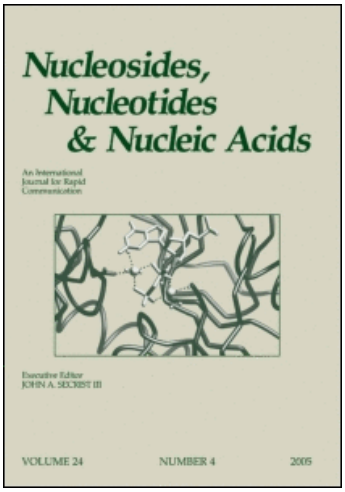

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF PHOSPHONOCARBOXYLATE DNA

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□ *Oligodeoxynucleotides containing internucleotide phosphonoacetate esters are taken up irreversibly by cells in culture in the absence of cationic lipids. These oligonucleotides also are active in stimulating RNase H and are stable toward nucleases.*

Keywords Phosphonocarboxylate oligonucleotides; passive delivery

INTRODUCTION

Readily available, rapidly synthesized oligodeoxyribonucleotides and their analogs (ODNs) have become indispensable tools for modern research in biology and biochemistry as these reagents are used to sequence DNA, probe genomes, direct mutagenesis, diagnose genetic activity, and manipulate the expression of genes. Although a large number of analogs have been prepared, essentially all possess various limitations that restrict their usefulness.[1] For example, most biologically active derivatives are anionic and thus require complexation with cationic lipids in order to be taken up by cells in culture. These complexes are extremely toxic to cells and require careful titration with each cell type in order to be used effectively. Even under these conditions, many cell types simply do not take up DNA or do so at extremely low levels. Additionally, once an analog has been transfected into cells using cationic lipids, it must not only be biologically useful (inhibit or activate gene expression, measure biological activity, etc.) but stable to endogenous nucleases. To date only a limited number of analogs (phosphorothioate, phosphorodithioate, cyclohexenenucleic acids, and 2′-fluoro-2′-deoxyarabinofuranosyl nucleic acids) have been shown to possess

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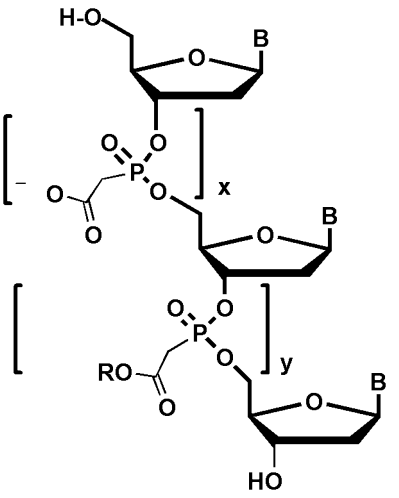

FIGURE 1 Chemical structure of phosphonoacetate DNA.

these properties. However, as outlined previously, all these derivatives have limited usefulness for various reasons.[2]

In this article, we present results with phosphonoacetate DNA (Figure 1) that addresses these challenges. We show that certain esters of phosphonoacetate DNA are taken up irreversibly by cells in culture and can be used to transport RNA into cells—both in the absence of cationic lipids.

RESULTS AND DISCUSSION

Transfections with Esterified Phosphonoacetate ODNs in the Absence of Cationic Lipids

Based on previous observations that small molecule phosphonocarboxylates[3] and oligodeoxynucleotide phosphate triesters[4] are taken up by cells, especially when esterified, we initially explored the transport of methyl and butyl thiophosphonoacetate ODNs.[5] These derivatives, which have acetate esters linked to synthetic DNA through one of the non bridging phosphorus-oxygen bonds (Figure 1), are found to be reversibly taken up by cells through a passive diffusion mechanism. This early work led us to develop new phosphonoacetate ODNs that potentially would be taken up by cells, preferably in the absence of cationic lipids, through a passive diffusion mechanism. Once inside the cell, these esters would be hydrolyzed by endogenous esterases and thus irreversibly retained either in the cytoplasm or nucleus (Figure 2). In order to explore this possibility, we first synthesized a series of esterified phosphonoacetate ODNs and tested them as substrates in vitro for porcine liver esterase.[6] Of these ODNs, three (POB, POM, and SPTE) are completely hydrolyzed within 12 hours (Figure 3). These three ODNs have been used for further transfection studies.

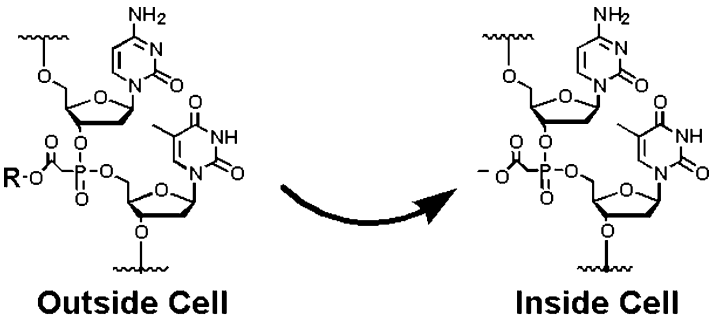

FIGURE 2 Potential pathway for the irreversible uptake of phosphonoacetate DNA by cells in culture.

Initially mixed sequence 21 mers are synthesized that have internucleotide linkages with 3 or 5 anionic phosphonoacetates and the remainder POM, POB, and SPTE esterified phosphonoacetates (Figure 4). A 5′-fluoroscein label is also attached in order to monitor results. These ODNs and a phosphorothioate of the same sequence (Figure 4, S1) are then added to cells at a concentration of 10 μM in Opti-Mem media. After 24 h, the cells are rinsed and uptake assessed with confocal microscopy and Fluorescence Assisted Cell Sorting (FACS) as a quantitative method. Figure 5 shows representative data with Jurkat Cells as acquired from FACS analysis. The

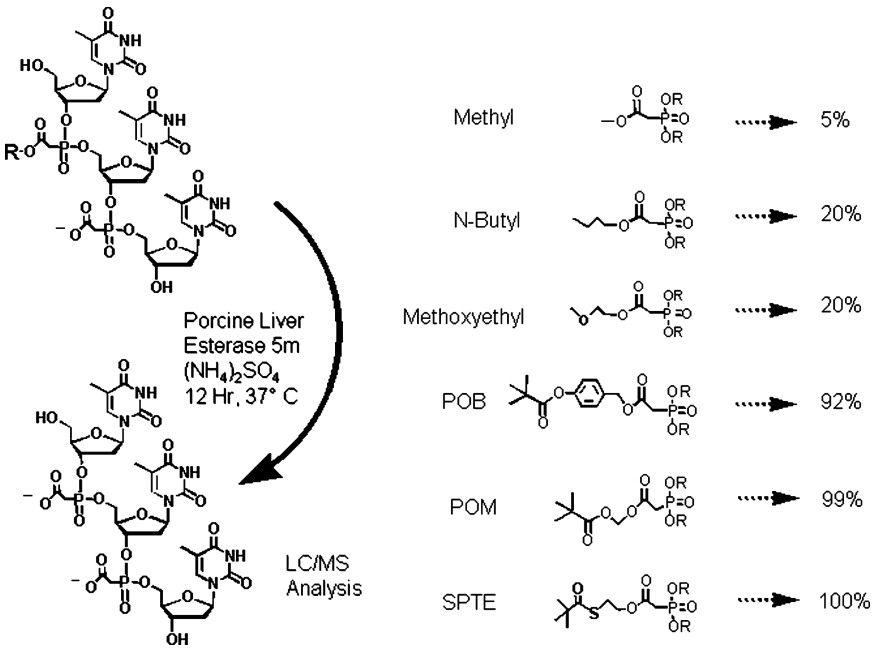

FIGURE 3 Assay describing the lability of various ODN phosphonoacetate esters toward porcine liver esterase in vitro.

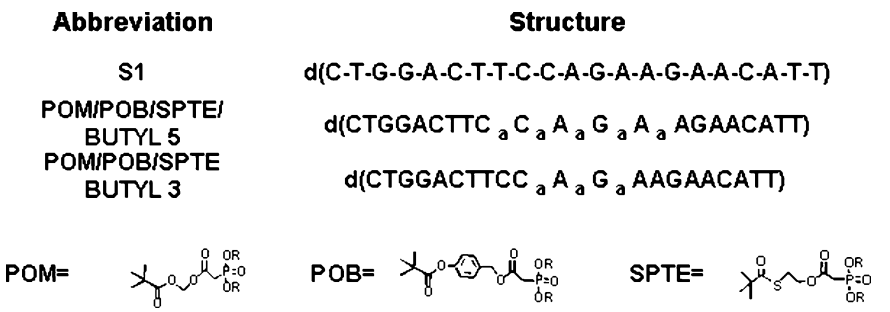

FIGURE 4 Structures of ODN analogs used for transfection experiments: (−), a phosphorothioate internucleotide linkage; a), a phosphonoacetate internucleotide linkage; no abbreviation between the bases means the internucleotide linkage is phosphonoacetate esterified with POM, POB, or SPTE.

left panel shows results with untreated cells having a small amount of background fluorescence. The middle panel displays results as observed with the phosphorothioate control in the presence of cationic lipids. Only perhaps 20% of the cells are transfected—a result which is expected with this cell line. In contrast and without cationic lipids, there is essentially no uptake of S1 by Jurkat cells in culture (data not shown). The right panel shows that all Jurkat Cells, in the absence of cationic lipid, are transfected by an ODN (21mer) having 5 anionic phosphonoacetate and the remainder being neutral SPTE internucleotide linkages (SPTE 5). Similar results in the absence of cationic lipids (data not shown) are obtained with the other ODNs

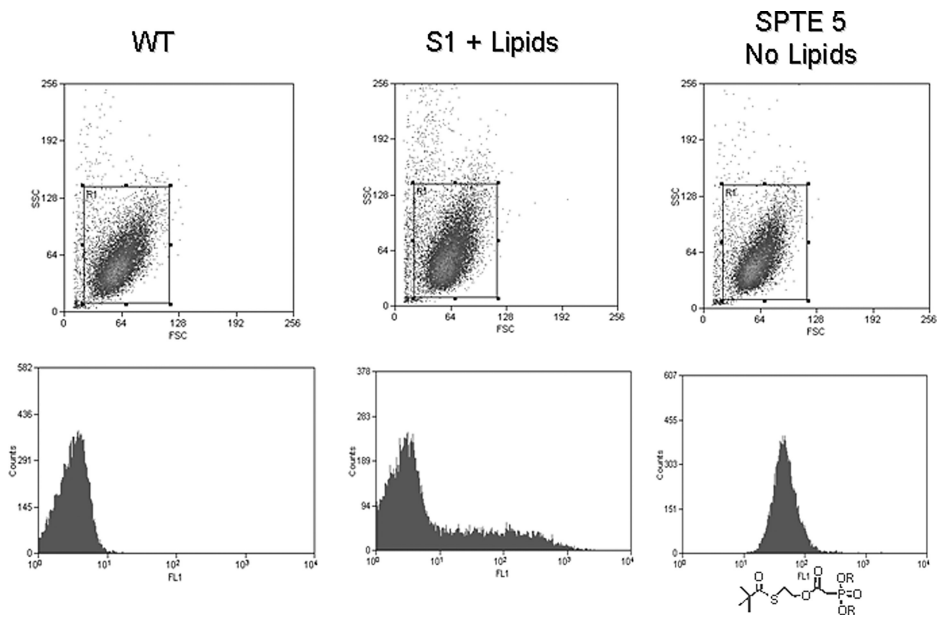

FIGURE 5 FACS analysis of Jurkat cells transfected with SPTE 5 ODN.

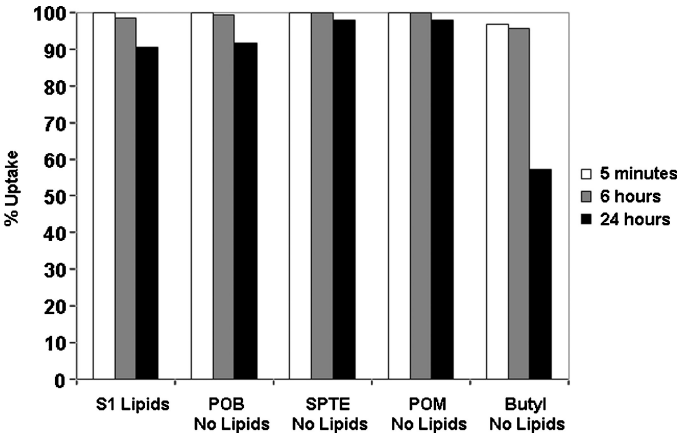

FIGURE 6 Irreversible uptake of esterified ODNs in HeLa cells.

(POM, POB, BUTYL 5 and POM, POB, SPTE, BUTYL 3). Moreover these eight ODNs also are taken up in the absence of cationic lipids by several other cell lines including HeLa, MDCK, K562, and WM35 (data not shown). When examined by confocal microscopy, these ODNs are predominantly localized in the cytoplasm (data not shown). Previous studies have shown that the butyl and methyl esterified phosphonoacetate ODNs are freely diffusable between the cells and media.[5] In contrast, when these same ODNs are esterified with POB, SPTE, and POM, they are irreversibly taken up by cells (Figure 6). Cells are incubated with fluorescein labeled ODN, the media changed, and after 24 hours; the remaining fluorescence measured (FACS). As can be seen from these data, fluorescence remains in all esterified ODNs tested with the exception of the butyl ester—a result with the butyl ester that is consistent with previous results.[5] This means that the POM, POB, and SPTE are hydrolyzed within the cell which generates the phosphonoacetate ODN. These anionic oligomers are then irreversibly retained within the cell. Presumably a biochemically active ODN would have enhanced activity due to this irreversibility.

Transfections of Esterified Phosphonoacetate ODNs/RNA Duplexes in the Absence of Cationic Lipids

We then wanted to assess if a complementary, unmodified RNA strand hybridized to the esterified ODNs also could be transfected into cells without cationic lipids. This experiment was carried out using a 5′-fluorescein labeled RNA (no internucleotide modifications) that is complementary to the six esterified phosphonoacetate ODNs as shown in Figure 4. Duplexes are formed between this RNA and each of the six ODNs, the duplexes added at 10 μM in Opti-MEM media to HeLa cells in culture, and uptake analyzed by confocal microscopy and FACS. Results by FACS analysis are shown in

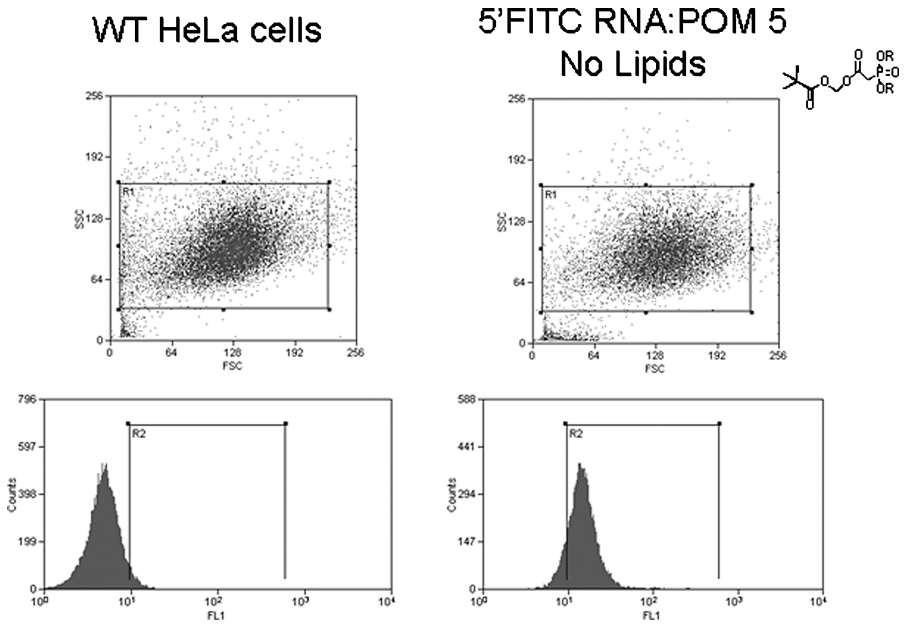

FIGURE 7 FACS analysis of HeLa cells transfected with a duplex composed of POM ODN and a complementary 5′-fluorescein labeled RNA.

Figure 7. The left panel displays only the low level of background fluorescence as observed from HeLa cells untreated with ODN. When these duplexes are added in the absence of cationic lipid (results shown in right panel), approximately 85% of the cells show an uptake of the complementary RNA strand. When a fluorescein labeled, non-complementary RNA is added with each of these oligomers, there is no RNA uptake. Thus, a duplex must form in order for transfection to occur. When uptake is examined by confocal microscopy (data not shown), uptake of fluorescence labeled RNA is similar to that observed in the presence of cationic lipid with a complementary phosphorothioate ODN.

These results indicate that esterified phosphonoacetate DNA will transfect cells in the absence of cationic lipid and that the same ODNs also can be used to transport unmodified RNA into cells. The results are encouraging relative to the use of phosphonoacetate ODNs for studying biological activity by antisense and siRNA pathways.

EXPERIMENTAL

Synthesis of Esterified Phosphonoacetate, Phosphonoacetate, and Phosphodiester DNAs

Phosphonoacetate and phosphorothioate ODNs are synthesized using standard procedures.[2] Chimeric ODNs containing phosphonoacetate

and esterified phosphonoacetate internucleotide linkages are synthesized on a 1.0 μmole Q-support (Glen Research, Sterling, VA, USA).[7] Three synthons having appropriate base protection and joined at the 3′-oxygen as phosphoramidites are used to generate stable, esterified phosphonoacetate internucleotide linkages. They are prepared using published methods.[2] These are 3′-O-acetic acid, [bis(N,N- diisopropylamino)phosphino]pivaloyloxymethylene ester; acetic acid, [bis(N,N-diisopropylamino)phosphino]pivaloyloxybenzyl ester; and acetic acid, [bis(N,N-diisopropylamino)phosphino]-S-thiopivaloylethylene ester. These three synthons are used with the standard 3′-O-acetic acid, [bis(N,N-diisopropylamino)phosphino]-1,1-dimethyl-2-cyanoethylester to generate chimeric ODNs having various combinations of esterified phosphonoacetate and phosphonoacetate internucleotide linkages (Figure 1). A 5′-fluorescein phosphoramidite (Glen Research) is used to generate a 5′-fluorescein label on ODNs. Following condensation, the PIII 5′-fluorescein linkage is sulfurized in order to generate a phosphorothioate and thus be resistant to nuclease degradation. Postsynthesis the support is treated with 1.5% DBU in anhydrous acetonitrile for 1 hour at room temperature to remove the cyanoethyl or dimethylcyanoethyl group from phosphodiester or phosphonoacetate linkages, respectively. The support is then rinsed with acetonitrile for 60 seconds and flushed with a stream of argon until dry. Supports used to prepare standard phosphodiester linked ODNs are poured into one-dram vials and treated with 40% methylamine (aq.) for 15 minutes at 55°C to remove exocyclic amine protecting groups and cleave the ODN from CPG. Supports derivatized with the Q-linker are poured into one-dram vials, a TEMED-HF solution (20% TEMED, 10% HF (aq.) in acetonitrile) added, the vials sealed with a Teflon cap, and then agitated for 4 hours at room temperature. All supernatants are separated from CPG by filtration and solvents removed in vacuo. These reaction mixtures are then redissolved in water and ODNs isolated by reverse phase HPLC.[2]

Biochemical Studies with ODNs

Cells are propagated in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin (10 units ml^{-1}. Cells are regularly passaged to maintain exponential growth.

FACS is carried out using the following procedure. Twenty-four hours before transfection at 50–80% confluency, 75,000 cells are seeded per well in a 24 well plate in 500 μL of FBS containing media. Following removal of media, transfections are carried out at a concentration of 10 μM FITC labeled ODN in reduced serum free Opti-MEM (Gibco) either in the presence of cationic lipids as described by the manufacturer for adherent cell lines (Lipofectamine 2000, Invitrogen) or without lipid. After a set time,

cells are counted and the fluorescence detected with a 488 nm argon laser using a Dako Cytomation MoFlo cell sorter (Dako, Carpintería, CA, USA).

Confocal microscopy is carried out according to the following procedure. Twenty-four hours before transfection at 50–80% confluency, 75,000 cells are seeded per well in a 24 well plate in 500 μL of FBS containing media. Following removal of media, ODN transfections are carried out at 10 μM 5′-FITC labeled oligomer in serum-free Opti-MEM (Gibco) either in the presence of cationic lipid (Lipofectamine). For duplexes having one oligomer with a 5'-FITC label, the single strands (10 μM) are incubated at 95°C for 1 minute and then allowed to hybridize for 30 minutes at 4°C. The duplex is then introduced to cells either in the presence or absence of cationic lipids (Lipofectamine 2000). The microscope is an automated Leica DMRXA/RF4/V with a Cooke Sensi-Cam digital camera. Images are collected and subjected to no neighbors or nearest neighbor deconvolution algorithms using the Slidebook software package (Intelligent Imaging Innovations, Denver, CO, USA).

Exonuclease digestion experiments are completed as described by Cummins.[8]

REFERENCES

1. Micklefield, J. Backbone modification of nucleic acids: Synthesis, structure and therapeutic applications. *Curr. Med. Chem.* **2001**, 8, 1157–1179.
2. Dellinger, D.J.; Sheehan, D.M.; Christensen, N.K.; Lindberg, J.G.; Caruthers, M.H.; Solid phase chemical synthesis of phosphonoacetate and thiophosphonoacetate oligodeoxynucleotides. *J. Am. Chem. Soc.* **2003**, 125, 940–950.
3. Valazquez, S.; Lobaaton, E.l.; DeClercq, E.; Koontz, O.L.; Mellors, J.W.; Balzarini, J.; Camarasa, M.-J. Hybrids of [TSAO-T]-[Foscarnet]: The first conjugate of foscarnet with a non-nucleoside reverse. transcriptase inhibitor. *J. Med. Chem.* **2004**, 47, 3418–3426.
4. Vives, E.; Dell'Aquila, C.; Bologna, J.D.; Morvan, F.; Rayner, B.; Imbach, J.L. Lipophilic prooligonucleotides are rapidly and efficiently internalized in hela cells. *Nucleic Acids Res.* **1999**, 27, 4071–4076.
5. Sheehan, D.; Lunstad, B.; Yamada, C.M.; Stell, B.G.; Caruthers, M.H.; Dellinger, D.J. Biochemical properties of phosphonoacetate and thiophosphonoacetate Oligodeoxyribonucleotides. *Nucleic Acids Res.* **2003**, 31, 4109–4118.
6. Briggs, A.D.; Camplo, M.; Freeman, S.; Lundstrom, J.; Pring, B.G. Acyloxymethyl and 4-acetoxybenzyl diester prodrugs of phosphonoformate. *Tetrahedron* **1996**, 52, 14937–14950.
7. Pon, R.T.; Yu, S. Hydroquinone-O,O′-diacetic acid ('O-linker') as a replacement for succinyl and oxalyl linker arms in solid phase oligonucleotide synthesis. *Nucleic Acids Res.* **1997**, 25, 3629–3635. ¡pg=10¿
8. Cummins, L.; Graff, D.; Beaton, G.; Marshall, W.S.; Caruthers, M.H. Biochemical and physicochemical properties of phosphorodithioate DNA. *Biochemistry* **1996**, 35, 8734–8741.