Biochemical Pharmacology, Vol. 58, pp. 709–714, 1999.
© 1999 Elsevier Science Inc. All rights reserved.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00138-0

9-(2-Phosphonylmethoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine (cpr-PMEDAP) as a Prodrug of 9-(2-Phosphonylmethoxyethyl)guanine (PMEG)

Mindy L. Compton, John J. Toole† and Lisa R. Paborsky*
GILEAD SCIENCES, INC., FOSTER CITY, CA 94404, U.S.A.

ABSTRACT. 9-(2-Phosphonylmethoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine (cpr-PMEDAP) is an acyclic nucleotide analog of the [9-(2-phosphonylmethoxyethyl)-] (PME) series containing a cyclopropyl substituent on the N^6 position of the 2,6-diaminopurine (DAP) base. Growth inhibition assays in a broad range of tumor cell lines demonstrated that this analog had potent antiproliferative activity with IC_{50} values similar to those of the structurally related guanine analog 9-(2-phosphonylmethoxyethyl)guanine (PMEG). A substantially lower growth inhibitory effect was observed for the 2,6-diaminopurine analog, PMEDAP. To dissect the basis for these varying potencies, the metabolism of the three analogs was examined in a human pancreatic carcinoma cell line, BxPC-3. HPLC analysis of the intracellular metabolites demonstrated that the cpr-PMEDAP was deaminated to PMEG and subsequently phosphorylated to PMEG mono- and diphosphates (PMEGp and PMEGpp). The level of PMEGpp generated from cpr-PMEDAP-treated cells was 50% greater than the level generated from cells incubated with PMEG. The presence of PMEG in the DNA of cells incubated with cpr-PMEDAP confirmed that the cpr-PMEDAP was converted to PMEG. In contrast, PMEDAP was not deaminated to PMEG, but directly phosphorylated to PMEDAPp and PMEDAPpp. The adenylate deaminase inhibitor 2′-deoxycoformycin (dCF) inhibited the conversion of cpr-PMEDAP in a rat liver cytosolic extract and increased the IC50 value for growth inhibition by 40-fold. The antiproliferative activities of PMEG and PMEDAP were unaffected by dCF. Thus, it appears that cpr-PMEDAP, but not PMEDAP, is converted by an adenylate deaminase-like enzyme and functions as a prodrug of PMEG. BIOCHEM PHARMACOL **58**;4:709–714, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. acyclic nucleotide analog; prodrug; N^6-cyclopropyl-2,6-diaminopurine; N^6-deamination of a 2,6-diaminopurine nucleotide; antiproliferative activity

The acyclic nucleoside phosphonates (phosphonates) are a novel class of nucleotide analogs characterized by a highly stable carbon–phosphorus bond between the acyclic nucleoside and the phosphate moiety. Intracellular conversion via two steps of phosphorylation generates the nucleoside analog diphosphate [1–3]. As structural derivatives of the natural nucleoside 5′-triphosphates, the nucleotide analog diphosphates are recognized as alternative substrates for viral DNA synthesis [4–6]. The broad-spectrum antiviral activities of several members of this class have been well described [7]. The oral prodrug of PMEA‡, bis (POM)-PMEA (adefovir dipivoxil, Preveon 228), currently is being evaluated in clinical trials for both HIV and hepatitis B virus infections. PMEDAP (Fig. 1), another member of this class, has biological activities similar to those of PMEA [8].

We recently described the potent antiproliferative activity of the guanine analog PMEG (Fig. 1). PMEG inhibits the growth of a wide range of solid tumor cell lines and induces cells to undergo apoptosis after blocking them in the S phase of the cell cycle [9]. Furthermore, PMEG was shown to increase the life-span of mice bearing both B16 melanoma and P388 leukemia [10]. Experiments with the purified cellular DNA polymerases α, δ, and ϵ demonstrated that PMEGpp was a competitive inhibitor of dGTP incorporation and that all three replicative enzymes could recognize PMEGpp as a substrate and incorporate the analog into DNA. Due to the lack of a 3′ hydroxyl group, which is required for further DNA chain elongation, PMEG functions as an absolute chain terminator following incorporation into DNA [11–14]. Taken together, these results indicated that PMEG may have potential as a cancer chemotherapeutic agent.

Cyclopropyl-PMEDAP (cpr-PMEDAP, Fig. 1) contains a cyclopropyl substituent at the N^6 position of the 2,6-diaminopurine ring and is analogous to 1592U89 (abacavir)

* Current address: Axys Pharmaceuticals, Inc., 180 Kimball Way, South San Francisco, CA 94080.

† Corresponding author: Dr. Jay Toole, Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA 94404. Tel. (650) 573-4751; FAX (650) 522-5611; E-mail: jay_toole@gilead.com

‡ *Abbreviations*: dCF, 2′-deoxycoformycin; HIV, human immunodeficiency virus; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEDAP, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine; PMEDAPp, PMEDAP monophosphate; PMEDAPpp, PMEDAP diphosphate; cpr-PMEDAP, 9-(2-phosphonylmethoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine; cpr-PMEDAPp, cpr-PMEDAP monophosphate; cpr-PMEDAPpp, cpr-PMEDAP diphosphate; PMEG, 9-(2-phosphonylmethoxyethyl)guanine; PMEGp, PMEG monophosphate; and PMEGpp, PMEG diphosphate.

Received 5 August 1998; accepted 28 December 1998.

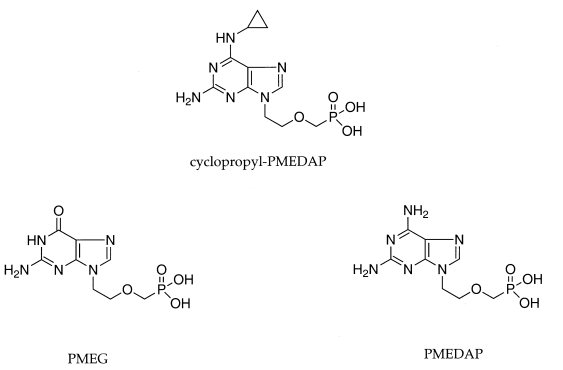

FIG. 1. Structures of PMEG, PMEDAP, and cpr-PMEDAP.

monophosphate. Abacavir is a selective inhibitor of HIV reverse transcriptase being developed for the treatment of acquired immune deficiency syndrome. A recent report [15] describing the intracellular activation of this nucleoside analog claimed that it first was phosphorylated to the monophosphate and then deaminated by a novel cytosolic enzyme to the guanine analog, carbovir monophosphate.

In this study, we describe the cellular metabolism of cpr-PMEDAP and provide evidence that this analog is functioning as a prodrug of PMEG. The same enzyme involved in the activation of abacavir monophosphate may be responsible for the deamination of cpr-PMEDAP.

MATERIALS AND METHODS

Materials

PMEG, PMEDAP, and cpr-PMEDAP along with their mono- and diphosphates were synthesized at Gilead Sciences. [^{3}H]PMEG, [^{3}H]PMEDAP, and [^{3}H]cpr-PMEDAP were radiolabeled by Moravek Biochemicals, Inc. with purities of $>$ 98, 98, and 93%, respectively. dCF was obtained from SuperGen, Inc. DNase I, proteinase K, alkaline phosphatase, and the protease inhibitor mixture were obtained from Boehringer Mannheim. Phosphodiesterase I was obtained from Worthington, and RNace-It ribonuclease preparation from Stratagene.

Growth Inhibition Assay

All cell lines were obtained from the American Type Culture Collection and were maintained according to their recommendations. Cell growth was analyzed using the supravital stain neutral red as previously described [16]. In short, cells were seeded at a density ensuring log phase growth through the incubation period and allowed to adhere prior to the addition of the phosphonates. Following 96 hr at 37°, 0.1 mg/mL of neutral red was added and incubated for 1.5 hr. The cells were washed with PBS and then solubilized in 50% EtOH containing 50 mM NaH_2PO_4. Absorbance was quantitated using a UVmax microplate reader at a test wavelength of 560 nm and a reference wavelength of 650 nm. In calculating the IC_{50} values, quadruplicate absorbance values corresponding to the initial cell numbers were subtracted from the 96-hr values. The resulting absorbance values were normalized to the control (no phosphonate) and were plotted as percent of control absorbance. The IC_{50} value was defined as the concentration of phosphonate required to inhibit cell growth by 50%.

For enzyme inhibitor experiments, a final concentration of 1 μM dCF was added to the cells along with the phosphonates.

Metabolism of cpr-PMEDAP, PMEDAP, and PMEG in BxPC-3 Cells

BxPC-3 cells were seeded at 1.5×10^4 cells/mL, and after 24 hr of growth, the cultures were treated with 1 μM [^{3}H]cpr-PMEDAP (14.1 Ci/mmol), [^{3}H]PMEDAP (13.8 Ci/mmol), or [^{3}H]PMEG (15.5 Ci/mmol) and incubated for 96 hr. Subsequently, the cells were collected, washed with PBS, and counted. Next, the cells were extracted with 500 μL of ice-cold methanol for 15 min. The extraction was repeated, and the supernatants were combined, centrifuged to remove cellular debris, and vacuum-dried overnight. The PMEG and cpr-PMEDAP metabolites were analyzed using a Partisil-10 SAX (4.6 × 250 mm) column eluted at 1 mL/min with a 50-min gradient of 20–200 mM KPO_4, pH 5.0. The PMEDAP metabolites were analyzed using a 40-min gradient of 25–250 mM KPO_4, pH 5.0, on the same column. One-milliliter fractions were collected, and the radioactivity in each fraction was determined by liquid scintillation counting. Radioactive peaks were identified by comparing their retention times to those of authentic standards. The counting efficiency was determined by measuring the radioactivity of a tritium standard (Wallac) in the elution buffers.

To convert picomoles/10^6 cells to molar concentrations, a cellular volume of BxPC-3 cells treated with a 1 μM concentration of each phosphonate for 96 hr was estimated as previously described [17] using [^{3}H]H_2O (1 mCi/mL) and inulin[^{14}C]carboxylic acid (6.4 mCi/mmol) obtained from Amersham. A volume of 5.0, 2.4, and 5.0 μL of intracellular water was determined per million BxPC-3 cells treated with PMEG, PMEDAP, and cpr-PMEDAP, respectively.

DNA Incorporation Studies

The insoluble material collected following the methanol extraction described above was resuspended in 10 mM Tris–HCl, pH 7.5, 0.5% SDS, 100 mM NaCl, 25 mM EDTA and incubated overnight at 50° with 0.2 mg/mL of proteinase K. The material was extracted twice by phenol: chloroform:isoamyl alcohol (25:24:1) and once by chloroform. After ethanol precipitation, the RNA was removed during a 3-hr incubation with 100 U/mL of RNace-It ribonuclease preparation at 37°. The phenol extraction and ethanol precipitation steps were repeated, and the resulting DNA (~ 60 μg) was digested for 1 hr at 37° with DNase I

TABLE 1. Antiproliferative activity of PMEG, cpr-PMEDAP, and PMEDAP

Cell line	Cell type	IC_{50}* (μM)		
		PMEG	cpr-PMEDAP	PMEDAP
BxPC-3	Pancreas	0.77 ± 0.03	3.3 ± 0.6	31 ± 4
SK-HEP-1	Liver	2.0 ± 0.1	11 ± 1	90 ± 14
SiHa	Cervix	2.9 ± 0.1	3.3 ± 0.8	329 ± 24
C33A	Cervix	2.1 ± 0.4	5.8 ± 0.8	52 ± 8
mCF07	Breast	15 ± 3	10 ± 1	66 ± 4
CEM	T lymphoblastoid	0.87 ± 0.12	1.4 ± 0.2	8.6 ± 1.6

*Results are means ± SD (N = 3).

(500 U/mg of DNA) in buffer containing 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, and 2 mM $CaCl_2$. The digest was repeated, and then the pH was adjusted to 9. The digested DNA was incubated twice for 1 hr with 2 U of phophodiesterase I and 5 U of alkaline phosphatase at 37°. To determine whether the DNA digestion was complete, approximately 10% of the sample was analyzed by reverse-phase chromatography on a C_{18} column (Vydac) eluted at 1 mL/min for 15 min with a gradient of 2–6% acetonitrile in 10 mM KPO_4, pH 5.0. The UV absorbance of the column effluent was monitored at 254 nm. To identify the phosphonate in the DNA digest, the remaining sample was loaded onto a Partisil-10 SAX (4.6 × 250 mm) column and eluted at 1 mL/min for 30 min with a gradient of 20–120 mM KPO_4, pH 5.0. Radioactivity in each 1-mL fraction was determined by liquid scintillation counting.

Preparation of Rat Liver Cytosolic Extract

A cytosolic extract was prepared from a fresh rat liver received from the vivarium at Gilead Sciences, Inc. Two grams of liver was minced and resuspended in 5 mL of 0.15 KCl containing protease inhibitor mixture and then Dounce-homogenized. After homogenization, the extract was centrifuged at 100,000 *g* for 1 hr. The supernatant then was collected and used fresh or was aliquoted and stored at −80° for future use.

Inhibition of cpr-PMEDAP Deamination with dCF in Rat Liver Extract

[^{3}H]cpr-PMEDAP or [^{3}H]PMEDAP (0.6 μM) in 20 mM HEPES buffer, pH 7.4, containing 10% of the rat cytosolic extract and increasing concentrations of dCF was incubated overnight at 37°. The reactions were stopped by the addition of acetonitrile, and the samples were centrifuged for 10 min before the supernatant was collected and vacuum-dried. The dried pellet was resuspended in 30 μL of water and then analyzed on a SAX ion-exchange column eluted at 1 mL/min for 30 min with a gradient of 50–250 mM KPO_4, pH 5.0. The UV absorbance of the column effluent was monitored at 254 nm, and fractions were collected at 1-min intervals. The radioactivity in each collected fraction was determined by liquid scintillation counting. The radioactive peaks were identified by their retention times using authentic standards.

RESULTS AND DISCUSSION

The ability of the three phosphonates (Fig. 1) to inhibit tumor cell growth was tested against a variety of solid tumor cell lines as well as a T lymphoblastoid cell line (Table 1). BxPC-3, a human pancreatic carcinoma cell line, was the most sensitive, with an IC_{50} value of 0.7 μM following a 96-hr incubation with PMEG. For the entire cell panel, the IC_{50} values for cpr-PMEDAP ranged from 1.4 to 11 μM, and were comparable to those of PMEG. In contrast, the IC_{50} values for the 2,6-diaminopurine analog, PMEDAP, ranged from 9 to 330 μM, a 5- to 120-fold decrease in the growth inhibitory effect compared with PMEG and cpr-PMEDAP. The relative antiproliferative potencies of the three phosphonates were PMEG ≥ cpr-PMEDAP ≫ PMEDAP.

Previous work has demonstrated that PMEG is transported into cells and then phosphorylated by cellular kinases to PMEGpp [1]. To determine the activation pathway for cpr-PMEDAP, BxPC-3 cells were incubated for 96 hr with 1 μM [^{3}H]cpr-PMEDAP. The intracellular metabolites were analyzed by ion exchange chromatography. A typical radioactivity elution profile is shown in Fig. 2A. The radioactive peaks at 11, 20, 22, and 29 min corresponded to PMEG, PMEGp, cpr-PMEDAP, and PMEGpp, respectively, based on the overlapping retention times with authentic standards. There was no evidence of PMEDAP or its phosphorylated metabolites. Neither cpr-PMEDAPp nor cpr-PMEDAPpp was detected following the incubation of cells with [^{3}H]cpr-PMEDAP. These results suggested that cpr-PMEDAP was converted to PMEG and then phosphorylated to the mono- and diphosphate of PMEG. As a comparison, the profile of intracellular metabolites produced in BxPC-3 cells treated for 96 hr with 1 μM [^{3}H]PMEDAP is shown in Fig. 2B. The identities of PMEDAP, PMEDAPp, and PMEDAPpp were based on the retention times for the authentic standards. In contrast to cpr-PMEDAP, PMEDAP was not deaminated to PMEG but was phosphorylated directly to PMEDAPp and PMEDAPpp. Neither PMEG nor phosphorylated metabo-

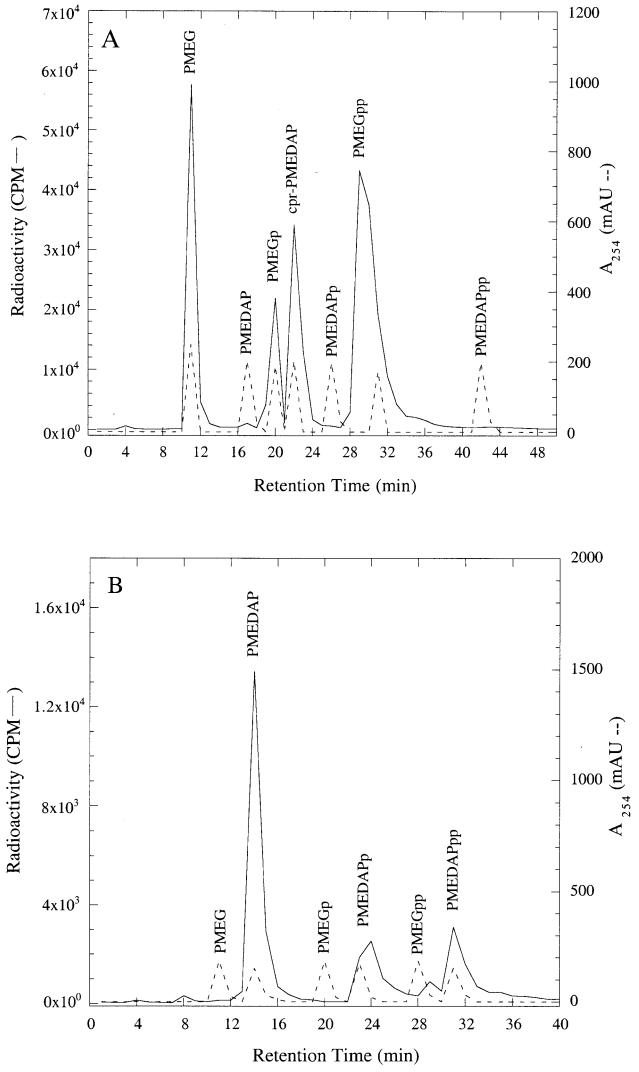

FIG. 2. Ion-exchange HPLC elution profiles of intracellular metabolites. BxPC-3 cells were incubated with (A) 1 μM [^{3}H]cpr-PMEDAP or (B) 1 μM [^{3}H]PMEDAP for 96 hr. The cells were collected, extracted with 60% methanol, and the metabolites were analyzed by SAX HPLC as described in Materials and Methods.

lites of PMEG were detected following the incubation of cells with [^{3}H]PMEDAP.

The intracellular levels of each metabolite generated from the incubation of BxPC-3 cells with 1 μM phosphonates are shown in Table 2. Examination of the distribution of the metabolites generated by each phosphonate suggested that in the case of PMEG and cpr-PMEDAP metabolism, the second phosphorylation step, i.e. the generation of PMEGpp from PMEGp, appeared to be rate-limiting to the accumulation of PMEGpp. For PMEDAPpp formation, the rate-limiting reaction was the initial phosphorylation step. The volume of the BxPC-3 cells treated with phosphonates was determined in order to convert picomoles to molar concentrations. The level of diphosphate generated was 240, 360, and 180 nM for cells treated with PMEG, cpr-PMEDAP, and PMEDAP, respectively. One apparent

TABLE 2. Levels of intracellular metabolites in BxPC-3 cells incubated for 96 hr with 1 μM [^{3}H]PMEG, [^{3}H]cpr-PMEDAP, or [^{3}H]PMEDAP

	Level of metabolites* (pmol/10^6 cells)		
Metabolities	**[^{3}H]PMEG**	**[^{3}H]cpr-PMEDAP**	**[^{3}H]PMEDAP**
cpr-PMEDAP	ND	1.3 ± 0.0	ND
PMEG	0.9 ± 0.4	0.7 ± 0.0	ND
PMEGp	1.7 ± 0.4	2.3 ± 0.1	ND
PMEGpp	1.2 ± 0.1	1.8 ± 0.0	ND
PMEDAP	ND	ND	1.0 ± 0.2
PMEDAPp	ND	ND	0.3 ± 0.1
PMEDAPpp	ND	ND	0.4 ± 0.1

*Results are measn ± range (N = 2). ND, not detected.

inconsistency in these data is the lack of correlation between the IC_{50} values and the level of diphosphates generated in BxPC-3 cells. The IC_{50} value for PMEG was 40-fold lower compared with the IC_{50} value for PMEDAP even though the level of diphosphate formed was comparable. The decreased growth inhibitory activity observed for the 2,6-diaminopurine analog may be due to the decreased affinity of PMEDAPpp compared with PMEGpp for the replicative enzymes. Studies with rat DNA polymerase α indicated that PMEGpp was a more potent inhibitor than PMEDAPpp [14]. However, the level of PMEGpp formed following incubation of the cells with cpr-PMEDAP was 50% greater than the level generated following PMEG incubation, which does not correlate with the 4.5-fold lower IC_{50} value for PMEG compared with the IC_{50} value for cpr-PMEDAP. Experiments are currently underway to explain this discrepancy.

The metabolism studies indicated that BxPC-3 cells deaminated cpr-PMEDAP to generate PMEG. To determine whether this corresponds with incorporation into DNA, the DNA from BxPC-3 cells incubated with [^{3}H]cpr-PMEDAP was purified and digested. To identify the incorporated phosphonate, the digested DNA was divided and analyzed by both reverse-phase and ion-exchange chromatography. Because the phosphonate bond is resistant to the alkaline phosphatase treatment used to convert the 2′-deoxynucleoside 5′-monophosphates to 2′-deoxynucleosides, the resulting analog retains its charged phosphonate group and is bound by the ion-exchange column. The reverse-phase elution profile demonstrated that the DNA digestion was complete and generated the four natural 2′-deoxynucleosides (Fig. 3, inset). The peak eluting at 8 min on the ion-exchange elution profile (Fig. 3) corresponded to PMEG. No peak was observed at the retention time for cpr-PMEDAP. The radioactive peak with a retention time identical to the 2′-deoxynucleosides was most likely the result of the accumulation of a trace amount of a labeled purine contaminant in the [^{3}H]cpr-PMEDAP preparation. Based on HPLC analysis, the radiolabeled cpr-PMEDAP had less than 1% of a purine contaminant (data not shown). A single peak of radioactivity coeluting with authentic PMEG was observed, thus verifying that cpr-

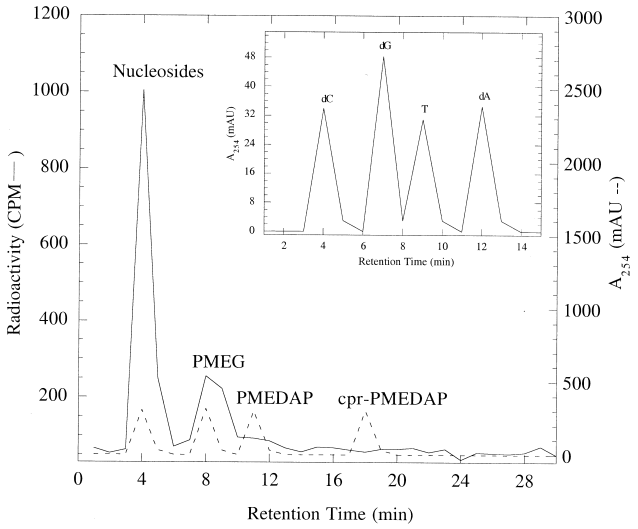

FIG. 3. Incorporation of PMEG into DNA. BxPC-3 cells in log phase growth were incubated with 1 μM [^{3}H]cpr-PMEDAP for 96 hr. Following the extraction of the intracellular metabolites, the DNA was purified. The digested DNA was analyzed by reverse-phase HPLC (inset) and SAX ion-exchange chromatography as described in Materials and Methods. The radioactivity in the collected peaks was used to identify the incorporated analog.

PMEDAP was converted to the parent PMEG, phosphorylated, and then incorporated into cellular DNA. Our previous results indicated that PMEGpp inhibited DNA synthesis by competing with dGTP for incorporation into elongating DNA strands *in vitro* [12, 13]. The absence of a surrogate 3′-OH group renders the analog incapable of further elongation; hence, it functions as a true DNA chain terminator [11–14]. Incorporation into cellular DNA is believed to be the mechanism responsible for the antiproliferative activity of other nucleoside analogs including gemcitabine [18] and fludarabine [19]. The prevention of DNA chain elongation and the competition between PMEGpp and dGTP for the nucleotide-binding site on the replicative DNA polymerases are the most likely mechanisms of action for this nucleotide analog. The appearance of PMEG in the DNA of cells grown in the presence of cpr-PMEDAP suggested that cpr-PMEDAP may be functioning by the same mechanism *in vivo*.

To determine whether the conversion of cpr-PMEDAP was required for its growth inhibitory activity, the effect of the adenylate deaminase inhibitor dCF on the antiproliferative activity of cpr-PMEDAP was examined. PMEG and PMEDAP were used as controls. BxPC-3 cells were grown in the presence of 1 μM dCF along with increasing concentrations of the three phosphonates. The resulting IC_{50} values generated are displayed in Table 3. As expected, the inhibitor had no effect on the antiproliferative activity of PMEG. The presence of dCF resulted in an increase in the IC_{50} value for cpr-PMEDAP, rendering it virtually without antiproliferative activity. Since dCF blocks metabolic deamination, these results demonstrated that deamination of cpr-PMEDAP was required for its antiproliferative activity. In contrast, adenylate deaminase inhibition did not have an effect on the antiproliferative activity of PMEDAP, demonstrating that deamination of this compound was not required for its growth inhibitory activity. These data are consistent with our metabolism results, demonstrating that PMEDAP was not converted to PMEG but directly phosphorylated to PMEDAP phosphates.

TABLE 3. Effect of dCF on the antiproliferative activity of PMEG, cpr-PMEDAP, and PMEDAP

Treatment	IC_{50}* (μM, 96 hr)
pMEG	0.7 ± 0.2
+ 1 μM dCF	0.9 ± 0.2
cpr-PMEDAP	3.3 ± 0.8
+ 1 μM dCF	136.2 ± 23.8
PMEDAP	30.2 ± 5.8
+ 1 μM dCF	28.4 ± 8.2

*Results are means ± SD (N = 3).

The abolition of the antiproliferative activity of cpr-PMEDAP in the presence of dCF implied the involvement of an adenylate deaminase in the activation pathway. As a large-scale source for this enzyme, a rat liver cytosolic extract was prepared. cpr-PMEDAP, but not PMEDAP, was converted to PMEG in a concentration- and time-dependent manner (data not shown). The conversion of 0.6 μM [^{3}H]cpr-PMEDAP with 10% of rat liver cytosolic extract was inhibited 96% by the presence of 50 μM dCF (Fig. 4). The data demonstrated that a deaminase activity present in the rat liver extract converted cpr-PMEDAP, but not

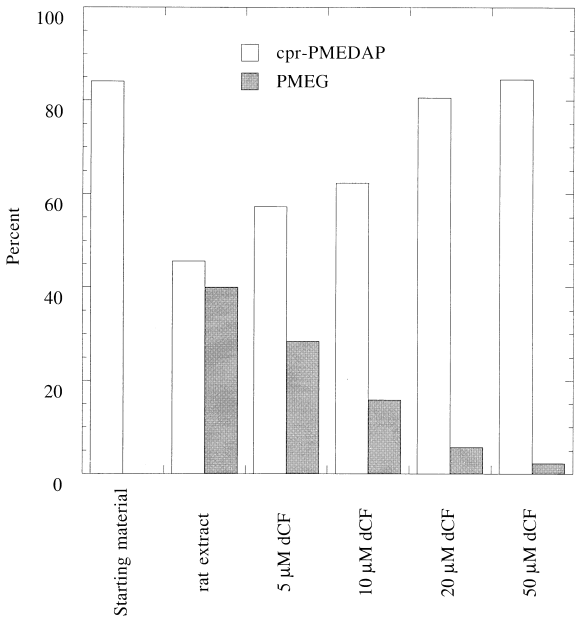

FIG. 4. Effect of dCF on the activation of cpr-PMEDAP. Rat liver cytosolic extract (10%) was incubated overnight with [^{3}H]cpr-PMEDAP (0.6 μM) along with the adenylate deaminase inhibitor dCF (0–50 μM). The percent of radioactivity shown as cpr-PMEDAP or PMEG was determined as described in Materials and Methods (N = 2). One hundred percent represents a 0.6 μM concentration of the phosphonate

PMEDAP, to PMEG and that the conversion was inhibited by the adenylate deaminase inhibitor, dCF.

In summary, our results indicated that cpr-PMEDAP functions as a prodrug of the guanine nucleotide analog PMEG, and that the conversion of this prodrug is required for its antiproliferative activity. Abacavir monophosphate, which has a structure similar to cpr-PMEDAP, is converted to a guanine analog by a cytosolic adenylate deaminase [15]. It is possible that cpr-PMEDAP may be activated by the same enzyme. Experiments currently are underway to purify this deaminase.

References

1. Ho HT, Woods KL, Konrad SA, De Boeck H and Hitchcock MJM, Cellular metabolism and enzymatic phosphorylation of 9-(2-phosphonylmethoxyethyl)guanine (PMEG), a potent antiviral agent. In: *Innovations in Antiviral Development and the Detection of Virus Infection* (Eds. Block T, Jungkind D and Crowell R), pp. 159–166. Plenum Press, New York, 1992.
2. Merta A, Votruba I, Jindřich J, Holý A, Cihlář T, Rosenberg I, Otmar M and Herve TY, Phosphorylation of 9-(2-phosphonomethoxyethyl)adenine and 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine by AMP(dAMP) kinase from L1210 cells. *Biochem Pharmacol* **44**: 2067–2077, 1992.
3. Cihlář T, Votruba I, Horska K, Liboska R, Rosenberg I and Holý A, Metabolism of 1-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)cytosine (HPMPC) in human embryonic lung cells. *Collect Czech Chem Commun* **57**: 661–672, 1992.
4. Balzarini J, Hao Z, Herdewijn P, Johns DG and De Clercq E, Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxyethyl)adenine, a potent anti-human immunodeficiency virus compound. *Proc Natl Acad Sci USA* **88**: 1499–1503, 1991.
5. Merta A, Votruba I, Rosenberg I, Otmar M, Hřebabacký H, Bernaerts R and Holý A, Inhibition of herpes simplex virus DNA polymerase by diphosphates of acyclic phosphonylmethoxyalkyl nucleotide analogues. *Antiviral Res* **13**: 209–218, 1990.
6. Votruba I, Trávníček M, Rosenberg I, Otmar M, Merta A, Hřebabecký H and Holý A, Inhibition of avian myeloblastosis virus reverse transcriptase by diphosphates of acyclic phosphonylmethyl nucleotide analogues. *Antiviral Res* **13**: 287–294, 1990.
7. De Clercq E, Broad spectrum anti-DNA virus and anti-retrovirus activity of phosphonylmethoxyalkylpurines and -pyrimidines. *Biochem Pharmacol* **42**: 963–972, 1991.
8. Naesens L, Balzarini J, Rosenberg I, Holý A, and De Clercq E, 9-(2-Phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP): A novel agent with anti-human immunodeficiency virus activity *in vitro* and potent anti-Moloney murine sarcoma virus activity *in vivo*. *Eur J Clin Microbiol Infect Dis* **8**: 1043–1047, 1989.
9. Paborsky LR, Mao CT and Toole JJ, Potent antiproliferative activity of a nucleotide analog, PMEG, against human solid tumor cell lines. *Proc Am Assoc Cancer Res* **38:** 100, 1997.
10. Rose WC, Crosswell AR, Bronson JJ and Martin JC, *In vivo* antitumor activity of 9-[(2-phosphonylmethoxy)ethyl]guanine and related phosphonate nucleotide analogues. *J Natl Cancer Inst* **82:** 510–512, 1990.
11. Cihlář T and Chen MS, Incorporation of selected nucleoside phosphonates and anti-human immunodeficiency virus nucleotide analogues into DNA by human DNA polymerases α, β and γ. *Antiviral Chem Chemother* **8:** 187–195, 1997.
12. Pisarev VM, Lee SH, Connelly MC and Fridland A, Intracellular metabolism and action of acyclic nucleoside phosphonates on DNA replication. *Mol Pharmacol* **52:** 63–68, 1998.
13. Kramata P, Downey KM and Paborsky LR, Incorporation and excision of 9-(2-phosphonylmethoxyethyl)guanine (PMEG) by DNA polymerases δ and ϵ *in vitro*. *J Biol Chem* **273**: 21966–21971, 1998.
14. Kramata P, Votruba I, Otová B and Holý A, Different inhibitory potencies of acyclic phosphonomethoxyalkyl nucleotide analogs toward DNA polymerases α, δ, and ϵ. *Mol Pharmacol* **49**: 1005–1011, 1996.
15. Faletto MB, Miller WH, Garvey EP, St. Clair MH, Daluge SM and Good SS, Unique intracellular activation of the potent anti-human immunodeficiency virus agent 1592U89. *Antimicrob Agents Chemother* **41:** 1099–1107, 1997.
16. Lowik CWGM, Alblas MJ, van de Ruit M, Papapoulos SE and van der Pluijm G, Quantification of adherent and nonadherent cells cultured in 96-well plates using the supravital stain neutral red. *Anal Biochem* **213:** 426–433, 1993.
17. Wohlhueter RM and Plagemann PGV, Measurement of the transport versus metabolism in cultured cells. *Methods Enzymol* **173**: 714–733, 1989.
18. Huang P, Chubb S, Hertel LW, Grindey GB and Plunkett W, Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* **51:** 6110–6117, 1991.
19. Huang P, Chubb S and Plunkett W, Termination of DNA synthesis by 9-β-arabinofuranosyl-2-fluoroadenine. A mechanism for cytotoxicity. *J Biol Chem* **265:** 4033–4040, 1990.