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A Human T Lymphoid Cell Variant Resistant to the Acyclic Nucleoside Phosphonate 9-(2-Phosphonylmethoxyethyl)adenine Shows a Unique Combination of a Phosphorylation Defect and Increased Efflux of the Agent

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SUMMARY

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is a new antiviral agent with activity against herpes viruses and retroviruses, including human immunodeficiency virus, but its metabolism and mechanism of action remain unclear. We have isolated a human T lymphoid cell line (CEMr-1) that is resistant to the antiproliferative effects of PMEA. The antiviral effects of PMEA against human immunodeficiency virus-1 infection were also greatly reduced in CEM-r1 cells, compared with the parental cells. This mutant showed cross-resistance to the related acyclic nucleoside phosphonates 9-(2-phosphonylmethoxyethyl)diaminopurine and 9-(2-phosphonylmethoxyethyl)guanine and the lipophilic prodrug bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine-(bispom-PMEA), as well as partial resistance to the purine nucleosides 2-chlorodeoxyadenosine, 2-fluro-9-β-D-arabinosylfuranosyladenine, and adenosine, but did not show resistance to 2′-deoxyadenosine or 9-β-D-arabinosylfuranosyladenine. We compared the uptake and metabolism of [^{3}H]PMEA and [^{3}H]-bispom-PMEA in the mutant and parental cells. The analysis of radioactive products by high pressure liquid chromatography revealed marked alterations in the ability of the mutant cell line to accumulate PMEA and its anabolites, compared with the parental cells. Accumulation of PMEA, PMEA monophosphate, and PMEA bisphosphate (major metabolites formed with either PMEA or bispom-PMEA) decreased by 50, 95, and 97%, respectively. Compared with the parental cells, the variant cells showed a ~7-fold increase in the rate of efflux of PMEA and a 2-fold decrease in the activity of adenylate kinase. In contrast, other enzymes of nucleotide metabolism, such as adenosine kinase, deoxycytidine kinase, and 5-phosphoribosyl-1-pyrophosphate synthetase, showed no significant change in the two cell lines. Overall, these results suggest that the mutation in this resistant cell line is of a novel type, involving an alteration in the cellular efflux of PMEA as the major basis for the resistant phenotype.

The acyclic nucleoside phosphonate PMEA is a new broad-spectrum antiviral agent that demonstrates potent antiviral activity against various DNA viruses and retroviruses, including the HIVs (1–14). The *in vivo* antiviral efficacy of PMEA has been documented in various animal retroviral models, including murine leukemia/sarcoma virus infections in mice, feline immunodeficiency virus infections in cats, and simian immunodeficiency virus infections in macaques (5, 7, 9, 10). In a recent report, PMEA was shown to be particularly effective in preexposure prophylaxis of simian immunodeficiency virus infections in macaques and was found to completely suppress viremia and disease symptoms in all of the treated animals (14). PMEA and its lipophilic prodrug bispom-PMEA (15) have currently entered phase I clinical trials as treatment for HIV infections (16, 17). PMEA acts as a stable monophosphate analog of AMP and dAMP, and its antiviral activity is thought to require activation to the diphosphate derivative PMEApp, which then acts to inhibit viral DNA polymerases with relative sparing of cellular DNA replication (8, 11, 18). However, the exact mode of metabolism and action of PMEA and related acyclic phosphonate analogs remains unclear. Studies have suggested that PMEA enters cells via endocytosis and is further

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ABBREVIATIONS: PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMEAp, 9-(2-phosphonylmethoxyethyl)adenine monophosphate; PMEApp, 9-(2-phosphonylmethoxyethyl)adenine bisphosphate; HIV, human immunodeficiency virus; RT, reverse transcription; PMEG, 9-(2-phosphonylmethoxyethyl)guanine; PMPDAP, 9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine; PMEDAP, 9-(2-phosphonylmethoxyethyl)diaminopurine; bispom-PMEA, bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine; monopom-PMEA, pivaloyloxymethyl-9-(2-phosphonylmethoxyethyl)adenine; MDR, multiple drug-resistant; PRPP, 5-phosphoribosyl-1-pyrophosphate; HPMPA, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)adenine; HPLC, high pressure liquid chromatography.

metabolized to PMEAp and PMEApp by cellular enzymes (19). An initial report suggested that the anabolism of PMEA may involve direct conversion to PMEApp via PRPP synthetase, although direct evidence for this mechanism in intact cells has not been obtained (11). We now describe studies carried out with a human T lymphocytic cell line that was selected after exposure to cytotoxic concentrations of PMEA. In these studies we have examined the resistance profile and metabolism of PMEA and its lipophilic prodrug bispom-PMEA. Our results show that the variant cells, termed CEMr-1, were limited in their ability to accumulate PMEA and its metabolites and exhibited enhanced cellular export of PMEAp. A modest alteration in phosphorylation of PMEA was also observed in the variant cells.

Materials and Methods

Chemicals. PMEA, PMEDAP, PMEG, PMEApp, bispom-PMEA, and monopom-PMEA were kindly provided by Dr. Norbert Bischofberger, Gilead Sciences (Foster City, CA). [2,8-^{3}H]PMEA (17 Ci/mmol) and [^{3}H]bispom-PMEA (21 Ci/mol) were obtained from Moravek Biochemicals (Brea, CA). The radioactive compounds were repurified before each experiment. All other nucleoside/nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and virus. The human T lymphoid cell line CEM-SS and HIV-1_{IIIB} were obtained from the National Institutes of Health/National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Ogden BioServices, Rockville, MD). CEM-r1, a PMEA-resistant variant of CEM-SS, was selected as described below. All cells were maintained in modified Eagle's medium (BioWhittaker, Walkersville, MD) containing 10% (v/v) heat-inactivated (56°, for 0.5 hr) newborn bovine serum (HyClone, Logan, UT) and 2 mM L-glutamine. HIV-1_{IIIB} was propagated in CEM-SS cells, and cell-free virus stocks were stored in the vapor phase of liquid N_2 cylinders until further use.

Cytotoxicity assays. All assays were performed in 24-well tissue culture plates (Costar, Cambridge, MA). CEM-SS or CEM-r1 cells were seeded at a density of 4×10^5 cells/well, in the presence or absence of the test compounds, and were allowed to grow for 48 hr (approximately two doublings) at 37° in a humidified CO_2-controlled atmosphere. At the end of the incubation, the cell concentrations, sizes, and volumes were counted in a Coulter counter (Coulter Electronics, Hialeah, FL). The data were used to calculate the 50% growth inhibitory concentrations (IC_{50}) of the various test compounds.

RT assays. The antiviral effects of the different drugs were monitored by RT assays according to previously described procedures (20, 21). CEM-SS or CEM-r1 cells were infected with an inoculum of HIV-1_{IIIB} standardized to contain 1 RT cpm/cell, and the virus-infected cells were seeded at a concentration of 0.2×10^6 cells/ml in medium containing varying concentrations of PMEA (or other test compounds). After 5 days of incubation, the RT activity of the culture supernates was determined by previously described procedures (22). Briefly, the reactions were carried out in a total volume of 50 μl, using 5 μg/ml poly(A)$^+$, 1.6 μg/ml oligo(dT)$_{12-18}$, and 1 μCi of [^{3}H]TTP in 50 mM Tris, pH 7.8, 75 mM potassium chloride, 5 mM magnesium chloride, 2 mM dithiothreitol, 0.05% Nonidet P-40. The reaction was initiated by addition of 10 μl of virus-containing tissue culture supernatant. After 1 hr of incubation, 10 μl of the reaction mixture were spotted on a Whatman DE-81 filter paper, air dried, and washed four times with 2× standard saline citrate (0.3 M NaCl, 0.03 M sodium citrate). The filter papers were dried, transferred to a plastic bag containing scintillation cocktail, sealed, and counted in an LKB Betaplate reader. The drug concentrations yielding half-maximal RT activity (ED_{50}) was calculated as a measure of the antiviral efficacy of the test compounds, using the nonlinear curve-fitting software Enzfitter (Elsevier Biosoft, Cambridge, UK).

Uptake and metabolism of [^{3}H]PMEA and [^{3}H]bispom-PMEA. Exponentially growing CEM-SS and CEMr-1 cells were harvested by centrifugation, resuspended at 1×10^6 cells/ml in fresh medium in the presence of [^{3}H]PMEA or [^{3}H]bispom-PMEA, and incubated at 37°. At the indicated time points, aliquots of the cells were removed and centrifuged through Nyosil 50 (W. F. Nye, Inc., New Bedford, MA) at 13,000 × *g* for 60 sec at 4°. When bispom-PMEA was investigated, an aliquot of the cell-free medium was also removed and analyzed by HPLC for determination of extracellular metabolites. The cell pellet was extracted with 70% ice-cold methanol and the aqueous phase was collected and analyzed for intracellular tritiated PMEA or tritiated bispom-PMEA and their metabolites by using HPLC analysis, essentially as described previously (23, 24). Peaks were identified by chromatography of authentic standards.

Efflux of [^{3}H]bispom-PMEA and its metabolites. CEM-SS and CEMr-1 cells were preincubated at 1×10^6 cells/ml with [^{3}H]bispom-PMEA (2 μCi/ml) for 15 min, washed with ice-cold medium by centrifugation, resuspended at the same concentration in drug-free medium, and maintained at 37°. At the indicated times, extracts were prepared from the cells and the medium, and the prodrug and its various nucleoside/nucleotide metabolites were analyzed by HPLC as described previously (24).

Results

Selection of PMEA-resistant cells. Selection for PMEA resistance was carried out by exposing CEM-SS cells in culture to increasing concentrations of PMEA, over several months. Of the various lines that grew in the presence of high concentrations of PMEA, one (designated CEMr-1) was selected and cultured for at least 10 generations in PMEA-free medium before subsequent studies. CEMr-1 cells, cultured in the presence or absence of PMEA, were morphologically indistinguishable from the parent CEM-SS cells. However, as shown in Fig. 1, CEM-r1 cells were ~250-fold more resistant to the cytotoxic effects of PMEA, compared with the parental cells. This drug resistance of CEM-r1 cells has remained stable for at least 3 months of cell culturing in the absence of PMEA. We also compared the antiviral efficacy of PMEA against HIV-1_{IIIB} replication in CEM-r1 and parental cells. As shown in Table 1, the ED_{50} of PMEA and its derivative PMPDAP was at least 20-fold higher in CEM-r1 cells than in parental CEM-SS cells.

Uptake and metabolism of [^{3}H]PMEA. Because PMEA requires activation to the diphosphorylated derivative PMEApp

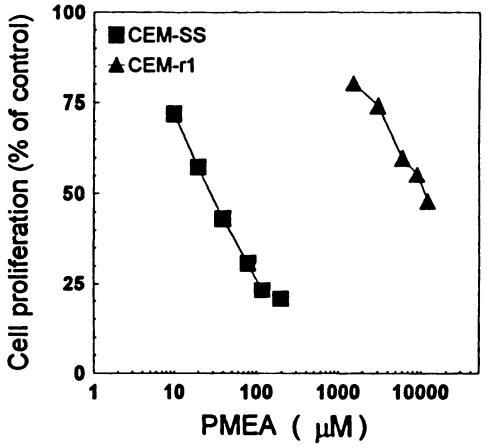

Fig. 1. Cytotoxicity of PMEA in CEM-SS and CEM-r1 cells. Exponential cultures were incubated with PMEA, and the increases in cell numbers were monitored after 48-hr incubations, using a Coulter counter, as described in Materials and Methods. The results are the mean from two independent experiments done in duplicate.

TABLE 1

Anti-HIV activity of PMEA and PMPDAP in CEM-SS and CEM-r1 cells

The antiviral efficacy of the drugs against HIV-1_{IIIB} was determined as described in Materials and Methods, using an RT assay to monitor virus yield. Data shown are for one representative experiment, which has been repeated with essentially identical results.

Drug	ED_{50}	
	CEM-SS	CEM-r1
	μM	
PMEA	5	>40
PMPDAP	0.625	>10

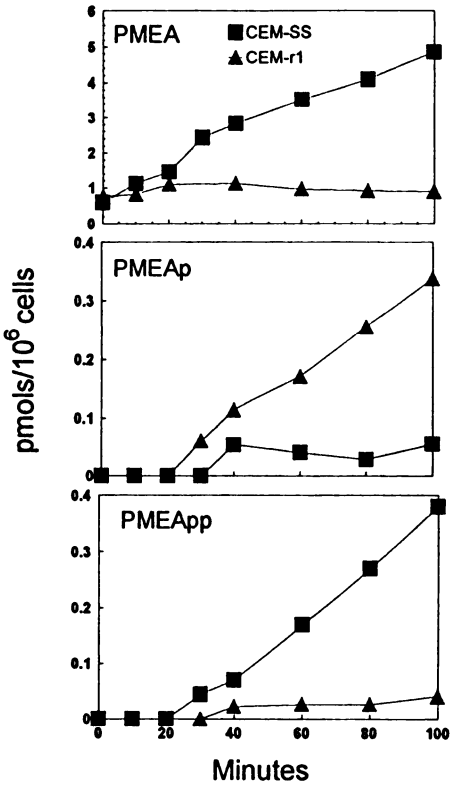

Fig. 2. Uptake and metabolism of [^{3}H]PMEA in CEM-SS and CEM-r1 cells. Exponentially growing cultures were incubated with 10 μM [^{3}H]-PMEA, and at various intervals the cell extracts were analyzed for PMEA and its metabolites by ion exchange HPLC, as described in Materials and Methods. These results are from an experiment that was repeated twice, with identical results.

for its activity, we determined whether the mutant cells exhibit altered drug anabolism. The mutant and parental cells were incubated with 10 μM [^{3}H]PMEA and at various times were analyzed for intracellular levels of PMEA and its metabolites by Partisil SAX HPLC. In the parental cells the accumulation of PMEA and its anabolites PMEAp and PMEApp was dependent on the concentration of PMEA in the external medium at least up to 2 mM, with no evidence of saturation in either the uptake or phosphorylation of the drug (data not shown). The parental CEM-SS cells accumulated PMEA and its anabolites PMEAp and PMEApp in a relatively linear fashion for at least 100 min, during which measurements were made. In contrast, CEMr-1 cells accumulated markedly lower levels of PMEA than did the parental cells and there were virtually no anabolites, PMEAp and PMEApp, detectable within this time period (Fig. 2). The metabolism of [^{3}H]PMEA was also examined in both cell lines after longer periods (i.e., 8 and 12 hr) of incubation with 10 μM [^{3}H]PMEA. As shown in Table 2, in wild-type cells intracellular PMEA levels measured after 8 and 12 hr reached 1.7 and 2.5 μM, respectively, and PMEAp and PMEApp concentrations increased to 0.23 and 0.41 μM, respectively, after a 12-hr incubation period. In contrast, PMEA levels in CEMr-1 cells after 12 hr reached only 1 μM (40% of the wild-type level) and PMEAp and PMEApp levels were only 2–3% of parental cell levels after 12 hr.

Uptake and metabolism of [^{3}H]bispom-PMEA. These lower levels of PMEA and anabolites in CEMr-1 cells could result from decreased uptake, increased excretion, and/or decreased activation of the drug. However, the level of radioactive PMEA accumulating in CEMr-1 cells was too low for a detailed analysis of the specific mechanism involved. We, therefore, investigated the metabolism of [^{3}H]bispom-PMEA (15), a lipophilic prodrug of PMEA, in the two cell lines to further examine the mechanism of resistance in the mutant cell line. We have previously shown that bispom-PMEA, unlike PMEA, is rapidly taken into cells and converted by cellular esterases to unmodified PMEA (24). Fig. 3 shows data on the early time course (120 sec) of 1 μM [^{3}H]bispom-PMEA entry into the parental and CEMr-1 cells. As can be seen, there was no significant difference between the two cell lines in their initial uptake of bispom-PMEA. However, when the cells were incubated for longer periods with [^{3}H]bispom-PMEA, significant differences in the accumulation of drug metabolites were observed. Table 3 depicts results from an experiment in which

TABLE 2

Intracellular accumulation of PMEA, PMEAp, and PMEApp in CEM-SS and CEM-r1 cells

Exponentially growing cultures were incubated with 10 μM [^{3}H]PMEA for 8 or 12 hr and the cell extracts were analyzed for PMEA and its metabolites by ion exchange HPLC, as described in Materials and Methods.

	Intracellular levels			
	CEM-SS		CEM-r1	
	8 hr	12 hr	8 hr	12 hr
	μM			
PMEA	1.7	2.5	1.0 (56%)	0.99 (40%)
PMEAp	0.13	0.23	0.005 (3.8%)	0.005 (2.2%)
PMEApp	0.22	0.41	0.008 (3.6%)	0.01 (2.4%)

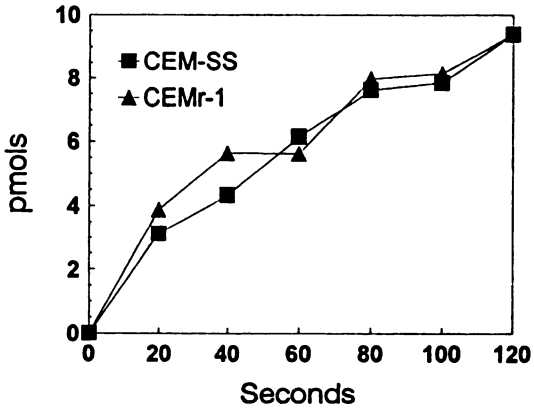

Fig. 3. Uptake of [^{3}H]bispom-PMEA in CEM-SS and CEM-r1 cells. Exponentially growing cultures were incubated with 1 μM [^{3}H]bispom-PMEA, and at the indicated times incubations were terminated by centrifugation of cells through an inert oil phase. The amounts of bispom-PMEA internalized at various intervals of incubation are shown. The data are from a typical experiment, which has been repeated several times with identical results.

TABLE 3

Metabolism of [^{3}H]bispom-PMEA in CEM-SS and CEMr-1 cells

Exponentially growing cultures were incubated with 1 μM [^{3}H]bispom-PMEA for 15 min and the various metabolites in the cell extracts and media were analyzed by ion exchange HPLC, as described in Materials and Methods. Data are shown for one individual experiment; essentially similar results were obtained in a second independent experiment.

Metabolite	Metabolite levels			
	Cells		Medium	
	CEM-SS	CEM-r1	CEM-SS	CEM-r1
	μM		nM	
PMEA	33.1	13.3	4.8	23.2
PMEAp	0.48	0.17	ND[a]	ND
PMEApp	0.16	0.07	ND	ND
Bispom-PMEA	0.03	0.04	490	429
Monopom-PMEA	4.6	4.1	185	166

[a] ND, not determined.

the two cell lines were incubated with 1 μM [^{3}H]bispom-PMEA for 15 min. As shown previously (24), the extent of accumulation of PMEA and its metabolites PMEAp and PMEApp in the parental CEM-SS cells was much higher after a 15-min incubation with 1 μM bispom-PMEA than after an 8-hr incubation with 10 μM PMEA (compare Tables 2 and 3). In the resistant CEM-r1 cells, however, incubation with 1 μM [^{3}H]-bispom-PMEA resulted in levels of tritiated PMEA, PMEAp, and PMEApp that were only about one third of those seen in parental cells. Only ~2% of the radioactivity present in the cells after incubation could be detected as the intact prodrug and this was similar in parental and resistant cells, indicating that there was no significant difference in the hydrolysis of the prodrug to the unmodified parental drug in the two cell lines.

The results in Table 3 also include the levels of various metabolites in the medium of cells incubated with tritiated [^{3}H]-bispom-PMEA. Most of the radioactivity remaining in the medium was associated with bispom-PMEA and monopom-PMEA, and this was comparable for the two cell lines. However, a significant difference was seen in the levels of extracellular PMEA, which were ~5-fold higher with the mutant CEM-r1 cells than with the parental CEM-SS cells. Control experiments revealed that under these conditions direct extracellular hydrolysis of bispom-PMEA in the medium did not contribute significantly to PMEA in the medium (data not shown). Thus, this observation suggested that the appearance of PMEA in the medium was due to the excretion from cells of the drug derived from intracellular metabolism of bispom-PMEA.

Measurements of efflux of [^{3}H]PMEA. To examine whether the resistant cells could be altered in their capacity to retain the phosphonate analog, we compared the clearance of [^{3}H]PMEA and its metabolites from CEM-SS and CEMr-1 cells. CEM-SS or CEMr-1 cells were incubated for 15 min with 1 μM or 2 μM [^{3}H]bispom-PMEA, respectively, in an attempt to accumulate comparable levels of intracellular PMEA and anabolites, and were then incubated in drug-free medium. Aliquots of cells and medium sampled at different intervals over a period of 2 hr were analyzed by HPLC, and the results are shown in Fig. 4. The intracellular levels of radioactivity associated with PMEA declined gradually in the parental cells, and half-maximal clearance was attained within about 60 min. Some of this clearance was probably due to the anabolism of PMEA, because the PMEAp- and PMEApp-associated radioactivity continued to accumulate within the cells, but the

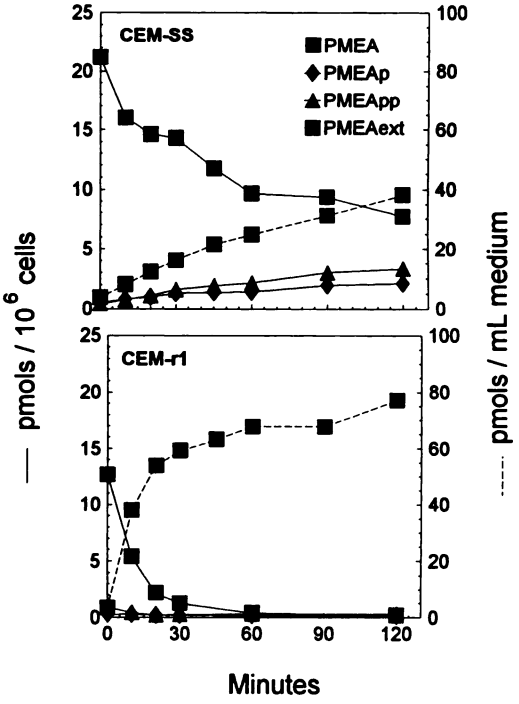

Fig. 4. Efflux of PMEA from [^{3}H]bispom-PMEA-treated cultures. Exponentially growing cultures of CEM-SS and CEM-r1 cells were incubated for 15 min with 1 μM or 2 μM [^{3}H]bispom-PMEA, respectively. The cell extracts and media were analyzed by ion exchange HPLC as described in Materials and Methods.

majority of the radioactivity was excreted into the medium as PMEA. In contrast to the results with parental cells, loss of intracellular PMEA-associated radioactivity in the resistant CEM-r1 cells was extremely rapid and the majority of the radioactivity within the cells was excreted into the medium within 20–30 min of incubation (Fig. 4). From the increases in the extracellular radioactivity accumulated in the medium, one can calculate that the rate of efflux of PMEA was increased ~7-fold to 2.55 pmol/min/10^6 in resistant CEM-r1 cells, from 0.4 pmol/min/10^6 cells in parental CEM-SS cells.

Cross-resistance to other agents. The relative sensitivity of the wild-type and CEM-r1 cells to structurally related and unrelated analogs has also been examined. As shown in Table 4, the CEMr-1 cells are highly cross-resistant to the prodrug bispom-PMEA and the 2,6-diaminopurine derivative PME-DAP. Only partial cross-resistance was observed against two related nucleoside phosphonates, HPMPA and PMEG (Table 4). Interestingly, the mutant was also partially resistant to a number of unrelated purine nucleosides, including adenosine, 2-chlorodeoxyadenosine, and 2-fluoroarabinosyladenine, but not to arabinosyladenine, 2′-deoxyadenosine, hydroxyurea, or the adenine nucleotide precursor 5-amino-4-imidazolecarboxamide riboside. CEMr-1 cells were also not resistant to vinblastine and colchicine, two agents that are characteristic of the MDR phenotype in a number of mutant cell lines selected against these agents (25). It should be noted that the MDR mutant CEM/VLB_{100} (26) did not exhibit any cross-resistance to PMEA (data not shown).

Enzyme levels. Cytosolic extracts of CEM-SS and CEM-r1 cells were also examined for differences in enzyme activity of potential importance for nucleoside/nucleotide analog metabolism. The two cell lines had indistinguishable activities of

TABLE 4

Comparative resistance of CEM and CEMr-1 cells to various agents

The IC_{50} values were obtained after 2 days of culture. Each result is the mean of one representative experiment performed in duplicate, as described in Fig. 1. The cultures with the purine nucleosides adenosine, 2′-deoxyadenosine, and arabinosyladenine contained 5 μM deoxycoformycin to inhibit adenosine deaminase activity.

Compound	IC_{50}		Relative resistance
	CEM-SS	CEM-r1	
	μM		
PMEA	34	6200	182
PMEDAP	2.3	1200	533
Bispom-PMEA	0.26	32	123
PMEG	0.7	19	30
HPMPA	92	1100	12
Adenosine	38	205	5.4
2-Chlorodeoxyadenosine	0.08	0.4	5.5
2-Fluoroarabinosyladenine	1.2	4.4	3.7
Arabinosyladenine	0.7	0.9	~1
2′-Deoxyadenosine	1.7	2.5	~1
5-Amino-4-imidazolecarboxamide	130	106	~1
Hydroxyurea	55	50	~1
Vinblastine	0.0018	0.0021	~1
Colchicine	0.014	0.013	~1

TABLE 5

Purine nucleoside-phosphorylating enzymes in CEM-SS and CEM-r1 cells

Mean values from at least two independent determinations are shown. The different enzyme activities, adenosine kinase (24), deoxycytidine kinase (24), PRPP synthetase (11), and adenylate kinase (25), were determined according to previously described procedures.

Enzyme	Activity	
	CEM-SS	CEM-r1
	nmol/hr/mg of protein	
Adenosine kinase	143	168
Deoxycytidine/deoxyadenosine kinase	4.5	4.9
PRPP synthetase	492	480
Adenylate kinase	2675	1442

adenosine kinase and deoxycytidine/deoxyadenosine kinase, enzymes that phosphorylate the various purine nucleosides described above to their 5′-phosphorylated derivatives (27–30), and PRPP synthetase, which has been implicated in the phosphorylation of PMEA (11) (Table 5). However, the resistant CEM-r1 cells did exhibit a 2-fold decrease in the activity of adenylate kinase, compared with the wild-type CEM-SS cells (Table 5), suggesting that this enzyme may be important for the phosphorylation of PMEA.

Discussion

The PMEA-resistant cell line CEMr-1 is the first example of a human cell line selected specifically for resistance to the acyclic nucleoside phosphonates. Compared with the parental CEM-SS cells, the CEMr-1 variant was ~180-fold more resistant to PMEA and also showed varying degrees of cross-resistance to related phosphonate derivatives and a number of purine nucleoside analogs. Biochemical characterization of the CEMr-1 variant revealed that the resistance to PMEA was associated with a novel mechanism involving both an anomaly in its capacity to accumulate the phosphonate analog and reduced phosphorylation to the higher anabolites PMEAp and PMEApp.

Previous studies of PMEA and its pyrimidine derivative (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine have shown that these compounds enter cells via a process of fluid-phase endocytosis in a variety of cell types, including CEM-SS (19, 23). This is consistent with the very slow permeation of PMEA into CEM-SS cells and the lack of saturability in uptake of PMEA seen in the present study. The availability of a lipophilic prodrug of PMEA, bispom-PMEA, in which the charges associated with the phosphonyl groups of PMEA are masked by hydrolyzable bispivaloyloxymethyl groups, facilitated additional studies to characterize the nature of the defects in the CEMr-1 cells. Consistent with earlier studies (24), bispom-PMEA was rapidly taken into cells, and the entry was similar in both sensitive and resistant cells. However, the level of PMEA accumulation from the prodrug in the resistant cells was markedly decreased, compared with that in the sensitive cells. The decrease in the intracellular concentration of the drug was associated with a ~5–6-fold increase in the appearance of PMEA in the medium of the resistant cells, compared with the parental cells. These data therefore suggested that CEMr-1 cells can remove PMEA from the cells, thus precluding its accumulation to high intracellular concentrations. Evidence for this efflux mechanism was provided by the direct demonstration of an ~7–8-fold increase in the excretion of radiolabeled PMEA into the medium from cells preincubated with [^{3}H]-bispom-PMEA.

The nature of this efflux pathway for PMEA has not yet been established but is likely to be distinct from the P-glycoproteins encoded by the *MDR* gene locus, because (i) CEM-r1 cells were not cross-resistant to vinblastine and colchicine, two agents that are characteristic of the MDR phenotype in a number of mutant cell lines (25), and (ii) CEM/VLB_{100}, a cell line overexpressing the *MDR* gene product (26), did not exhibit any cross-resistance to PMEA (data not shown). Experiments implying outward transport of nucleoside monophosphates from mammalian cells have been reported for cAMP and a 5′-phosphate of tricyclic 7-deazapurine nucleoside (31, 32). Thus, it seems possible that PMEA export may be mediated by a transport system for cAMP or, alternatively, it may represent a previously undefined efflux system that may limit the accumulation of toxic anionic metabolites. A detailed investigation of this potentially important mechanism is currently underway.

The PMEA-resistant CEMr-1 cells also displayed a 50% decrease in adenylate kinase activity, compared with the parental CEM-SS cell line. Compared with the parental CEM-SS cells, the accumulation of PMEAp and PMEApp in CEM-r1 cells was decreased ~8–9 fold, whereas the PMEA levels showed a ~2-fold decrease. Thus, these results indicate that the decrease in the formation of PMEA anabolites in the CEMr-1 cells can be attributed partly to an increased efflux of PMEA from the cytosol and partly to reduced phosphorylation mediated by the decrease in adenylate kinase activity. The enzyme responsible for the phosphorylation of PMEA (and related phosphonates) in intact cells has not been established. In an initial report, Balzarini *et al.* (11) showed that PMEA can be phosphorylated *in vitro* directly to the diphosphorylated form PMEApp by PRPP synthetase, using PRPP as the pyrophosphate donor. In subsequent studies, Merta *et al.* (33) showed that murine L1210 cell adenylate kinase is able to phosphorylate PMEA. The emergence under selective PMEA pressure of a deficiency in adenylate kinase activity, with a concomitant

decrease in PMEA phosphorylation, provides evidence for adenylate kinase as the activating (phosphorylating) enzyme for PMEA.

It could be argued that the increased efflux of PMEA from CEMr-1 cells is a secondary effect arising from the decreased phosphorylation of PMEA and thus may not involve any mutations involving an efflux pathway. This explanation, however, appears unlikely for several reasons. The CEMr-1 cells were selected by stepwise increases in PMEA concentrations, ranging from 0.8 to 10 mM, and the alteration in adenylate kinase did not parallel the changes in the excretion of PMEA. A ~2-fold reduction in adenylate kinase levels was observed after selection in 0.8 mM PMEA, after which no further change was noted, whereas PMEA efflux showed progressive increases with increased drug resistance. Also, if AMP kinase were the only change in the resistant cells, then this might be expected to bring about an increase, rather than the observed decrease, in the levels of PMEA as a result of the decreased phosphorylation. Moreover, the fact that CEM-r1 cells are cross-resistant to PMEG, which is not phosphorylated via adenylate kinase, further points to distinct changes in the CEM-r1 cells. Therefore, our results seem to be most consistent with the hypothesis that PMEA resistance in CEM-r1 cells involves two independent mutations, one leading to a 50% reduction in the adenylate kinase activity, which determines partial resistance to PMEA and probably to a variety of other adenine nucleoside analogs, and a second mutation that activates a novel efflux mechanism that contributes to the high level of resistance of CEM-r1 cells to phosphonates.

The 8–9-fold decrease in PMEApp levels observed in CEMr-1 cells may not explain quantitatively the 180-fold resistance that is observed in these cells. The reason for this apparent discrepancy is not clear; however, a similar phenomenon has been seen with the MDR phenotype (25). Possible explanations for this discrepancy may include (i) changes in the intracellular nucleotide pools that compete with PMEApp for binding to its cellular target(s) or (ii) sequestration of PMEA in a vesicular compartment such as the lysosome or an exocytic vesicle (as a part of the efflux pathway), which renders it unavailable at the nucleus to exert its inhibitory effect.

In summary, these studies identify several mechanisms in the metabolism of PMEA and related nucleoside monophosphate analogs, including efflux of the analog and phosphorylation by the enzyme adenylate kinase, in CEM cells. Although the specific nature of this efflux remains uncertain, the possibility that the regulation of nucleotide accumulation may be mediated in cells by such a process has not been previously recognized. Finally, PMEA and related compounds have demonstrated potent antiviral activity against both HIV and DNA viruses *in vitro* and *in vivo*, and elucidation of the biochemical pathways that influence their uptake, accumulation, and metabolism is likely to provide rational approaches for their clinical use and the development of more effective agents.

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