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Spontaneous and Protein-Mediated Intermembrane Transfer of the Antiretroviral Liponucleotide 3'-Deoxythymidine Diphosphate Diglyceride

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ABSTRACT: Phospholipid conjugates of antiretroviral nucleosides show activity against the human immunodeficiency virus in vitro [Hostetler, K. Y., Stuhmiller, L. M., Lenting, H. B. M., Van den Bosch, H., & Richman, D. D. (1990) J. Biol. Chem. 265, 6112–6117]. In order to gain insight into the membrane association and the spontaneous and protein-mediated intermembrane transfer of these compounds, we have synthesized the fluorescent analog 3'-deoxythymidine diphosphate 1-myristoyl-2-(10-pyren-1-yl-decanoyl)glycerol. The compound readily incorporated into ethanol-injection vesicles, but the stability of the fluorescent probe (10% of total lipid) in the lipid bilayer was less than that of 1-myristoyl-2-(10-pyren-1-yl-decanoyl)phosphatidylcholine. Using a donor–acceptor vesicle assay system, half-times for spontaneous transfer at 25 and 37 °C were 20 and 100 min, respectively. The liponucleotide was rapidly transferred between membranes by the nonspecific lipid-transfer protein at a rate at least 10-fold that of the corresponding phosphatidylcholine. Depletion of the liponucleotide from the outer monolayer of vesicles by a large excess of nonspecific lipid-transfer protein indicated a transbilayer distribution similar to the mass distribution of phospholipids. Essentially no flip-flop of the inner monolayer liponucleotide was observed.

**D**ideoxy nucleosides comprise a family of nucleoside analogs, several members of which have in vitro and in vivo activity against the human immunodeficiency virus (HIV) (Yarchoan et al., 1989; de Clerq, 1990), the causative agent of the acquired immunodeficiency syndrome (Barré-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984). The compounds exert their antiviral activity after anabolic phosphorylation to their triphosphates, which are specific inhibitors of the viral enzyme reverse transcriptase (Huang et al., 1990; Parker et al., 1991). Zidovudine (3'-azido-3'-deoxythymidine; AZT) is the first agent to be approved for treatment of HIV infection (Fischl et al., 1987). In recent reports, the synthesis, metabolism, and anti-HIV activity of phospholipid conjugates of some selected dideoxy nucleosides have been described (Hostetler et al., 1990, 1991; van Wijk et al., 1991a,b). Nucleoside diphosphate diglycerides are one class of antiviral phospholipids we synthesized and examined for anti-HIV activity in vitro. These conjugates are analogs of cytidine diphosphate diglyceride (CDP-DG), the naturally occurring intermediate in anionic phospholipid biosynthesis (van den Bosch, 1974). A potential advantage of this type of conjugate is the release of an antiviral nucleoside monophosphosphate, as a result of substitution of the liponucleotide in the metabolism of CDP-DG. This drug release mechanism would bypass thymidine kinase activities, reported to be relatively weak in macrophages (Richman et al., 1987), which are an important reservoir of HIV infection (Meltzer et al., 1990). Dideoxyctidine (ddC) diphosphate diglyceride, 3'-azido-3'-deoxythymidine (AZT) diphosphate diglyceride, and 3'-deoxythymidine (3dT) diphosphate diglyceride all show anti-HIV activity in vitro (Hostetler et al., 1990; unpublished observations) and can be metabolized with the concomitant release of ddC monophosphate, AZT monophosphate, and 3dT monophosphate (van Wijk et al., 1991a,b).

To gain further information on some properties of antiviral nucleoside diphosphate diglycerides, we synthesized 3'-deoxythymidine diphosphate 1-myristoyl-2-(10-pyren-1-yl-decanoyl)glycerol (3dTDP-pyrDG; Figure 1) as a model

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**Figure 1:** Structure of 3'-deoxythymidine diphosphate diglyceride and phosphatidylcholine both containing an sn-1-myristoyl-2-(10-pyren-1-yl-decanoyl)glycerol 3-phosphate moiety. When X = X₁, the structure is that of pyrPC; when X = X₂, the structure is that of 3dTDP-pyrDG.

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1 Abbreviations: HIV, human immunodeficiency virus; AZT, 3'-azido-3'-deoxythymidine; 3dT, 3'-deoxythymidine; ddC, 2',3'-dideoxyctidine; 3dTDP-DG, 3'-deoxythymidine diphosphate diglyceride; 3dTDP-pyrDG, 3'-deoxythymidine diphosphate 1-myristoyl-2-(10-pyren-1-yl-decanoyl)glycerol; pyrPC, 1-myristoyl-2-(10-pyren-1-yl-decanoyl)phosphatidylcholine; pyrPA, 1-myristoyl-2-(10-pyren-1-yl-decanoyl)phosphatidic acid; nsL-TP, nonspecific lipid-transfer protein; PC-TP, phosphatidylcholine-transfer protein; PI-TP, phosphatidylinositol-transfer protein; DOPC, dioleylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; TNP-PE, N-(2,4,6-trinitrophenyl)phosphatidylethanolamine.
compound. This compound closely resembles the hydrophobicity of 3dTDP-dimyristoylglycerol, which we used in the metabolic and virus inhibition studies. Using the pyrene-labeled fluorescent probe, we studied its spontaneous intermembrane transfer in vitro, as a measure of its stability in liposomal formulations. A comparison is made with 1-myristoyl-2-(10-pyren-1-yldecanoyl)phosphatidylcholine (ppyPC), a compound with identical acyl chains but a less bulky headgroup (Figure 1). In addition, the protein-mediated transfer of 3dTDP-pyrDG and ppyPC was examined, using the nonspecific lipid-transfer protein (nSL-TP) from bovine liver, the phosphatidylinositol-transfer protein (PI-TP) from bovine brain, and the phosphatidylcholine-transfer protein (PC-TP) from bovine liver. From these experiments, we were able to determine the bilayer distribution, the half-time of spontaneous transfer at 37 °C, and the virtual absence of flip-flop of 3dTDP-pyrDG.

**Experimental Procedures**

**Materials**

Egg yolk phosphatidylcholine (eggPC), cholesterol, dioleoylphosphatidylcholine (DOPC), 1-myristoyl-lyso phosphatidylcholine (lysoPC), 10-pyren-1-yldecanoic acid, phospholipase D, and 3'-deoxythymidine were obtained from Sigma Chemical Co., St. Louis, MO. (Dimethylamino) pyridine, silica gel 60 (230–400 mesh), TLC and HPTLC plates (silica 60 F254), ethanol (Uvasol), and chromatographic solvents (Lichrosolv) were all from Merck, Darmstadt, FRG. Morpholine and dicyclohexylcarbodiide were products from Aldrich Chemical Co., Milwaukee, WI, and dimyristoylphosphatidic acid was obtained from Avanti Polar Lipids, Inc., Alabaster, AL. All other chemicals used were of analytical grade. N-(2,4,6-trinitrophenyl)phosphatidylethanolamine (TNP-PE) was prepared from egg yolk PE and trinitrobenzenesulfonic acid (Van Duyn et al., 1985). 1-Myristoyl-2-(10-pyren-1-yldecanoyl)phosphatidylcholine (ppyPC) was synthesized and purified as described (Sommerharju et al., 1985). The nonspecific lipid-transfer protein (Van Amerongen et al., 1989), phosphatidylcholine-transfer protein (Westerman et al., 1983), and phosphatidylinositol-transfer protein (Van Paridon et al., 1987) were isolated as described.

**Methods**

**Synthesis and Characterization of 3dTDP-pyrDG**. The phosphorylation of 3dT to 3dT 5'-monophosphate was performed essentially as described by Yoshikawa et al. (1967). The crude reaction mixture was fractionated by anion-exchange chromatography (Q-Sepharose fast flow; Pharmacia, Uppsala, Sweden), and 3dT 5'-monophosphate was eluted isocratically at 0.1 M ammonium hydrogencarbonate as a single compound. Its purity was determined by HPTLC and HPLC and was greater than 95% (Van Wijk et al., 1992). The activated ppyPA was then isolated, lyophilized, and condensed with two equivalents of 3dT 5'-monophosphate (H⁺-form) in anhydrous pyridine (Van Wijk et al., 1992). After 3 h, the reaction was stopped by evaporation of pyridine and the crude mixture was extracted (Bligh & Dyer, 1959). The combined chloroform layers were evaporated to dryness, and the residue was dissolved in a minimum amount of n-hexane/2-propanol/25% ammonia/water (43:57:3:7 v/v). This solution was subjected to HPLC using a silica column (Waters; μPorasil 0.39 × 30 cm; flow, 1 mL/min; detection at 206 nm) and eluted with the above solvent mixture. The product, 3dTDP-pyrDG, eluted as a single component with a retention time of 10 min, well separated from ppyPA, a major contaminant. Fractions containing product were pooled, evaporated to dryness, lyophilized, and redissolved in chloroform/methanol (1:1 v/v). HPTLC analysis showed a single phosphorus-positive (Dittmer & Lester, 1964) and ultraviolet-positive (irradiation at λ = 254 or 312 nm) spot with Rf values identical to that of 3dTDP-dimyristoylglycerol with both solvents A and B as the developing system. Its concentration was determined by phosphorus (Rouser et al., 1970) and UV analysis (ε342 nm = 42 000 M⁻¹ cm⁻¹ in ethanol) (Sommerharju et al., 1985). The compound was readily hydrolyzed by a mitochondrial pyrophosphatase activity (Van Wijk et al., 1991a).

**Fluorescence Measurements**. Fluorometric spectroscopy was performed on a SLM-Aminco SPF-500C spectrofluorometer equipped with a stirring device and thermostated cuvette holder. The temperature was kept at 25 or 37 °C. Excitation and emission wavelengths were 346 and 377 nm respectively, with corresponding band-passes of 1 and 10 nm. The small excitation band-pass ensured minimal photolysis of the pyrene probes. Measurements were performed in buffer (filtered through 0.22-μm millipore filter) consisting of 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl, pH 7.4 (buffer A).

**Donor Vesicle Preparation**. A mixture of DOPC/cholesterol/TNP-PE/3dTDP-pyrDG or ppyPC (5:3:1 mol/mol) was evaporated to dryness under a gentle stream of N₂. The dried lipids were redissolved in ethanol at a final concentration of 250 μM of total lipid. From this stock solution, 10 μL was injected into 1.7–2.0 mL of buffer A under continuous stirring. The vesicles thus prepared (Batzi & Korn, 1973) showed essentially completely quenched fluorescence (>98%).

**Acceptor Vesicle Preparation**. Acceptor vesicles consisting of egg yolk PC/cholesterol/DMPA (5:3:2 mol/mol) were prepared by suspending a lyophilized mixture of the lipids in buffer A (final concentration 2 mM of total lipid), followed by ultrasonication (N₂ stream, 0 °C, 2 × 3 min, 50 W) yielding a clear solution. Acceptor vesicle preparations were freshly made every day.

**Spontaneous Transfer Assay**. Donor vesicles were prepared as described above. After a stable quenched signal from the
donor vesicles was obtained (generally within 60 s). 50 μL (100 nmol) of acceptor vesicles was added to give a final volume of 2.0 mL. The spontaneous transfer of 3dTDP-pyrDG and pyrPC from donor to acceptor membranes was monitored continuously, by measuring the increase of pyrene-monomer fluorescence at 377 nm (excitation at 346 nm) as a function of time up to 80 min. The pyrene-monomer fluorescence was calibrated by use of an acceptor vesicle preparation containing 0.05% of 3dTDP-pyrDG or pyrPC (Homan & Pownall, 1988; Gadella & Wirtz, 1991). Potential degradation of the probes was routinely checked by the addition of three to four aliquots of 20 μL of a solution containing 10% Triton X-100, which completely dequenched the probes. After a stable signal was obtained, the quantum yield in Triton X-100 (Qv) was determined and compared with the quantum yield in vesicles (Qv). Degradation of the probes due to photolysis was less than 5% in all experiments as judged by the Triton X-100 dequenching procedure, and no spectral changes were observed in the pyrene fluorescence spectrum.

Transfer and Binding Assays. Protein-mediated transfer of 3dTDP-pyrDG and pyrPC was measured as described above for spontaneous transfer, except that after a linear signal was achieved, lipid-transfer proteins were added (see legends to the figures). Binding of 3dTDP-pyrDG and pyrPC by nsL-TP was determined by measuring the increase of pyrene-monomer fluorescence. Quenched donor vesicles were prepared by injecting 10 μL from the ethanol stock solution into buffer A, and aliquots of nsL-TP were added after a stable donor vesicle signal was obtained. The quantum yield of pyrene-lipid bound to nsL-TP (Qv) was calibrated as described by Gadella and Wirtz (1991). This allowed us to quantify the amount of lipid bound to nsL-TP at equilibrium and to obtain information on bilayer distribution and flip-flop rate of 3dTDP-pyrDG.

RESULTS AND DISCUSSION

Synthesis and Characterization of 3dTDP-pyrDG. Following the route described in the experimental section, 3dTDP-pyrDG was synthesized successfully. The structure of the probe (Figure 1) was verified in several ways. All pyrene-labeled intermediates in the synthesis coeluted with unlabeled authentic samples on HPTLC using solvent A or B as developing system. The final product had the same Rf value as 3dTDP-dimyristoylDG (Rf = 0.3 or 0.5 with solvent A or B, respectively). The 1H NMR spectrum of the latter in CDC13/MeOD (1:1 v/v) with tetramethylsilane as internal standard showed the following chemical shifts: 0.88 (CH3, acyl chains), 1.27 (CH3, acyl chains), 1.61 (β-CH2, acyl chain), 2.31 (α-CH2, acyl chain), 4.25 (CH2, sn-3-glycerol), 4.43 (CH2, sn-1-glycerol), 5.25 (CH, sn-2-glycerol), 6.07, 20.4, 0.07, 4.51, and 3.90 for 1H1, 1H2, 1H3, 1H4, and 1H5 of deoxyribose, respectively, and 1.96 and 7.77 for CH3 and H6 of thymine.

Results and Discussion

The quantum yield of pyrene-monomer fluorescence bound to nsL-TP at equilibrium and to obtain information on bilayer distribution and flip-flop rate of 3dTDP-pyrDG. In Figure 2, both spectra are essentially identical with spectra of pyrene-labeled probes published by others (Somerharju et al., 1985; Hresko et al., 1985). In the absorption spectrum the typical maxima at 311, 325, and 342 nm are observed. The pyrene-monomer fluorescence spectrum has the typical maxima at 377 and 397 nm. Upon omission of TNP-PE from the donor vesicle preparation, the emission spectrum displayed the characteristic pyrene excimer fluorescence between 380 and 600 nm (data not shown), indicating close proximity of the pyrene moieties in the vesicle bilayer structure.

Spontaneous Transfer Studies. As a measure of the stability of the association of 3dTDP-pyrDG with a liposomal structure, we determined its intermembrane transfer from donor vesicles to a 40-fold excess of acceptor vesicles. Figure 3 shows the spontaneous transfer of 3dTDP-pyrDG (curves I and II) and pyrPC (curves III and IV) at 25 and 37 °C. Compared to pyrPC, the spontaneous transfer of 3dTDP-pyrDG is at least 15-fold faster, with estimated half times (t1/2) of 20 min at 37 °C and of 100 min at 25 °C. The time scale of these experiments was too short to determine the t1/2 values for pyrPC, but these will be on the order of hours to days as has been determined by others (Homan & Pownall, 1988; Hresko et al., 1985). In control experiments, the spontaneous transfer of [3H]3dTDP-DG from donor vesicles to multilamellar liposomes consisting of egg PC/cholesterol/DMPA (6:3:1 mol/mol) was determined according to Banks et al.
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A. G. Gadella (1988). A $t_{1/2}$ value of 20–30 min at 37°C was found, confirming that the relatively high rate of spontaneous transfer for this type of compound is not a peculiar property of the pyrene-labeled analog.

The spontaneous transfer of 3dTDP-pyrDG at 37°C could easily be fitted into a single-exponential equation $F(t) = A(1 - e^{-kt}) + C$ (Homan & Pownall, 1988). In this equation, $F(t)$ represents the percent fluorescent signal at time $t$ (in minutes), $A =$ percent of probe available for transfer, and $C =$ constant, reflecting background fluorescence, and $k =$ rate constant (in minutes$^{-1}$). From curve-fitting of the fluorescence increase, we could estimate that 60% of the 3dTDP-pyrDG was available for spontaneous transfer (see also below), with a calculated $t_{1/2}$ at 37°C of 20.4 ± 0.9 min. This value is in excellent agreement with the experimentally derived $t_{1/2}$ value. If the 60% value represents 3dTDP-pyrDG in the outer monolayer of the donor vesicles, the remaining 40% should be in the inner monolayer. In view of the small vesicles that are generally obtained by the ethanol-injection method (Batzri & Korn, 1973), this bilayer distribution is as expected and indicates that 3dTDP-pyrDG follows the mass phospholipid distribution (van Paridon et al., 1988). These measurements also indicate that the rate of spontaneous transfer is much faster than the transbilayer movement. A considerable flip-flop of 3dTDP-pyrDG from the inner to the outer monolayer of the donor vesicles would result in an amount of the probe available for intermembrane transfer that is larger than 60% and in an additional exponential term in the kinetic description of the experimental data (Homan & Pownall, 1988). Slow rates of flip-flop in lipid model systems is a general phenomenon that results from the insolubility of the polar headgroup in the hydrophobic membrane interior (Homan & Pownall, 1988; Lange, 1986). This certainly applies to the bulky negatively charged headgroup of 3dTDP-pyrDG. On the other hand, both bulkiness and charge of the polar headgroup are known to decrease the stability of phospholipids in the membrane (Homan & Pownall, 1988; Lange, 1986). These factors must be responsible for the less stable association of 3dTDP-pyrDG with liposomal membranes when compared with pyrPC (Figure 3), since both compounds have identical acyl constituents. Manipulation of the acyl chain lengths might greatly increase the $t_{1/2}$ values for spontaneous transfer. Studies performed by Homan and Pownall (1988) have shown that an increase of the sn-1-acyl chain length with two methylene groups reduced the rate of spontaneous transfer approximately 8-fold. By extrapolation, it can be predicted that substitution of the myristoyl chain at the sn-1-position of 3dTDP-pyrDG by a palmitoyl or stearoyl chain would give rise to $t_{1/2}$ values of 160 or 1280 min, respectively. We are currently working on the synthesis of these species to test this prediction.

**Protein-Mediated Transfer.** A comparison between the nsl-TP-mediated intermembrane transfer of 3dTDP-pyrDG and pyrPC is shown in Figure 4 (curves I and II, respectively). The results clearly demonstrate that nsl-TP also catalyzes the intermembrane transfer of the class of CDP-DG analogs of which 3dTDP-pyrDG is a representative. In fact, it can be calculated that the amount of 3dTDP-pyrDG transferred by nsl-TP, after correction for spontaneous transfer, exceeds that of pyrPC at all time points. From the shape of the curve it appears that upon addition of nsl-TP 3dTDP-pyrDG is at first rapidly bound by the protein and then transferred to the acceptor vesicles. By contrast, pyrPC is not rapidly extracted from the donor vesicles by nsl-TP but is steadily transferred to the acceptor vesicles. These findings closely resemble observations made in a recent study on the mode of action of nsl-TP (Gadella & Wirtz, 1991). By using the negatively charged phospholipids phosphatidylinositol, phosphatidylinositol phosphate, and phosphatidylinositol bisphosphate, it was shown that binding of these lipids to nsl-TP increased with increasing negative charge and that those phospholipids were preferred substrates when compared to phosphatidylcholine.

The nearly instantaneous extraction of a considerable part of 3dTDP-pyrDG from the donor vesicle membranes by nsl-TP, as observed in Figure 4, suggested an additional method for determination of the transbilayer distribution of this liponucleotide in donor vesicles by the use of a large excess of nsl-TP. Under these conditions, an almost instantaneous and maximal transfer of 3dTDP-pyrDG from donor to acceptor vesicles was observed (Figure 5, trace I). By addition of nsl-TP (Gadella & Wirtz, 1991). By using the negatively charged phospholipids phosphatidylinositol, phosphatidylinositol phosphate, and phosphatidylinositol bisphosphate, it was shown that binding of these lipids to nsl-TP increased with increasing negative charge and that those phospholipids were preferred substrates when compared to phosphatidylcholine.
of Triton X-100 (not shown) and the use of a standard acceptor vesicle preparation containing 0.05 mol % 3dTDP-pyrDG for calibration, we were able to calculate the amount of 3dTDP-pyrDG transferred from donor to acceptor membranes by correlating the quantum yield in Triton X-100 (QT) to the quantum yield in vesicles (Qv); i.e., QT = 1.6Qv. These calculations showed that the plateau of trace I (Figure 5) represented 155 pmol of the total 250 pmol of 3dTDP-pyrDG in the donor vesicles that became transferred under these conditions by the large excess of nsL-TP. This value again indicates that approximately 62% of 3dTDP-pyrDG is located in the outer monolayer of the donor vesicles. The stable plateau signal further indicates that there is no appreciable flip-flop of 3dTDP-pyrDG. In addition, the transfer of 3dTDP-pyrDG from donor to acceptor vesicles in the presence of phosphatidylinositol-transfer protein (Figure 5, trace III) or phosphatidylinositol-choline-transfer protein (Figure 5, trace II) is shown. As expected, on the basis of the specificity of these transfer proteins (Wirtz & Gadella, 1990), no protein-mediated transfer of 3dTDP-pyrDG is apparent. The slow increase of pyrene-monomer fluorescence can be fully accounted for by spontaneous transfer.

Binding of 3dTDP-pyrDG and pyrPC to nsL-TP. The rapid extraction of 3dTDP-pyrDG from the outer monolayer of donor vesicles by binding to nsL-TP was confirmed in the absence of acceptor vesicles (Figure 6). After donor vesicles were prepared, the addition of nsL-TP (arrow B) resulted in rapid (<30 s) binding of 3dTDP-pyrDG as judged by the appearance of pyrene-monomer fluorescence. After equilibrium was reached, a 40-fold excess of acceptor vesicles was added (arrow C). This resulted in a decrease in pyrene-monomer fluorescence due to a rapid release of nsL-TP-bound 3dTDP-pyrDG into the acceptor vesicles and a lower quantum yield in the vesicles. Thereafter, a linear increase in fluorescence with time is observed due to protein-mediated transfer of 3dTDP-pyrDG that was still associated with the outer leaflet of the donor vesicles. By extrapolating this protein-mediated transfer to the acceptor vesicles to zero-time, i.e., at addition of nsL-TP, the quantum yield of 3dTDP-pyrDG bound to the protein (Qp) can be correlated to that in vesicles (Qv, see Figure 6). The fluorescence quantum yield of 3dTDP-pyrDG in nsL-TP was found to be 1.3 times that in vesicles. This correlation and a calibration of the pyrene-monomer fluorescence in vesicles using an acceptor vesicle preparation containing 0.05 mol % 3dTDP-pyrDG allowed us to calculate the amount of 3dTDP-pyrDG that was bound to nsL-TP. Experiments as those depicted in Figure 6 were performed with a range of nsL-TP concentrations, and the results are summarized in Table I. An increase in the amount of nsL-TP resulted in increased extraction of 3dTDP-pyrDG from the donor vesicles up to 55.7% of the total amount of probe present. This is close to the amount of probe available in the outer leaflet, i.e., 62% (see above). Thus, at a molar ratio of nsL-TP to 3dTDP-pyrDG of 13.6 essentially all of the probe is extracted from the outer leaflet by binding to nsL-TP. The maximal percentage of nsL-TP molecules that carried a probe molecule amounted to 12.9% attained at the molar ratio of nsL-TP to total probe of 0.14. This value of 12.9% compares favorably to the 8.1% and 0.6% for the binding of the pyrene-labeled analogs of phosphatidylinositol 4,5-bisphosphate and phosphatidylycholine, respectively (Gadella & Wirtz, 1991). This shows that, inherent to its low-affinity lipid binding site, the binding of lipid to nsL-TP remains limited even in the presence of a 7-fold molar excess of 3dTDP-pyrDG. On the other hand, nsL-TP appears very well suited to transfer the antiviral liponucleotide to vesicle membranes. It remains to be established whether nsL-TP will also transfer this liponucleotide to intact cells.

In conclusion, pyrene-labeled analogs of liponucleotides are very convenient probes for assessing the stability of these prodrugs in vesicles and, hence, for determining the optimal liponucleotide vesicle formation in prodrug delivery.

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