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Metabolism and In Vitro Antiretroviral Activities of Bis(Pivaloyloxymethyl) Prodrugs of Acyclic Nucleoside Phosphonates

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Bis(pivaloyloxymethyl) [bis(pom)] derivatives of various acyclic nucleoside phosphonates—9-(2-phosphonylmethoxyethyl)adenine (PMEA), 9-(2-phosphonylmethoxypropyl)adenine (PMPA), and 9-(2-phosphonylmethoxypropyl)diaminopurine (PMPDAP)—were found to exhibit 9- to 23-fold greater antiviral activity than their corresponding unmodified compounds. The cytotoxicity of the bis(pom) analogs was also increased by various degrees, thus altering the therapeutic indexes of these compounds. Metabolic studies using [^{3}H]bis(pom)PMEA and [^{3}H]PMEA as model compounds suggested a >100-fold increase in the cellular uptake of the bis(pom) derivative and formation of active diphosphorylated metabolite. However, the bis(pom) derivatives were chemically unstable and highly susceptible to serum-mediated hydrolysis, factors which limit their potential utility for intracellular drug delivery.

Acyclic nucleoside phosphonate analogs of both purine and pyrimidine bases show a broad-spectrum antiviral activity against several RNA and DNA viruses (reviewed in reference 4). The adenine analog, 9-(2-phosphonylmethoxyethyl)adenine (PMEA), is active against human immunodeficiency virus type 1 (HIV-1) and other retroviruses, including HIV-2, simian immunodeficiency virus, feline immunodeficiency virus, and Moloney murine sarcoma virus. PMEA is also active against various herpesviruses, including herpes simplex virus type 1 (HSV-1), HSV-2, cytomegalovirus, and Epstein-Barr virus. Thus, PMEA is of interest both as a potential antiretroviral drug for HIV-1 infections and also for the treatment of some of the opportunistic infections associated with AIDS, and it is currently undergoing a phase I and II trial for the evaluation of its toxicity and/or efficacy in AIDS patients. The related phosphonate analogs 9-(2-phosphonylmethoxypropyl)adenine (PMPA) and 9-(2-phosphonylmethoxypropyl)diaminopurine (PMPDAP) exhibit potent anti-HIV activity, although these compounds are less effective against herpesviruses. The phosphonyl groups exhibit a negative charge at the physiological pH, and hence the cellular uptake and bioavailability (in rats) of these molecules with oral drug administration are relatively poor (8). Recently, bis(pivaloyloxymethyl) [bis(pom)] esters of the antitumor nucleotide analogs 5-fluoro-2′-deoxyuridine were shown to function as membrane-permeable prodrugs and inhibit proliferation of both wild-type and thymidine kinase-deficient murine leukemia cells (5, 9). Similarly, a bis(pom) ester of PMEA was found to have increased anti-herpes simplex virus activity in vitro compared with PMEA (11). However, the mechanisms involved in the increased activity of the bis(pom) derivative were not determined. In addition, little is known about the stability of the bis(pom) derivatives or their bioconversion to the active intracellular metabolites. In this study, we have evaluated the anti-HIV activity of bis(pom) esters of PMEA, PMPA, and PMPDAP and their metabolism in human lymphoid cell lines.

Table 1 summarizes the anti-HIV and cytotoxic activities of the various phosphonates and their bis(pom) derivatives. The antiviral activities of the different compounds against HIV-1_{IIIB} replication in MT-2 cells were monitored by XTT assays performed as previously described (12). The cytotoxicities of these compounds against MT-2 cells were evaluated by a dye conversion-cell viability assay (12). Compared with the unmodified analogs, the bis(pom) derivatives were biologically more active and showed enhanced antiviral activities. The cytotoxicities of these compounds were also enhanced to various degrees (Table 1). The activities of these compounds are likely to be greater than the values shown in Table 1, since the XTT assay is relatively insensitive compared with other methods. The antiviral versus cytotoxic activities were enhanced to various degrees after bis(pom) modification, thus altering the therapeutic index (ratio of cytotoxicity to antiviral efficacy) of the unmodified and bis(pom)-modified compounds. Bis(pom)PMEA and bis(pom)PMPDAP showed approximately fourfold lower therapeutic indexes than their unmodified analogs, while the therapeutic index of bis(pom)PMPA was comparable with that of PMPA. The differences in the extent of accumulation of the intracellular metabolites from these analogs and their inhibitory potencies against the target enzymes may account for the observed effects.

To gain some understanding of the biochemical basis for the potency of the bis(pom) derivatives, we compared the metabolisms of bis(pom)PMEA and PMEA in MT-2 cells. The metabolisms of PMEA and bis(pom)PMEA were studied by previously described procedures (1–3, 6). Exponentially grown cultures of MT-2 cells were incubated with 10 μM [^{3}H]PMEA or 1 μM [^{3}H]bis(pom)PMEA. After 2 h, the cells were extracted in 70% methanol and analyzed by anion-exchange high-performance liquid chromatography

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TABLE 1. Comparison of the antiviral and cytotoxic effects of PMEA, PMPA, PMPDAP, and their bis(pom) derivatives

Compound	Anti-HIV activity (ED_{50})[a] (μM)	Cytotoxicity (IC_{50})[b] (μM)	Selectivity (IC_{50}/ED_{50})
PMEA	16 ± 6[c]	160 ± 10	10
Bis(pom)PMEA	0.5 ± 0.2 (32)[d]	2 ± 0.3 (80)	4 (0.25)
PMPA	11 ± 2	440 ± 10	40
Bis(pom)PMPA	0.5 ± 0.3 (22)	40 ± 10 (11)	80 (2)
PMPDAP	2 ± 1	350 ± 10	175
Bis(pom)PMPDAP	0.2 ± 0.1 (10)	9 ± 1 (39)	45 (0.25)

[a] ED_{50}, 50% effective dose.
[b] IC_{50}, 50% inhibitory concentration.
[c] Each figure represents the mean ± the standard deviation of three or more experiments.
[d] Figures in parentheses indicate the fold difference between the activity of the bis(pom) derivative and the activity of its unmodified analog.

(HPLC). Nearly 21% of the [^{3}H]bis(pom)PMEA added to the medium appeared within the cells, and essentially all the intracellular radioactivity was associated with PMEA and its metabolites, PMEA monophosphate (PMEAp) and PMEA diphosphate (PMEApp); little or no radioactivity could be found as intact prodrug under these conditions (Fig. 1A). By contrast, only a small proportion (<1%) of the label, which was distributed primarily in PMEA, PMEAp, and PMEApp, appeared within PMEA-treated cells (Fig. 1B). However, a small proportion of the label was found to be incorporated into additional metabolites, primarily ATP; we attribute this to some radioactive adenine present as a contaminant.

The time courses of intracellular metabolism of bis(pom)PMEA and PMEA in MT-2 cells are shown in Fig. 2. Cells incubated with 1 μM bis(pom)PMEA showed a rapid accumulation of PMEA which after 2 h reached a peak concentration of 200 pmol/10^6 cells (Fig. 2A). The intracellular PMEA level decreased thereafter, but relatively high levels (~25 pmol/10^6 cells) were seen even after 8 h of incubation. By contrast, much lower levels of PMEA were detected in the MT-2 cells incubated with a 10-fold higher concentration of PMEA (Fig. 2B). The intracellular concentrations of PMEAp and PMEApp formed from bis(pom)PMEA increased during the initial 6 h of incubation and plateaued at concentrations of 25 and 80 pmol/10^6 cells, respectively. This contrasts sharply with the low levels of PMEAp and PMEApp accumulated in cells incubated with PMEA. At any time point tested, the intracellular level of bis(pom)PMEA or mono(pom)PMEA, a presumed intermediate (5), was <1.0% of the level of PMEA or its metabolites (Fig. 2A, inset).

We also determined the extracellular concentrations of the drug or drug metabolites in [^{3}H]bis(pom)PMEA-treated MT-2 cell cultures (Fig. 3A). Bis(pom)PMEA was rapidly cleared from the medium (half-life, ~100 min). A large amount of mono(pom)PMEA accumulated in the culture medium within 30 min, and the levels declined gradually thereafter. This accumulation of mono(pom)PMEA in medium contrasts with the very low level of the mono(pom)PMEA detected in the cells (Fig. 2A, inset) and indicates that the formation of mono(pom)PMEA probably occurs predominantly extracellularly. PMEA was also detected in the culture medium, and PMEA levels progressively increased with time. However, at any given time point, the extracellular PMEA level constituted only a small fraction of the intracellular concentration. Finally, the stability of [^{3}H]bis(pom)PMEA in cell-free medium was examined in the presence or absence of serum. As shown in Fig. 3B, bis(pom)PMEA hydrolyzed primarily to the mono(pom) derivative with a half-life of about 4 h in the absence of cells or serum. Further breakdown of mono(pom)PMEA to PMEA was observed only in the presence of cells or serum (data not shown). The metabolisms of the other two phosphonate analogs, PMPA and PMPDAP, and their bis(pom) derivatives were not examined because of the nonavailability of radiolabeled compounds.

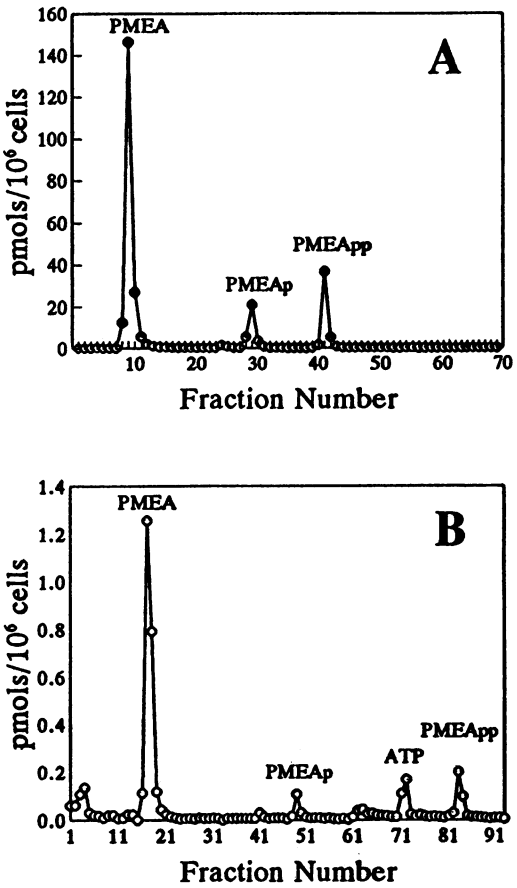

FIG. 1. HPLC profiles of PMEA metabolites in MT-2 cells incubated with radioactive PMEA or bis(pom)PMEA. MT-2 cells were incubated with 1 μM [^{3}H]bis(pom)PMEA (A) or 10 μM [^{3}H]PMEA (B). After 2 h, cell extracts were prepared and analyzed by HPLC. (A) The elution was performed on a Hamilton PRP-1 reverse-phase column (15 by 0.41 cm) with a linear gradient of 0.05 M ammonium phosphate buffer (0.05 M tetraacetyl ammonium phosphate and 5 to 30% acetonitrile) (pH 7.1), at a flow rate of 1 ml/min. These conditions were necessary to resolve bis(pom)PMEA from PMEA and its metabolites, PMEAp and PMEApp. (B) A Whatman Partisil-10 SAX ion-exchange column (25 by 0.46 cm) was eluted with a linear gradient of 0.05 M ammonium phosphate buffer (pH 3.5) to 0.7 M ammonium phosphate buffer (pH 3.5). Elution of nucleotides was monitored by UV A_{254}. The retention times of authentic standards and controls are indicated.

We show here that masking the charges associated with the phosphonyl group by alkylation with pivaloyloxymethyl groups increases the cellular uptake and biological activities of various acyclic nucleoside phosphonates. Using [^{3}H]bis(pom)PMEA as the model compound, we have demonstrated that the prodrug is rapidly hydrolyzed into the parent

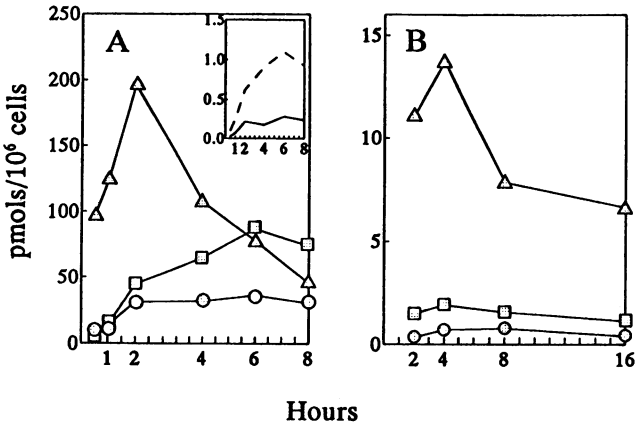

FIG. 2. Kinetics of intracellular metabolite formation in cells incubated with [^{3}H]PMEA or [^{3}H]bis(pom)PMEA. MT-2 cells were incubated with 1 μM [^{3}H]bis(pom)PMEA (A) or 10 μM [^{3}H]PMEA (B). At different intervals, cell extracts were prepared and analyzed by HPLC for drug metabolites as described in the legend for Fig. 1. The concentrations of PMEA (triangles), PMEAp (circles), and PMEApp (squares) are indicated. (Inset) The solid line shows the levels of bis(pom)PMEA, while the dashed line corresponds to mono(pom) PMEA.

compound, PMEA, within the cells and further metabolized into PMEAp and biologically active PMEApp. The results also indicate that bis(pom)PMEA is relatively unstable at the physiological pH and breaks down to the mono(pom) derivative or the parent compound in the absence or presence of serum. The chemical instability points to a previously unrecognized limitation of bis(pom) esters as an effective approach to deliver phosphorylated drugs inside cells. Earlier studies (5, 9) with bis(pom) esters of nucleotide derivatives examined their activity in vitro but did not determine the chemical or enzymatic stability of these prodrugs in cells and medium.

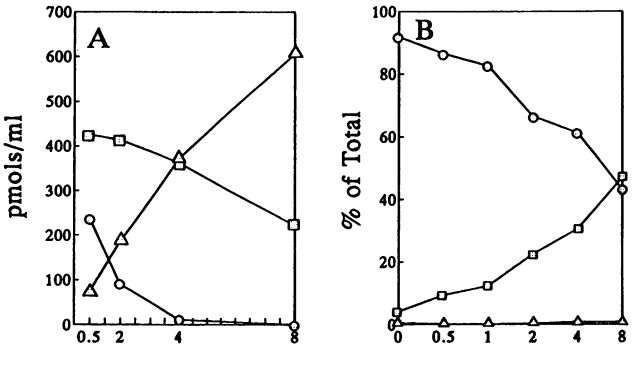

FIG. 3. Extracellular levels of bis(pom)PMEA and its metabolites. (A) HPLC profile of bis(pom)PMEA and its metabolites in supernatants of MT-2 cell cultures with 1 μM [^{3}H]bis(pom)PMEA. At different intervals, culture supernatants were analyzed by HPLC for bis(pom)PMEA, mono(pom)PMEA, and PMEA. (B) Distribution of radioactivity among bis(pom)PMEA and its metabolites when [^{3}H]bis(pom)PMEA was incubated in serum-free RPMI-1640 medium at 37°C. The concentrations of bis(pom)PMEA (circles), mono(pom)PMEA (squares), and PMEA (triangles) are shown.

Despite their susceptibility to spontaneous and serum-mediated hydrolysis, the bis(pom) esters may prove useful to deliver the parent compound into the plasma through oral administration. Bis(pom)PMEA is stable in buffer at acidic pH (pH 2.0) with an estimated half-life of over 24 h (11). It is therefore likely to be stable in the gastric environment when administered by the oral route and may be rapidly absorbed because of its lipophilicity. Once in circulation, it is likely to be hydrolyzed immediately to PMEA. Indeed, in preliminary studies, following oral administration of bis(pom)PMEA to monkeys ~30% of the compound was found in circulation as PMEA, whereas only ~6% of the orally administered PMEA was found in circulation (1a). It should be noted that good bioavailability following oral administration to humans has been demonstrated with bis(pom) esters of ampicillin and cephalosporin for the treatment of bacterial infections without any deleterious effects (7, 10). Optimizing the delivery of these active nucleoside phosphonates remains an important goal for the successful treatment of viral infections with these agents.

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