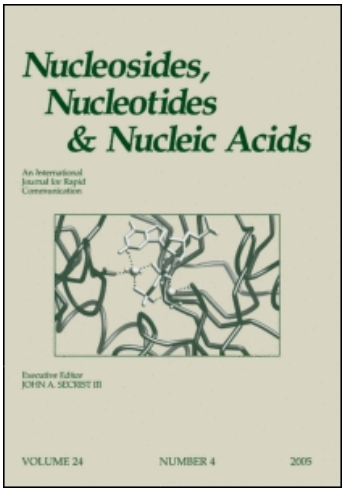

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ENHANCED INHIBITION OF HIV-1 REPLICATION IN MACROPHAGES BY ANTISENSE OLIGONUCLEOTIDES, RIBOZYMES AND ACYCLIC NUCLEOSIDE PHOSPHONATE ANALOGS DELIVERED IN pH-SENSITIVE LIPOSOMES

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ABSTRACT

An antisense oligodeoxynucleotide against the human immunodeficiency virus type 1 (HIV-1) Rev response element, a ribozyme complementary to the HIV-1 5′-LTR, and the reverse transcriptase inhibitors 9-(2-phosphonylmethoxyethyl) adenine (PMEA) and(R)-9-(2-phosphonylmethoxypropyl)-adenine (PMPA) inhibited virus replication in monocyte-derived macrophages more effectively when delivered in pH-sensitive liposomes compared to the free drugs.

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INTRODUCTION

Despite the optimism generated by the availability of a number of drugs that inhibit the human immunodeficiency virus (HIV) reverse transcriptase and protease, the emergence of drug-resistant strains during treatment is a major impediment to the control of virus replication in infected individuals. The development of new inhibitors against drug-resistant viral enzymes, or new drugs against alternative viral targets is likely to take long periods of time. In contrast, the identification of viral gene sequences amenable to hybridize with antisense oligonucleotides (ODN) or ribozymes (1–3) appears to be a more straightforward approach to the development of new drugs against multi-drug-resistant viral strains. The problem of the emergence of any viral strains resistant to these drugs may be addressed by the introduction of corresponding changes in the sequences of the drugs (4,5). Furthermore, the generation of mutants resistant to antisense oligonucleotides is likely to be slower compared to the emergence of those resistant to reverse transcriptase or protease inhibitors, since a single mutation (which can confer resistance to reverse transcriptase or protease inhibitors) may not be sufficient for escape from antisense inhibition, due to the complementarity of a large number of base pairs between the oligonucleotide and its target sequence (6). Despite the potential advantages of antisense ODN and ribozymes, the intracellular delivery of these macromolecular drugs *in vivo* presents an important challenge (2). These agents are thought to be taken up by receptor-mediated endocytosis, a relatively inefficient route requiring high concentrations of the drugs to be active. Although the complexation of antisense ODN or ribozymes with cationic liposomes can enhance their intracellular delivery (7,8), intravenous injection of the complexes can result in their rapid uptake by the liver, making delivery to other organs problematic (9).

Liposomes that destabilize and deliver their contents into the cytoplasm at the mildly acidic pH achieved in endosomes (10,11) have been utilized as carriers for the intracellular delivery of oligonucleotides (12,13). Although first-generation pH-sensitive liposomes are cleared rapidly from the bloodstream following intravenous administration, the inclusion of a low mole fraction of poly(ethylene glycol)-phosphatidylethanolamine (PEG-PE) in the membrane of these liposomes results in prolonged circulation, without compromising their ability to deliver charged fluorescent molecules into macrophage-like cells (14). Here, we investigated the ability of antisense oligonucleotides and ribozymes encapsulated in pH-sensitive liposomes to inhibit virus production in HIV-infected macrophages derived from human peripheral blood monocytes. We also examined whether the anti-HIV activity of acyclic nucleoside phosphonate analogs (15), which are taken up by cells relatively slowly via an endocytosis-like process (16), could be enhanced by delivery in pH-sensitive liposomes. Some of our results have been presented earlier in preliminary form (17,18).

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MATERIALS AND METHODS

Dioleoylphosphatidylethanolamine (DOPE) and phosphatidylglycerol from egg (PG) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate (CHEMS) was obtained from Sigma (St Louis, MO). Polyethylene glycol (2000)-distearoylphosphatidylethanolamine (PEG-PE) was a gift from SEQUUS Pharmaceuticals, Inc. (Menlo Park, CA). An anti-REV-responsive element (RRE) 15-mer phosphorothioate ODN (5′-F-GTGCTTCCTGCTGCOT-3′) and a 15-mer ODN of non-specific sequence (5′-F-CCTATCAGGCAGTAOT-3′), where F and O represent fluorescein and biotin, respectively, were synthesized, purified by HPLC, and provided by Lynx Therapeutics, Inc. (Hayward, CA). A 38-mer, 5′ fluorescein-labeled, chimeric DNA-RNA hammerhead ribozyme (5′-ACACAACAcugaugaGTCCGTGAGGACgaaaCGGGC*A*C-3′, where *: phosphorothioate linkage; capital letters: deoxyribonucleotides; lower case letters: ribonucleotides) targeted to the HIV-1 5′ LTR was synthesized on an automated Applied Biosystems 394 RNA/DNA instrument. A modified ribozyme lacking catalytic activity (5′-CAAACAACcugaugaGTCCGTGAGGACgaaaACCGG*G*C-3′) was used as a control.

pH-sensitive (CHEMS/DOPE, 4:6), word sterically-stabilized pH-sensitive (CHEMS/DOPE/PEG-PE, 4:6:0.3), and control non-pH-sensitive PG/DOPE (4:6) liposomes were prepared by reverse phase evaporation (19,20). Lipid mixtures dried from chloroform were dissolved in 780 μl of pre-washed diethyl ether, 260 μl of an ODN or ribozyme solution (0.2 mM in 100 mM HEPES buffer, pH 7.5, made isotonic with NaCl) was added and the mixture sonicated briefly under argon to form a stable emulsion. The ether was removed in a rotary evaporator under controlled vacuum until a gel was formed. An additional 240 μl of the ODN or ribozyme solution were placed on the gel, which was then disintegrated by vortexing. Evaporation was continued for 30 min to remove any residual ether. The acyclic nucleoside phosphonate analogs 9-(2-(phosphonylmethoxy) ethyladenine (PMEA) and 9-(2-phosphonylmethoxypropyl)-adenine (PMPA) were encapsulated similarly. The PMEA solution used for encapsulation was 5.4 mg/ml in 100 mM HEPES, pH 7.8, made isotonic with NaCl, and the PMPA solution was 5.8 mg/ml. The liposomes were extruded 21 times through two polycarbonate filters of 100 nm pore diameter, using a Liposo Fast device (Avestin, Inc., Ottawa, Canada), to obtain a uniform size distribution. To remove unencapsulated molecules, as well as to change the buffer, liposomes were subjected to dialysis in Spectra/Por (Spectrum, Houston, TX) dialysis bags (MW cut-off: 50,000) against 2 changes of 4 liters of 10 mM HEPES-buffered isotonic saline, pH 7.4, for 20 h each at 4°C. Liposomes were sterilized by filtration through 0.45 μm syringe filters (MSI, Westboro, MA). The amount of ODN or ribozyme associated with liposomes after dialysis was assessed by measuring their fluorescence and/or by evaluating the optical density at 260 nm after addition of a detergent ($C_{12}E_8$, final concentration 1 mg/ml). The amount of encapsulated PMEA or PMPA was determined by

fluoroscence after lysing the liposomes with detergent and reacting the drugs with chloracetaldehyde (21).

Human macrophages were isolated from HIV-seronegative buffy coats by centrifugation on Ficoll-Hypaque (Histopaque 1077, Sigma, St. Louis, MO) and adherence to plastic, as described previously (22). HIV-1_{BaL} was purchased from Advanced Biotechnologies and propagated in macrophages (22). The number of cells remaining at the end of the experiment was estimated by counting the nuclei after staining the cells with Naphthol Blue Black (23). Cell viability was measured by a modified Alamar blue assay (24). Macrophages plated in 48-well plates were infected with HIV-1_{BaL} at a multiplicity of infection of about 0.1 on day 7 after isolation, by incubation with 140–165 μl of virus-containing culture medium for 2 h at 37°C. The cells were then washed with fresh Dulbecco's Modified Eagle's Medium-high glucose (DME-HG) (Irvine Scientific, Santa Ana, CA) medium with 20% fetal bovine serum and incubation at 37°C was continued. Various dilutions of ODN or ribozyme preparations were made in the same culture medium, and added to the cells 24 h after infection. Fresh dilutions of treatments were added with two medium replacements until day 8 following infection. On this day fresh medium without treatments was added to the cells, and supernatants were saved for subsequent analysis. Viral p24 levels were then monitored in cell culture supernatants collected every 2–3 days, using an enzyme-linked immunosorbent assay (ELISA) as described (25). ELISA kits obtained from Coulter (Miami, FL) were also utilized in some experiments. Supernatants of uninfected cells were used as controls for the p24 determinations by ELISA.

RESULTS AND DISCUSSION

The free (unencapsulated) 15-mer anti-RRE phosphorothioate ODN was not active against HIV infection in macrophages, even at a dose of 3 μM (Fig. 1). When delivered in pH-sensitive CHEMS/DOPE liposomes, however it inhibited p24 production by 42% at 1 μM (data not shown), and by 91% at 3 μM (Fig. 1). The ODN was also effective when delivered in sterically stabilized pH-sensitive CHEMS/DOPE/PEG-PE liposomes, but not when encapsulated in non-pH-sensitive liposomes. The non-specific ODN encapsulated in pH-sensitive liposomes had no effect at 1 μm (data not shown), but at 3 μM it inhibited HIV infection by 53%. Other laboratories have also noted non-specific inhibition of HIV infection of lymphocyte cell lines by phosphorotioate ODN (26–28). This observation was attributed to the inhibition of virus binding to the CD4 receptor (29), or the non-specific inhibition of viral reverse transcriptase (27). In our experiments, however, the treatments were initiated 24 h after the initial infection step, excluding the possibility of inhibition of virus entry by the ODN in the medium. Control, buffer-loaded, pH-sensitive and sterically stabilized pH-sensitive liposomes affected HIV infection to some extent, but their effect was inconsistent from experiment to experiment.

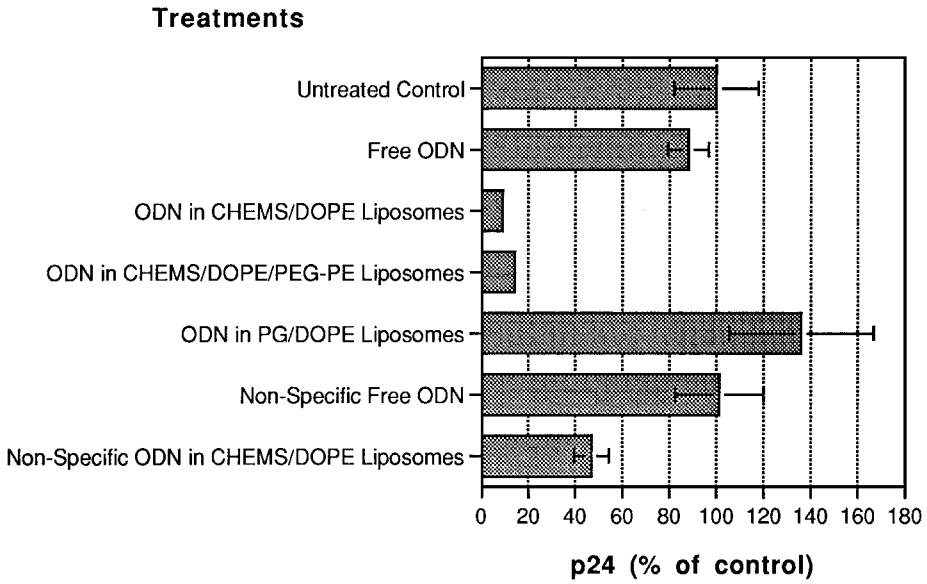

Figure 1. Inhibition of HIV-1 production in human macrophages by free and liposome-encapsulated anti-RRE 15-mer oligonucleotide (ODN) at a concentration of 3 μM. The viral p24 values are given as the percent of untreated controls.

Similar experiments were performed with a 38-mer chimeric ribozyme complementary to HIV 5′-LTR. Cationic liposome-mediated delivery of this ribozyme to HIV-1-infected cells was not effective in reducing virus production under conditions where the delivery method was not toxic to the cells (8). When this ribozyme was delivered to HIV-infected macrophages in pH-sensitive liposomes, virus production was inhibited by 88%, while the free ribozyme caused a decrease of only 10% (Fig. 2). A non-catalytic, modified ribozyme also had an inhibitory effect when delivered in pH-sensitive liposomes, reducing virus production by 73%, part of which may be attributed to an antisense-like action rather than ribozyme activity.

The potential of pH-sensitive liposomes to mediate intracellular delivery of anti-HIV agents was also illustrated by encapsulating the acyclic nucleoside phosphonates PMEA and PMPA which inhibit reverse transcription (15,16,30). Unlike nucleoside analogs, the intracellular phosphorylation of these compounds is not a limiting step for antiviral activity. The drawbacks of these antiviral drugs are their slow cellular uptake and their poor oral bioavailability, which have led to the development of prodrugs with improved uptake into cells and via the gastrointestinal tract (16). When PMEA and PMPA were delivered to HIV-infected macrophages in pH-sensitive liposomes the antiviral effect of the drugs was enhanced (Tables 1 and 2). The EC_{50} of the liposome-encapsulated PMEA was about 10-fold lower than that of the free antiviral. The encapsulated drugs were more effective in inhibiting HIV production by macrophages throughout the concentration range tested.

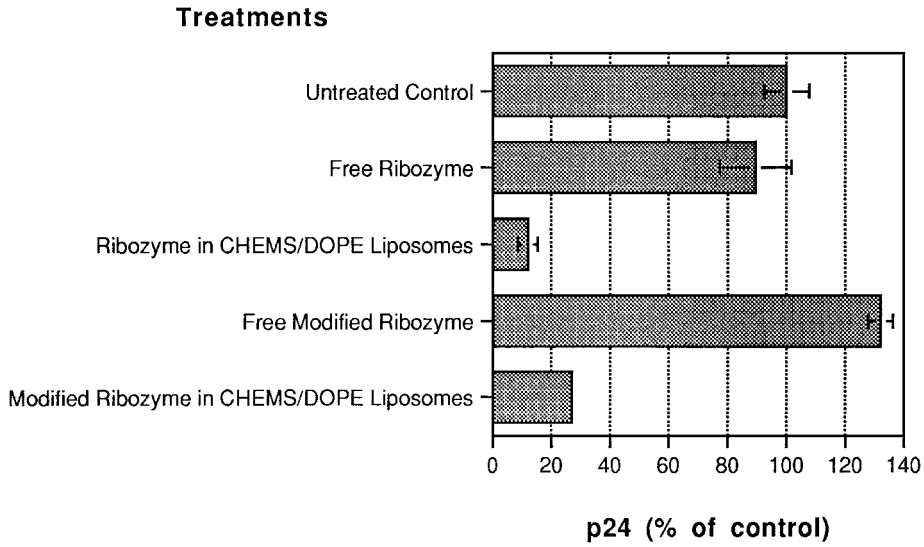

Figure 2. The effect of an anti-HIV 5′-LTR 38-mer chimeric (RNA-DNA) hammerhead ribozyme encapsulted in pH-sensitive liposomes on HIV-1 production in human macrophages. The ribozyme concentration was 3 μM. The data are expressed as the percentage of virus produced by untreated macrophages.

Phosphorothioate antisense ODN were shown to be delivered effectively into target cells *in vivo* even when they were administered systemically in the free form (31). However, the clinical use of these molecules may be limited because of the possible toxicity associated with non-sequence-specific effects (32,33). Drug carriers such as liposomes may decrease or eliminate this complication. Association of ODN with cationic liposomes resulted in rapid elimination of the complexes from

Table 1. Inhibition of Virus Production in HIV-1_{BaL}-Infected Macrophages by Free or Liposome-Encapsulated 9-(2-(phosphonylmethoxy)ethyladenine (PMEA)

	p24 (% of Control)	
Concentration (nM)	Free PMEA	Liposome-PMEA
0	100 ± 10.1	100 ± 15.7
2	91.8 ± 18.5	31.0 ± 11.1
10	80.7 ± 16.0	27.3 ± 2.7
20	24.1 ± 2.8	16.3 ± 2.7
100	ND	1.7 ± 0.05
200	3.03 ± 2.14	0.13 ± 0.34

The macrophages were infected by incubation with HIV-1_{BaL} for 2 h, and further incubated for 24 h. They were then treated for 8 days, and viral p24 in culture supernatants were determined on day 15 after infection. The results are given as the percentage of virus produced by untreated control cells. ND: not determined.

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Table 2. Inhibition of Virus Production in HIV-1_{BaL}-Infected Macrophages by Free or Liposome-Encapsulated (R)-9-(2-phosphonylmethoxypropyl)-adenine (PMPA)

	p24 (% of Control)	
Concentration (nM)	Free PMPA	Liposome-PMPA
0	100 ± 10.3	100 ± 10.3
2	91.8 ± 16.5	65.6 ± 7.9
10	66.2 ± 7.7	16.3 ± 0.6
20	40.2 ± 3.4	6.1 ± 0.4
50	18.7 ± 3.1	3.1 ± 0.5
100	6.5 ± 0.7	1.9 ± 0.5
200	2.5 ± 0.4	0.17 ± 0.1

The macrophages were infected by incubation with HIV-1_{BaL} for 2 h, and further incubated for 24 h. They were then treated for 8 days, and viral p24 in culture supernatants were determined on day 15 after infection. The results are given as the percentage of virus produced by untreated control cells.

the bloodstream by the reticuloendothelial system after early accumulation in the lung due to embolism (9). Thus, cationic liposomes in their current formulation may not be able to function as a useful delivery system *in vivo*. We have developed sterically stabilized pH-sensitive liposomes that can deliver their contents into cells and have prolonged circulation *in vivo* (14). The experiments presented here show that these liposomes can facilitate the antiviral effect of antisense ODN for the treatment of HIV infection of primary macrophages. Since these liposomes circulate for prolonged periods and can localize in lymph nodes after intravenous or subcutaneous injection (34), they may be useful for the delivery of antisense ODN, ribozymes and acyclic nucleoside phosphonates to lymph nodes where active HIV replication takes place (35–37).

The necessity for prolonged intravenous or subcutaneous administration of liposomal antivirals for the treatment of HIV infection is a major disadvantage of this delivery system. This disadvantage, however, has to be weighed against the potential of relatively infrequent administration of liposomal antivirals, localization in lymph nodes and the ability of liposomes to deliver novel macromolecular drugs against multi-drug-resistant HIV strains. Future applications of pH-sensitive liposomes in the treatment of HIV infection may include the delivery of triple helix-forming ODN to target the integrated provirus in the nucleus (38–39). If this approach becomes feasible, the necessity for freqent administration may also be obviated.

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