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

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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 nucleoside analogs 5-fluoro-2'-deoxycytidine 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 antiviral 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(LAI) replication in MT-2 cells were monitored by XTT assays 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 [3H]PMEA or 1 μM [3H]bis(pom)PMEA. After 2 h, the cells were extracted in 70% methanol and analyzed by anion-exchange high-performance liquid chromatography...
TABLE 1. Comparison of the antiviral and cytotoxic effects of PMEA, PMPA, PMPDAP, and their bis(pom) derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Anti-HIV activity (ED50) (µM)</th>
<th>Cytotoxicity (IC50) (µM)</th>
<th>Selectivity (IC50/ED50)</th>
</tr>
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<tbody>
<tr>
<td>PMEA</td>
<td>16 ± 6</td>
<td>160 ± 10</td>
<td>10</td>
</tr>
<tr>
<td>Bis(pom)PMEA</td>
<td>0.5 ± 0.2 (32)</td>
<td>2 ± 0.3 (80)</td>
<td>4 (0.25)</td>
</tr>
<tr>
<td>PMPA</td>
<td>11 ± 2</td>
<td>440 ± 10</td>
<td>40</td>
</tr>
<tr>
<td>Bis(pom)PMPA</td>
<td>0.5 ± 0.3 (22)</td>
<td>40 ± 10 (11)</td>
<td>80 (2)</td>
</tr>
<tr>
<td>PMPDAP</td>
<td>2 ± 1</td>
<td>350 ± 10</td>
<td>175</td>
</tr>
<tr>
<td>Bis(pom)PMPDAP</td>
<td>0.2 ± 0.1 (10)</td>
<td>9 ± 1 (39)</td>
<td>45 (0.25)</td>
</tr>
</tbody>
</table>

a ED50, 50% effective dose.
b IC50, 50% inhibitory concentration.
c Each figure represents the mean ± 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 [3H]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 (PMEA2p); 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 PMEA2p, 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 cell cultures were 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/10⁶ cells (Fig. 2A). The intracellular PMEA level decreased thereafter, but relatively high levels (~25 pmol/10⁶ 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 PMEA2p formed from bis(pom)PMEA increased during the initial 6 h of incubation and plateaued at concentrations of 25 and 80 pmol/10⁶ cells, respectively. This contrasts sharply with the low levels of PMEAp and PMEA2p 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 [3H]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 levels constituted only a small fraction of the intracellular concentration. Finally, the stability of [3H]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 halflife 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.

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

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 buffers at physiological pH (pH 7.4) 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 PMEApp 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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