Heteroatom fatty acid analogs of myristic acid containing oxygen or sulfur substituted for the alkyl methylene groups inhibit replication of the human immunodeficiency virus (HIV) in infected cells by acting as alternative substrates during the viral protein myristoylation event. In this class of compounds, 12-methoxydodecanoic acid is the most potent compound but is approximately 10^5-fold less active than azidothymidine. The antiviral activity of 12-methoxydodecanoic acid can be enhanced >40-fold by preparing L-α-phosphatidylethanolamine containing 12-methoxydodecanoic acid in both alkyl chains. In addition, the diacylated L-α-phosphatidylcholine analog containing 12-methoxydodecanoic acid in both alkyl chains (i) has a 15-fold better antiviral selectivity, (ii) is 7-fold more potent, and (iii) is 10–100-fold more synergistic with azidothymidine than 12-methoxydodecanoic acid. Because of potent synergism, the antiviral selectivity of the diacylated L-α-phosphatidylcholine analog is >10^4 when coadministered with azidothymidine. Phospholipid conjugates are chiral at the C-2 carbon of the glycerol backbone and most interesting is the observation that both the D- and L-isomers of phosphatidylcholine, phosphatidylglycerol, phosphatidic acid, and phosphatidyserine have approximately equal antiviral activity. Phospholipase A_2 stereospecifically hydrolyzes only the L-isomer of phospholipid analogs and similar activity for both the D- and L- phospholipid isomers suggests that phospholipase A_2 is not the rate-limiting enzyme for release of the drugs in vivo.

Although most retroviral genes do not encode for myristoylated proteins, the HIV-1 genome encodes for two myristoylated proteins; p17^{NMT} (1–5) and p27^{NMT} (4, 5). In situ myristoylation of these proteins is critical for the establishment and maintenance of HIV-I infection. Interfering with protein myristoylation has been established as a drug target site, and heteroatom analogs of myristic acid, containing oxygen or sulfur substituted for the alkyl methylene groups, have been reported to exhibit potent activity against HIV replication in infected cells (6, 7). N-Myristoyltransferase is the enzyme that cotranslationally transfers the myristoyl group to endogenous cellular and viral proteins (8, 9). Heteroatom analogs of myristic acid can exhibit inhibitory activity against viruses that produce myristoylated proteins by two general mechanisms. One mechanism involves competitively inhibiting the enzyme necessary for either activating the myristoyl group to the acyl-CoA derivative, or inhibiting N-myristoyltransferase from attaching the myristoyl group to the viral protein (10). The alternate mechanism "requires" incorporation of the myristoyl analog into the viral protein, but the myristoyl analog, tethered to the viral protein, alters the intracellular distribution or cellular processing of the protein (6, 7, 11). This second mechanism requires that the myristoyl analog be activated to the acyl-CoA intermediate and that the myristoyl-analog-CoA be a substrate for N-myristoyltransferase. Thus for anti-HIV activity, the first mechanism aims at inhibiting the myristoylation event, whereas the second mechanism requires the myristoylation event.

Heteroatom fatty acid analogs of myristic acid may interfere with HIV replication by either or both mechanisms. Some acyl-CoA derivatives of heteroatom fatty acid analogs have been shown to inhibit the N-myristoyltransferase reaction, whereas other analogs linked to host cell proteins by N-myristoyltransferase have been shown to alter the natural distribution of the protein (12, 13). In other words, acylation of proteins using heteroatom analogs of myristic acid shifts the protein distribution from the cell membrane to the cytosol and the amount of cellular redistribution depends on both the protein and the particular heteroatom fatty acid analog tethered to the protein.

HIV genes encode for only two myristoylated proteins, whereas the host cell produces hundreds of endogenous myristoylated proteins (7). 12-Methoxydodecanoic acid (12MO) is a fatty acid analog that apparently incorporates into many of the endogenous host cell myristylated proteins but only a small subset of the endogenous proteins containing 12MO undergo cellular redistribution from the cell membrane to the cytosol (7). However, 12MO incorporates into both HIV p17^{NMT} and HIV p27^{NMT} viral proteins, and it has been shown that 12MO tethered to the HIV gag polypeptide precursor inhibits proteolytic processing (7). 12MO thus selectively interferes with HIV myristoylated proteins yet produces minimal changes in the endogenous myristoylated proteins found in the host cells. This biochemical selectivity for HIV proteins

Charles Pidgeon, Robert J. Markovich, Min D. Liu, Timothy J. Holzer, Richard M. Novak, and Kay A. Keyer

From the Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy, Purdue University, West Lafayette, Indiana 47907, the Diagnostics Division, Hepatitis/Retrovirus Scientific Support 9YW, Abbott Laboratories, Abbott Park, Illinois 60064-3580, and the Department of Medicine, University of Illinois, Chicago, Illinois 60612

Antiviral Phospholipids

ANTIVIRAL DRUGS CONJUGATED TO THE GLYCEROBACKBONE OF PHOSPHOLIPIDS*

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† To whom correspondence should be addressed. Tel.: 317-494-6251; Fax: 317-494-6790.


The abbreviations used are: HIV, human immunodeficiency virus; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine; IAM, immobilized artificial membrane; 12MO, 12-methoxydodecanoic acid; FMO, N-(9-fluorenyl)methoxy carbonyl; PBMC, peripheral blood mononuclear cell; AZT, azidothymidine; PLA_2, phospholipase A_2.

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is critical for developing heteroatom fatty acids as HIV anti-

viral therapy. The endogenous source of myristic acid for protein myris-
toylation is unknown. The source may be from the free fatty acid pool obtained via de novo synthesis, or the pool may be from the endogenous lipid pools. The administration of fatty acids like 12MO to viable cells results in the rapid incorporation of the fatty acids into cellular lipid pools; the mono-, di-, and triglyceride pools accumulate ~70% of the dose, and the phospholipid pools accumulate ~30% of the dose (14-16). De novo fatty acid synthesis may be down-regulated when fatty acids are present as nutrients in the culture medium (17). Thus the potent anti-HIV activity of heteroatom analogs of myristic acid, which lasts for several days in T-cell cultures (7), is coupled to the rapid incorporation of heteroatom fatty acids into the host cell lipid pools (14-16). This suggests that in addition to de novo synthesis of fatty acids, an additional source of myristic acid for the N-

myristoyltransferase reaction may be the membrane lipids in HIV-infected cells. In this regard, membrane lipids would sequester the biologically active fatty acid in a manner similar to that for the storage of arachidonic acid in the alkyl chains of membrane lipids (18).

MATERIALS AND METHODS

Chemicals—L-α-Glycerophosphocholine (L-α-GPC) and L-α-glycerophosphoethanolamine (L-α-GPE) were purchased from Sigma. D-Glycerophosphocholine (D-GPC, unnatural configuration) was purchased from Synthetische Phosphor-Lipide (Bern, Switzerland). Glycerophosphocholine (D-GPC, unnatural configuration) was purified using silica gel chromatography. All other chemicals and solvents were obtained from established suppliers. 12-Methoxydodecanoic acid (dissolved in methanol) was adsorbed to IAM particles and dried under vacuum. They were then counted for number and viability using trypan blue exclusion, followed by infection with SFG7, a subclone of HIV-1 (IIIb) at a multiplicity of infection ranging from 0.04 to 2 in a volume of 0.2 ml culture supplemented with 20% FBS (HyClone Laboratories, Logan, UT). The cultures were refed every 24 h with complete media containing replacement drug and interleukin-2. Seven days after infection, samples were taken for the reverse transcriptase assay. These samples were frozen at ~70°C until analysis. Fifty μl of HIV culture supernatant were mixed with 50 μl of a 2 × RT assay buffer containing 0.1 M Tris pH 7.5, 0.5 mM KCl, 0.012 mg/mL dithiothreitol, 0.012 m M MgCl2, 1.2 mM reduced glutathione, 1 mM EDTA, 4% ethylene glycol, 10 μl of sterile distilled water, 0.2% Triton X-100, 1 μg/mL template primer poly(rA)-poly(dT) (0.05 μg/sample, Pharmacia), and 10 μCi of [3H]dTTP (Du Pont-New England Nuclear). Samples were incubated for 24 h at 37°C in microtiter plates, after which the reaction was stopped with 200 μl of cold 10% trichloroacetic acid containing 0.02 μM sodium PPi. The plates were washed 8 times in 5% trichloroacetic acid, 0.1% Triton X-100, 1 μg/mL template primer poly(rA)-poly(dT) (0.05 μg/sample, Pharmacia), and 10 μCi of [3H]dTTP (Du Pont-New England Nuclear). Samples were incubated for 24 h at 37°C in microtiter plates, after which the reaction was stopped with 200 μl of cold 10% trichloroacetic acid containing 0.02 μM sodium PP, The plate was then allowed to incubate for 2 h on ice, after which samples were harvested onto DE-81 filter paper discs (Whatman) using a cell harvester. The discs were washed 8 times in 5% trichloroacetic acid and 0.1% sodium dodecyl sulfate, and placed into scintillation vials. They were then counted on a β scintillation counter. Negative (uninfected cell supernatants) and positive (HIV-infected cell supernatants) controls were used to determine the background DNA polymerase activity, if any) and known positive controls were assayed simultaneously. Results measured in counts per minute (cpm) are plotted as percent of the control (i.e. cpm obtained for infected cells without drug).

Stability of Antiviral Phospholipids in Whole Blood—Whole blood was obtained from a human volunteer. Citrate was used as the anti-

coagulant for the blood. Lipid was quantitated using a scanning densitometer (Shimadzu CS 9000) operating in the reflectance mode. TLC plates were sprayed with Silica gel chromatography. Two diacylated phosphatidyl-

choline (PC) isomers (denoted as D-AC2 and L-AC2) contain 2 eq of 12MO.

Synthesis of Phosphatidylserine (PS), Phosphatidylethanolamine (PE), Phosphatidic Acid (PA), and Phosphatidylglycerol (PG) —D-AC2 and L-AC2 were converted into their corresponding phosphatidylserine (PS2 and L-PS2), phosphatidylethanolamine (D-PE2 and L-PE2), and phosphatidic acid (D-PA2 and L-PA2) analogs using phospholipase D (PC2) from Avanti Polar Lipids (Birmingham, AL), and the phospholipid conjugates in the crude enzyme reaction mixture were purified by silica gel chromatography. Racemic glycerol and L-
seryl were used for the phospholipase D-catalyzed transphosphatidy-
lation reactions using PC2 analogs as starting material. The phos-

phatidylethanolamine analog containing the unnatural configuration at the glycerol backbone was also enzymatically prepared from D-

AC2 by phospholipase D at Avanti Polar Lipids; however, L-AC2 was not used to prepare the phosphatidylethanolamine analog containing the natural glycerol backbone configuration. Briefly, the headgroup amine of L-AC2 was protected with N-(9-

flocovinyl)methylcarbonyl (FMOC) and the protected L-AC2 analog was diacylated using 12-methoxydodecanoic acid anhydride in dry chloroform using dimethyloxyproline as catalyst. The diacy-

lated L-GPE (FMOC) intermediate was purified using silica gel chroma-
tography, FMOC was removed using piperidine/CHCl3, and the final product (L-AC2) was purified using silica gel chromatography.

Materials and Methods

**Antiviral Assays**—Antiviral assays that monitor the inhibition of syncytia formation are described in the legend to Fig. 1. Reverse transcriptase assay preparations were performed as follows. Peripheral blood mononuclear cells (PBMC) were obtained from the whole blood of normal donors by Ficoll Hypaque (Pharmacia LKB Biotechnology Inc.) density gradient centrifugation. These cells were initially washed with buffer and then stimulated with phytohemagglutinin-M (GIBCO) for 72 h. The cells were then counted for number and viability using trypan blue exclusion, followed by infection with SFG7, a subclone of HIV-1 (IIIb) at a multiplicity of infection ranging from 0.04 to 2 in a volume of 0.2 ml culture supplemented with 20% FBS (HyClone Laboratories, Logan, UT). The cultures were refed every 24 h with complete media containing replacement drug and interleukin-2. Seven days after infection, samples were taken for the reverse transcriptase assay. These samples were frozen at ~70°C until analysis. Fifty μl of HIV culture supernatant were mixed with 50 μl of a 2 × RT assay buffer containing 0.1 M Tris pH 7.5, 0.5 mM KCl, 0.012 mg/mL dithiothreitol, 0.012 mM MgCl2, 1.2 mM reduced glutathione, 1 mM EDTA, 4% ethylene glycol, 10 μl of sterile distilled water, 0.2% Triton X-100, 1 μg/ml template primer poly(rA)-poly(dT) (0.05 μg/sample, Pharmacia), and 10 μCi of [3H]dTTP (Du Pont-New England Nuclear). Samples were incubated for 24 h at 37°C in microtiter plates, after which the reaction was stopped with 200 μl of cold 10% trichloroacetic acid containing 0.02 μM sodium PP, The plate was then allowed to incubate for 2 h on ice, after which samples were harvested onto DE-81 filter paper discs (Whatman) using a cell harvester. The discs were washed 8 times in 5% trichloroacetic acid and 0.1% sodium dodecyl sulfate, and placed into scintillation vials. They were then counted on a β scintillation counter. Negative (uninfected cell supernatants) and positive (HIV-infected cell supernatants) controls were used to determine the background DNA polymerase activity, if any) and known positive controls were assayed simultaneously. Results measured in counts per minute (cpm) are plotted as percent of the control (i.e. cpm obtained for infected cells without drug).
integrated intensity of the blood phosphatidylcholine spot varied very little (~10% increase) during 38 h of incubation at all temperatures tested. Thus the major degradation pathway of the HIV antiviral phospholipid compounds is not by formation of other blood phosphatidylcholines analogs. The percentage of drug remaining in the blood was calculated from \[ \frac{\text{Area}_{\text{drug}}}{\text{Area}_{\text{cpcl}}} \times 100\% \], where Area_{\text{drug}} and Area_{\text{cpcl}} are the integrated area under the densitometer peaks correspond to the HIV antiviral drug and the blood phosphatidylcholine lipids, respectively.

**RESULTS AND DISCUSSION**

L-AC2 is a phosphatidylcholine analog containing 2 eq of 12MO and was the most studied analog because it exhibited an excellent selectivity compared to the other compounds tested. Fig. 1 compares the HIV antiviral activity of L-AC2, 12MO, and AZT. The dose-response curves for each drug alone show that AZT is ~100 times more potent than L-AC2; however, AZT is ~10^4 to 10^6 times more potent than 12MO. In MT4 cells, L-AC2 is approximately 7-fold more potent than 12MO using antiviral syncytial cell assays (Fig. 1), but similar results were obtained using CEM cells and measuring the amount of inhibition of HIV-induced cell death (not shown). L-AC2 contains 2 eq of 12MO, and the 7-fold increased activity exceeds the expected 2-fold increased activity if the cellular availability and cellular disposition of free 12MO are identical to 12MO tethered to the phospholipid molecule. L-AC1 is a phosphatidylcholine analog that contains 1 eq of 12MO in the C-2 position (see structures in Fig. 1). However, relative to L-AC2, L-AC1 is virtually inactive. L-AC1 exhibits an IC_{50} ~ 200-300 \mu M in syncytial cell assays, which is approximately equal to the cellular toxicity of the compound.

Synergistic antiviral activity was evaluated by shifts in the dose-response curves of 12MO or LAC2 when an inactive antiviral concentration of AZT (0.5 nM) was coadministered to the HIV-infected cells. Synergy causes the antiviral dose-response curves of L-AC2 and 12MO to shift to lower concentrations. 12MO exhibited less than a factor of 10 increase in antiviral activity, which indicates minimal synergism when coadministered with AZT, whereas L-AC2 exhibited approximately 100-fold increased activity, indicating that L-AC2 is very synergistic with AZT (Fig. 1). This potent synergy was confirmed using the MACSYNERGY computer program (21). Fig. 1 also shows that 10 nM L-AC2 (an inactive antiviral drug concentration) shifts the dose-response curve of AZT approximately 100-fold. In separate experiments using noninfected MT4 cells, the direct cytotoxicity of either AZT or L-AC2 did not change when the compounds were coadministered to the cells. Thus the synergy observed in Fig. 1 for L-AC2 and AZT is not due to increased cellular toxicity from administering both drugs concurrently. The synergy is due to direct inhibition of HIV by two different mechanisms; AZT is a reverse transcriptase inhibitor, whereas L-AC2 putatively inhibits endogenous myristoylation of the HIV proteins.

Table I compares the selectivity index of L-AC2 to 12MO in syncytial cell assays. The selectivity index of a potential drug compares the toxic dose, TC_{50}, measured in uninfected cells, to the 50% inhibitory concentration, IC_{50}, in HIV-infected cells. Selectivity index values were calculated from toxicity data and HIV antiviral activity data obtained in the same cell line. L-AC2 was 2-fold less toxic than 12MO in MT4 cells. The antiviral activity data was measured in syncytial cell assays utilizing acutely infected MT4 cells cocultured with SupT1 cells. MT4 cells were infected for 2 h with HIV-1b at a multiplicity of infection of 0.5-2.5. The infected cells were then washed and incubated in the presence of the test compound for 6 days at 37°C in 5% CO_2, after which 10^6 MT4 cells were cocultured with an equal number of SupT1 cells in a volume of 200 \mu l in 96-well microtiter plates. Infected cells were titered to achieve the optimum number of syncytial cells to count. Syncytial cells were counted 18-24 h after coculture. The percent reactivation in syncytial cell number was determined relative to infected controls without drug. **All conditions were run in triplicate wells and experiments were performed at least 3 times.** ** corresponds to 0.5 nM AZT, which is inactive.**

The structures of myristic acid (Myr), 12-methoxydodecanoic acid (12MO), and glycerophosphatidylcholine phospholipids containing these fatty acids (L-AC2 and L-AC1) are shown. The stereochemical configuration at the C-2 carbon of the glycerol backbone is not shown. L-AC2 contains the natural configuration and D-AC2 contains the unnatural configuration at the glycerol C-2 carbon. Phospholipids and 12MO were dispersed at 40°C in isotonic buffer with intermittent vortexing.

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**FIG. 1. HIV antiviral activity of L-AC2 and synergy when AZT and L-AC2 are concurrently administered.** HIV-antiviral activity was measured in syncytial cell assays utilizing acutely infected MT4 cells cocultured with SupT1 cells. MT4 cells were infected for 2 h with HIV-1b at a multiplicity of infection of 0.5-2.5. The infected cells were then washed and incubated in the presence of the test compound for 6 days at 37°C in 5% CO_2, after which 10^6 MT4 cells were cocultured with an equal number of SupT1 cells in a volume of 200 \mu l in 96-well microtiter plates. Infected cells were titered to achieve the optimum number of syncytial cells to count. Syncytial cells were counted 18-24 h after coculture. The percent reactivation in syncytial cell number was determined relative to infected controls without drug. All conditions were run in triplicate wells and experiments were performed at least 3 times. ** corresponds to 0.5 nM AZT, which is inactive.
Table I: IC₅₀ and selectivity index of L-AC₂ and 12MO

<table>
<thead>
<tr>
<th>IC₅₀</th>
<th>Selectivity index</th>
<th>IC₅₀</th>
<th>Selectivity index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytotoxicity MT</td>
<td>50% reduction in</td>
<td>50% reduction in</td>
</tr>
<tr>
<td></td>
<td>assay*</td>
<td>syncytial cell</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>formation MT₄</td>
<td>activity PBMC*</td>
</tr>
<tr>
<td>12MO</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>140 (MT₄ cells)</td>
<td>8</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>340 (PBMC cells)</td>
<td>80 (MT₄ cells)</td>
<td>4</td>
<td>280</td>
</tr>
</tbody>
</table>

* Drug cytotoxicity in the absence of HIV was determined using MT₄ cells. The IC₅₀ is the concentration of drug that killed 50% of the cells during a 4-h incubation period.

** The IC₅₀ is the drug concentration that inhibited 50% of the maximum HIV response which was observed when no drug was present. The HIV responses that were measured are syncytial cell formation or reverse transcriptase activity.

The selectivity index was calculated as TC₅₀/IC₅₀.

* Syncytial cell assays were performed as described in legend to Fig. 1.

Therapeutic index ~15-fold. L-AC₂ is both more potent and less toxic than 12MO in T-cells, and this is the reason for the larger therapeutic window. Using reverse transcriptase antiviral assays and peripheral blood mononuclear cells (PBMCs), the therapeutic index for L-AC₂ was 37, whereas for 12MO the therapeutic index was 17. In contrast to MT₄ cells, there is no significant difference in toxicity for 12MO and LAC₂ in PBMCs. HIV antiviral activity of LAC₂ in PBMCs was confirmed at the National Institutes of Health Division of AIDS in their confirmatory testing division. In combination with an inactive concentration of AZT (0.5 nM), the IC₅₀ of L-AC₂ is 0.01 μM and the cellular toxicity (280 μM) of L-AC₂ does not change; this results in an enormous increase in therapeutic index (280 μM/0.01 μM) of ~28,000 for the L-AC₂ analog when combined with AZT compared to a therapeutic index of only ~280 for L-AC₂ when administered alone. Although when administered alone LAC₂ has good antiviral activity (IC₅₀ ~ 1 μM), when combined with AZT the selectivity of L-AC₂ is as good or better than many drugs currently under investigation.

L-AC₂ contains the natural configuration of glycerophosphocholine and is quantitatively hydrolyzed by bee venom phospholipase A₂ (PLA₂) within minutes. Because PLA₂s stereospecifically hydrolyze phospholipids, we prepared the D-isomer, i.e., D-AC₂, to test the hypothesis that endogenous PLA₂s are responsible for 12MO release from phosphatidylcholine analogs containing 12MO. Fig. 2 shows that the IC₅₀ for D-AC₂ is ~2 μM, which is not significantly different from the IC₅₀ of L-AC₂. Unlike L-AC₂, D-AC₂ is not hydrolyzed by PLA₂, and this suggests that PLA₂ is not a rate-limiting enzyme for 12MO release and HIV antiviral activity. Fig. 2 also shows the HIV antiviral activity of two phosphatidylethanolamine analogs L-P₄ and L-P₄; these analogs are chemically similar to L-AC₁ and L-AC₂ except the PC headgroup has been changed to the PE headgroup. The IC₅₀ of L-P₄ and L-P₄ are 6 and 0.2 μM, respectively. Compared to the PC analogs, this is approximately a 20–50-fold increase in activity. The HIV antiviral activity of L-P₄ is at least 40-fold more than 12MO (Fig. 2).

Table II compares the HIV antiviral activity, cellular toxicity, and selectivity index of 12MO (nonconjugated drug) to several phospholipid conjugates. As shown in Table II, L-AC₂ and L-P₄ improve the therapeutic index of 12MO approximately 15-fold, but analogs containing other lipid headgroups exhibit antiviral activity similar to 12MO with no increase in therapeutic index. The PG analogs have a similar therapeutic index or selectivity, and the PA and PS phospholipid conjugates have slightly less selectivity compared to 12MO. Very unexpectedly, we found that the stereoisomers of PE differ 25-fold in activity (Table II) indicating chiral recognition of the PE headgroup by membrane proteins. Our laboratory is currently preparing chromatographic immobilized artificial membrane (IAM) surfaces prepared from monolayers of membrane lipids (19, 22–24). Thus IAM surfaces prepared from chiral PE may be useful in both purifying and studying membrane proteins that participate in the antiviral activity of L-PE₂.

Drug development using phospholipids will require that the parent compound be stable in blood. The half-life of L-AC₂ in fresh blood is 4.6 h, and the half-life of D-AC₂ is 8.9 h, which is ~4 times longer (Fig. 3). The increased stability of the D-isomer in fresh blood is most interesting, because the equal antiviral activity of the L- and D- isomers is not complemented by equal stability in whole blood. Thus blood PLA₂ significantly participate in the degradation of the compounds in blood but PLA₂s are not the rate-limiting enzyme for drug release. Changing the lipid headgroup also significantly alters the stability in blood. For instance L-PE₂ has a half-life in fresh whole blood of 8.47 ± 0.89 h, which is

C. Pidgeon, unpublished observation.
Anti-HIV Drugs Conjugated To Glycerobackbone of Phospholipids

The average anti-HIV activity from 3 experiments and cellular toxicity in the absence of HIV infection were measured in the same cell line.

<table>
<thead>
<tr>
<th>Anti-HIV lipid*</th>
<th>Anti-HIV activity (IC&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cellular toxicity (TC&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Selectivity index (TC&lt;sub&gt;50&lt;/sub&gt;/IC&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12MO) 12-Methoxydodecanoic acid</td>
<td>6.8 µM</td>
<td>140 µM</td>
<td>21</td>
</tr>
<tr>
<td>(L-AC2) L-Phosphatidylcholine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0</td>
<td>280</td>
<td>280</td>
</tr>
<tr>
<td>(D-AC2) D-Phosphatidylcholine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.2</td>
<td>290</td>
<td>132</td>
</tr>
<tr>
<td>(L-PE2) L-Phosphatidylethanolamine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2</td>
<td>30</td>
<td>150</td>
</tr>
<tr>
<td>(D-PE2) D-Phosphatidylethanolamine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.4</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>(L-PG2) L-Phosphatidylglycerol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2</td>
<td>180</td>
<td>29</td>
</tr>
<tr>
<td>(D-PG2) D-Phosphatidylglycerol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0</td>
<td>240</td>
<td>48</td>
</tr>
<tr>
<td>(L-PA2) L-Phosphatidic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.7</td>
<td>70</td>
<td>10</td>
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<tr>
<td>(D-PA2) D-Phosphatidic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.5</td>
<td>68</td>
<td>19</td>
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<tr>
<td>(L-PS2) L-Phosphatidylserine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>(D-PS2) D-Phosphatidylserine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.5</td>
<td>32</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> L- denotes the natural and D- the unnatural conformation of the glycerol backbone. All phospholipids are diacylated and contained 2 eq of 12MO.

<sup>b</sup> Anti-HIV activity was measured in MT-2 cells infected with HIV-IIIB (see legends to Figs. 1 and 2 for additional details of the assay).

<sup>c</sup> Drug toxicity to cells in the absence of HIV infection was measured in MT-4 cells. Complete dose-response curves of toxicity versus drug concentration were obtained for each compound.

<sup>d</sup> PS and PC analogs were dispersed with culture media at 50 °C with vortexing.

<sup>e</sup> PE analogs were dispersed with culture media containing 15 wt % lysopalmitoylphosphatidylcholine at 40 °C with vigorous vortexing.

<sup>f</sup> PG and PA analogs were prepared by coprecipitation from chloroform of the phospholipid with ~30-35 wt % lysopalmitoylphosphatidylcholine. Redispersion with culture media, warming to 45 °C, and vortexing were needed to completely disperse the phospholipids.

Fig. 3. Stability of phospholipid analogs in whole blood. The t<sub>1/2</sub> values were calculated from 2 experiments and are the mean ± range.

approximately 2 times longer than L-AC2. L-PE1 and L-PE2 are identical except that the methylene group in the 13-position of the sn-2 alkyl chain has been replaced with an oxygen atom. L-PE1 has a very long half-life (t<sub>1/2</sub> > 50 h) compared to L-PE2, and thus the half-life of phospholipid conjugates will depend on both the lipid headgroup and the lipid alkyl chains. The significant increase in t<sub>1/2</sub> for L-PE1 compared to L-PE2 is unexpected because of the apparent trivial modification in the lipid alkyl chain. However, other lipids have shown similar increases in t<sub>1/2</sub> when apparent minor modifications exist in the alkyl chains. For instance, in myelin cell membranes, PC analogs containing 16:0-18:1 alkyl chains have a t<sub>1/2</sub> of 5.7 days, whereas PC analogs containing 18:0-18:1 alkyl chains have a t<sub>1/2</sub> of 25 days (25).

The importance of the lipid alkyl chains in the metabolic processing of membrane lipids by host cell enzymes may be similar to the importance of the lipid headgroups.

The HIV antiviral activity in syncytial cell assays ranged from >200 µM (L-AC1) to 0.2 µM (L-PE2 analog). The increased antiviral activity, particularly of L-AC2 and L-PE2 compared to 12MO, and the increased synergism of L-AC2 with AZT compared to 12MO and AZT are undoubtedly due
to the cellular disposition of phospholipid analogs. Differences in cellular processing of any drug will significantly influence both the activity and toxicity. When 12MO is delivered to cells as a free fatty acid, it is rapidly incorporated into triglycerides and membrane lipids and the t1/2 for incorporation is approximately 1–2 min; the mono-, di-, and triglyceride pools accumulate ~70% of the dose, and the phospholipid pools accumulate ~30% of the dose (14–16). Thus, HIV antiviral activity of heteroatom analogs of myristic acid persists for several days in T-cell cultures (7), with the concurrent rapid incorporation of heteroatom fatty acids into the host cell lipid pools, particularly the triglyceride pools (14–16). Triglycerides are usually considered as a fat storage depot that is used as an energy source for the cell. If intracellular triglycerides, containing 12MO, are used primarily as an energy source instead of a source of fatty acids for myristoylation of HIV proteins, then this may be the primary reason why the cellular availability, necessary for HIV antiviral activity of 12MO, is 10- or 100-fold less than L-AC2 and L-PE2, respectively.

The need to develop antiviral agents with a mode of action other than inhibition of reverse transcriptase remains the central problem in drug discovery in AIDS research. One primary reason for identifying antiviral agents that are not reverse transcriptase inhibitors is that the coadministration of two highly active antiviral drugs, which act by two different mechanisms, should generate potent synergism as was demonstrated for L-AC2/AZT combination therapy (Fig. 1). However, the potent HIV antiviral activity of L-AC2 and L-PE2 also supports the hypothesis that host cells can utilize phospholipids as one source for either myristic acid or heteroatom fatty acid antiviral analogs. In fact, the increased potency of L-AC2 and L-PE2 phospholipid conjugates compared to the nonconjugated drug 12MO suggests that phospholipid pools are the primary source of myristoyl groups instead of endogenous free fatty acid pools. There are many potential ways of utilizing phospholipid drug conjugates. For instance, serine is actively taken up by the brain, and phosphorylidyserine drug conjugates (or other amino acids tethered to lipid headgroups) may concentrate in the brain. In addition, since the D-isomers of phospholipid conjugates will not be degraded by gastric phospholipase A2, improved oral absorption of intact phospholipid conjugates may be possible with D-isomers. Earlier work on drug conjugation to glycerol itself (e.g. Refs. 26–29) or to the headgroup of phospholipids (30) has been reported. During the preparation of this manuscript, we became aware of an European Patent Office publication that describes the conjugation of anti-inflammatory drugs to the glycerol backbone of glycerophospholipids (31). The potent synergism of the anti-HIV phospholipid AC2 with AZT and the recent conjugation of anti-inflammatory compounds to glycerophospholipids suggests that this may be a general strategy for improving the therapeutic efficacy of biologically active compounds.

REFERENCES