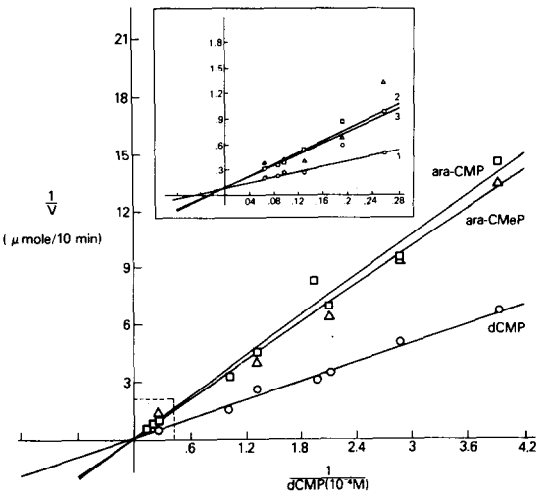

Fig. 2. Kinetic studies of dCMP kinase.

The chromatography system used to separate the mono-, di- and triphosphates does not distinguish specific nucleotides. Moreover, since all the nucleotide triphosphates remain at the origin, the system cannot distinguish dCTP, ara-CTP or ara-CMeTP from unreacted ATP. To exclude the possibility that ara-CMeP is selectively converted to the triphosphate, [8-^{14}C]labeled ATP was used. The rate of formation of [8-^{14}C]ADP measures all ATP-mediated phosphorylation in the reaction mixture and would detect the conversion of nucleoside monophosphate to triphosphates, a conversion which [γ-^{32}P]ATP would not detect. The use of [8-^{14}C]ATP showed no evidence of selective conversion of ara-CMeP to the triphosphate form.

Table 1. Effects of ara-C and ara-CMeP on P388 cells in culture*

Ara-C concn (μg/ml)	Per cent inhibition at 48 hr	Ara-CMeP concn (μg/ml)	Per cent inhibition at 48 hr
10	88.9	64	36.9
1	53.1	32	17.5
0.1	2.8	16	5.2
		1.6	3.5

* Leukemia P388 cells were maintained in static culture in RPMI No. 1630 medium with 10% fetal calf serum. Cell concentration was 10^5 cells/ml in a total volume of 5 ml. Cells were counted in a Coulter counter in duplicate.

DISCUSSION

Ara-CMeP was synthesized as an analogue of ara-CMP to circumvent resistance to ara-C as a result of deletion of CdR kinase. Based on the results of studies with 5-bromodeoxyuridine-5'-methylphosphonate [8], ara-CMeP was expected to have the following properties [9]: (a) to function as an analogue

of ara-CMP and be phosphorylated to the triphosphate form, (b) to penetrate cell membranes more easily than ara-CMP, and (c) to be resistant to phosphatase attack.

Growth of P388 leukemia *in vitro* is inhibited by the addition of ara-CMeP. However, concentrations of ara-CMeP 90-fold higher than ara-C were necessary for comparable inhibition. It is not clear whether this inhibition is due to ara-CMeP or to the breakdown of ara-CMeP to ara-C. Only a 1 per cent conversion of ara-CMeP to ara-C would account for the results obtained, a quantity too low to be detected by our chromatographic techniques. Conversely, the low level of ara-CMeP inhibition provides indirect evidence of the stability of ara-CMeP to serum phosphatase attack, supporting the report of Wigler and Lozzio [8] that such compounds are phosphatase resistant. The observed toxicity allows, at maximum, only a 1 per cent conversion of parent compound to ara-C since any greater conversion would have been reflected in greater cell kill. A possible reason for the phosphatase resistance is suggested by chemical mechanism studies on di-ionic phosphate monoesters which revealed that in hydrolysis the P—O bond is cleaved and that resonance stabilization of the leaving group is important [14]. Methyl group substitution would be expected to reduce this resonance stabilization and, consequently, make the P–O bond less susceptible to phosphatase attack.

If ara-CMeP functions as an analogue of ara-CMP, it must be capable of successive phosphorylation to the triphosphate form, thus requiring that it be a substrate for dCMP kinase. Ara-CMP is a substrate for this enzyme and competitively inhibits the phosphorylation of dCMP. Ara-CMeP competitively inhibits dCMP phosphorylation as effectively as ara-CMP, suggesting initially that ara-CMeP might be a substrate for this enzyme. However, chromatographic studies reveal that the γ-phosphate of [γ-^{32}P]ATP is not transferred to ara-CMeP.

The failure of ara-CMeP to be phosphorylated cannot be attributed to an inability to bind to the kinase because the kinetic studies show that the kinase has an equal affinity for both ara-CMeP, which it fails to phosphorylate, and ara-CMP, which it phosphorylates readily. Rather than indirectly affecting substrate–enzyme binding, the presence of a methyl group on the phosphate of ara-CMeP directly prevents further phosphate additions. The transfer of phosphate from ATP to a nucleoside monophosphate is thought to result from a nucleophilic attack by the acceptor molecule on the terminal phosphorus atom of ATP [15]. The detailed mechanisms of the enzymatic reaction have not been elucidated and the role played by the substitution of a methyl group for an oxygen atom in preventing the transfer is unclear [14].

Since ara-CMeP is resistant to further phosphorylation, it does not function as an ara-CMP analogue and, therefore, should be ineffective in the treatment of ara-C resistant tumors.

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