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Synthesis and in vitro evaluation of a phosphonate prodrug: bis(pivaloyloxymethyl) 9-(2-phosphonylmethoxyethyl)adenine

John E. Starrett, Jr., David R. Tortolani, Michael J.M. Hitchcock, John C. Martin[a] and Muzammil M. Mansuri

Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT 06492, USA

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Summary

9-(2-Phosphonylmethoxyethyl)adenine (PMEA; **1**) was acylated with chloromethyl pivalate to afford bis(pivaloyloxymethyl) PMEA (**2**). The ester prodrug demonstrated enhanced in vitro potency against HSV-2 greater than 150-fold higher than the parent compound. The antiviral activity of **2** was 50-fold better than PMEA against HSV-1, and equipotent against HIV and HCMV. The toxicity of **2** was studied in both resting and growing cells.

PMEA; Prodrug; Phosphonate transport; HIV; HSV; HCMV

Since their discovery in 1986 (De Clercq et al., 1986), the phosphonate nucleosides have generated considerable attention as broad spectrum antiviral agents. The adenine analogue, 9-(2-phosphonylmethoxyethyl)adenine (PMEA; **1**), has demonstrated in vitro activity against retroviruses such as human immunodeficiency virus (HIV) (Pauwels et al., 1988) and Rauscher murine leukemia virus (R-MuLV) (Bronson et al., 1989b), as well as DNA viruses such as herpes simplex virus (HSV) (De Clercq et al., 1989). PMEA has shown good in vivo antiviral activity against R-MuLV (Balzarini et al., 1991b), murine cytomegalovirus (MCMV) (Bronson et al., 1989b), HSV (De Clercq et al., 1989), simian immunodeficiency virus (SIV) (Balzarini et al., 1991b) and feline

Correspondence to: J.E. Starrett Jr., Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492, USA.

[a]*Present address*: Gilead Sciences, 344 Lakeside Drive, Foster City, CA 94404, USA.

immunodeficiency virus (FIV) (Egerbrink et al., 1990). This in vivo antiviral activity was observed when PMEA was administered intravenously, intraperitoneally, or intramuscularly. The oral bioavailability of PMEA has been reported to be $<1\%$ in monkeys (Balzarini et al., 1991b) and 11% in rats (Bronson et al., 1989a). This low oral bioavailability, which presumably results from the charged phosphonate functionality, could potentially limit its use as a chemotherapeutic agent in chronic treatment situations such as HIV. Similar difficulties in penetrating lipid membranes have been observed with other charged derivatives such as phosphates (Srivastva and Farquhar, 1984), phosphinates (Krapcho et al., 1988), and formyl phosphonates (Wondrak et al., 1988).

Masking the phosphonate functionality to allow passage through a membrane might potentially increase the potency of this class of molecules. Iyer et al. have recently prepared the acyloxyalkyl ester of phosphonoformate (PFA) in an attempt to increase the potency against HIV-1 (Iyer et al., 1989). This attempt was not successful, presumably due to the hydrolytic instability of the ester under the test conditions. PMEA has also been linked to a synthetic polymer bearing mannosylated residues (Midoux et al., 1990). In vitro evaluation of the PMEA/polymer complex showed it to be more efficient than free PMEA in preventing lysis of human macrophages by herpes simplex virus type 1.

By following a similar rationale to that described above, we reasoned that protection of the phosphonate moiety of PMEA as an ester or amide prodrug could enhance transport and possibly increase the biological activity of PMEA. In the current study a large number of derivatives were examined as potential prodrugs for PMEA (Starrett, 1992). The mono and bis alkyl phosphonate esters and amides were prepared, as well as mixed ester/amides. Both cyclic and acyclic variations were explored. In general, it was found that simple alkyl esters or amides failed as prodrugs because of either poor stability and/or poor activity. After examining derivatives which contained higher functionality than the esters and amides, it was found that the acyloxyalkyl esters of PMEA serve as a unique mask for the phosphonate group. In particular, bis(pivaloyloxymethyl)PMEA (piv_2PMEA; **2**) exhibits very interesting biological activity.

The synthesis of piv_2PMEA (**2**) is outlined in Fig. 1. PMEA (**1**) was prepared by previously reported methods (Holy and Rosenberg, 1987; Bronson et al., 1989b). Several different methods were explored in an attempt to prepare the bis ester **2**. Farquhar and co-workers prepared various bis(acyloxymethyl) esters of phosphates through alkylation of the phosphate silver salts by halomethylacylates (Srivastva and Farquhar, 1984). We felt that a similar approach might be used to prepare acyloxyalkyl ester prodrugs of phosphonates.

A heterogeneous mixture of the silver salt of PMEA (**1**), when treated with either chloromethyl pivalate or iodomethyl pivalate in acetonitrile, failed to provide any of the desired bisester. Presumably the insolubility of the disilver

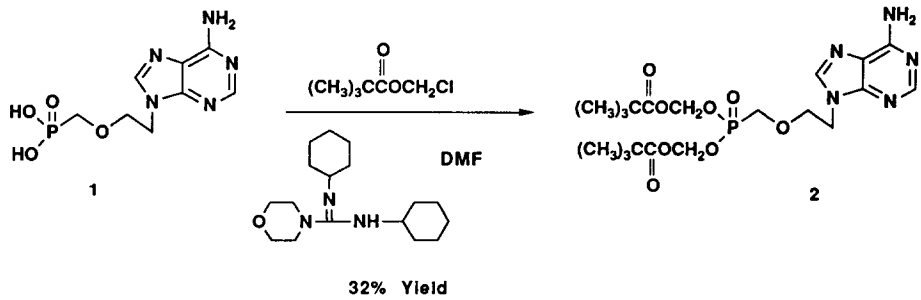

Fig. 1. Synthesis of bis(pivaloyloxymethyl)PMEA.

salt prevented the reaction from occurring. Similar negative results were obtained when the silver salts were formed via their disilyl esters (Iyer et al., 1989). Changing from the silver salts to various other inorganic (Li^+, K^+, Na^+, Cs^+) or organic (Et_3NH^+, $(iPr_2)NEtH^+$, Bu_4N^+) salts did not aid the desired acylation reaction. Bis(pivaloyloxymethyl) ester **2** was finally successfully prepared by homogeneous reaction of PMEA (**1**) with chloromethyl pivalate in the presence of the hindered base N,N'-dicyclohexyl-morpholine carboxamidine.

The antiviral activity of piv_2PMEA (**2**) was examined in vitro (Table 1) against human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), and herpes simplex virus (HSV) types 1 and 2. These assays were performed under 'standard conditions' where the drugs remained in contact with the cells for the duration of the assay. Piv_2PMEA and PMEA showed comparable activity against HIV (ID_{50} of 4 and 10 μM, respectively) and HCMV (ID_{50} of 65 and 117 μM, respectively). More dramatic differences were observed against HSV-1 and HSV-2. Piv_2PMEA was greater than 50-fold more potent than PMEA against HSV-1 (ID_{50} of 1.7 and 95 μM, respectively), and more than 150-fold more potent against HSV-2 (ID_{50} of 0.6 and 119 μM, respectively).

TABLE 1

In vitro antiviral activity of piv_2PMEA and PMEA

Compound	ID_{50} (μM)[1]			
	HIV	HCMV	HSV-1	HSV-2
piv_2PMEA (2)	4	65	1.7	0.6
PMEA (1)	10	117	95	119
AZT	1.1			
ACV		44	3.3	9

[1] The 50% inhibitory dose was determined in HIV infected CEM cells by XTT assay 6 days post infection. In HCMV-infected MRC-5 cells and HSV-infected vero cells, the ID_{50} was determined by plaque reduction assays after incubation for 10–14 days (HCMV) or 2 days (HSV). Virus strains: HSV-1, KOS; HSV-2, G; HCMV, AD-169.

TABLE 2

Persistence of antiviral activity against HSV-2 (strain G)

Compound	ID_{50} (μM)[1]	
	Standard assay[2]	Pretreatment assay[3]
piv_2PMEA	0.6	24
PMEA	119	470
Acyclovir	13	>400

[1] Dose inhibiting 50% of the number of plaques present in a drug-free control assay.
[2] Vero cell monolayers were infected, and then dilutions of compounds in media were added for 48 h. Plaques were then counted.
[3] Vero cell monolayers were exposed to dilutions of compounds in media for 24 h. The monolayers were then washed, infected, and incubated with drug-free medium for 48 h. Plaques were then counted.

Under the standard assay conditions employed above, the cells are continuously exposed to the test compound during the course of the assay. In a separate experiment, the persistence of antiviral activity was measured against HSV-2 (Table 2). In this assay the cells were pretreated with the drug for 24 h, washed to remove excess drug and then infected. This regimen measures residual antiviral activity since no new drug is added after the cells are infected. The effect of piv_2PMEA was only 20-fold improved over PMEA in this assay, compared to 50-fold if the drug was continuously present.

One explanation of the results observed in both of these assays is enhanced transport of the ester across cell membranes in both directions. In the standard assay, the intracellular concentration of PMEA is increased by faster uptake of piv_2PMEA. Once the esters are cleaved within the cell and PMEA is liberated, the charged nature of the phosphonate prohibits its rapid passage out of the cell. This accounts for the significantly enhanced activity (X50). In the pretreatment assay, the intracellular concentration of piv_2PMEA is more rapidly decreased by faster efflux once the extracellular drug is removed. This results in slight increases in the intracellular concentration of PMEA and hence a smaller increase in potency is observed over PMEA itself (when compared to the standard assay). Note that even in the pretreatment assay the activity is enhanced relative to PMEA. The lack of improvement in activity of piv_2PMEA compared in HIV and HCMV assays is not likely to be a differential effect of the virus. Most likely in these assays, which take longer to reach an endpoint than do the HSV assays, the advantage of more rapid uptake is not manifested.

The majority of the cell monolayer remained intact when stationary phase cultures of adherent cells were exposed to concentrations of piv_2PMEA as high as 200 μM. In contrast to the stationary phase cultures, striking differences were observed when measuring the effect of drug on generation time, the amount of time it takes for the cells to double in number (Table 3). Untreated cells have a doubling time of approximately 20 h. PMEA at 91 μM caused a 50% reduction in the growth rate (i.e., 39 h doubling time). By contrast

TABLE 3

Effect of piv_2PMEA and PMEA on doubling time of CEM cells

Conc. (μM)	G (h)[1]	
	PMEA	piv_2PMEA
0	21	17.5
0.10		19.2
0.2		18.0
0.4		41
2		>150
18.3	25	
91	39	

[1] G is the generation time (the amount of time needed for the cells to double). CEM cells were in log phase growth in Eagle's minimal essential medium. Cells were continuously exposed to various concentrations of PMEA or piv_2PMEA. Samples of the culture were removed daily and the cell concentrations were determined by coulter cell counter. Analyses were run in duplicate and averages are reported.

piv_2PMEA caused a 50% reduction in the growth rate at 0.4 μM. At a concentration of 2 μM piv_2PMEA virtually halted growth.

Plots of the effect of piv_2PMEA and PMEA on the growth rate of CEM cells are depicted in Fig. 2. At lower concentrations (0.1 and 0.2 μM), the toxicity of piv_2PMEA appeared reversible (control growth rates were achieved after a short lag). At 0.4 μM the growth rate was severely retarded, and at 2 μM the cells stopped duplicating. Since stationary phase cell cultures are relatively unaffected, the inhibitory effect appears to be cytostatic rather than cytotoxic.

PMEA has been shown to penetrate cells poorly, and intracellular concentrations are much lower than those in the extracellular medium (Palu

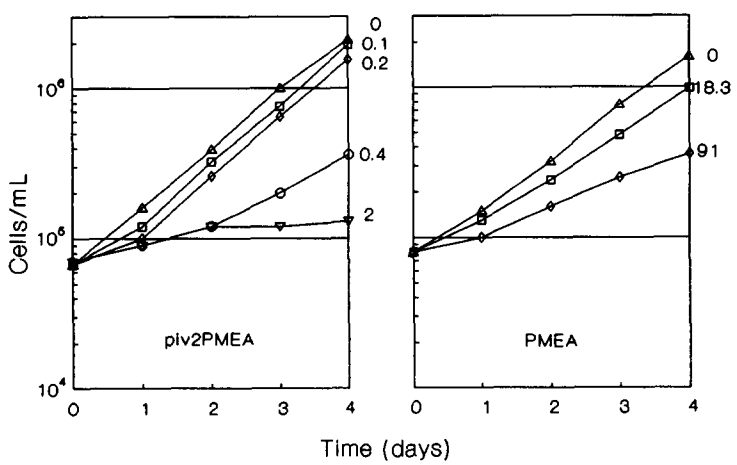

Fig. 2. Effect of piv_2 PMEA and PMEA on doubling time of CEM cells. Different concentrations in μM are enumerated on the right of each graph and are indicated by the different symbols.

et al., 1991). This is thought be to due to the negative charges on the phosphonate group at physiological pH, which make it a poor candidate for penetration through a lipid bilayer. To overcome these effects, a strategy to mask the charges with hydrolyzable esters was proposed. The present study demonstrates that the use of an ester prodrug of PMEA can greatly enhance the potency against HSV when compared to PMEA. Although the concentration of prodrug which kills the cell monolayers is >200 μM, much lower concentrations inhibit the replication rate of growing cells. The enhanced in vitro potency found with piv_2PMEA suggests that it should be examined further for advantages in vivo, including oral bioavailability, especially because acid stability studies indicate that the half-life of piv_2PMEA is greater than 24 h at pH 2 (Starrett, 1992).

During the course of the revision of this manuscript, Farquhar and co-workers described a study of bis(pivaloyloxymethyl) 2',3'-dideoxyuridine 5'-monophosphate (piv_2 ddUMP) as a method to deliver ddUMP across the cell membrane (Sastry et al., 1992). The piv_2 designation for bis(pivaloyloxymethyl) was taken from that communication.

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