P-CHIRAL OLIGONUCLEOTIDES. EFFECT OF CONFIGURATION AT PHOSPHORUS ON TRANSPORT OF TETRA(THYMIDINE METHYLPHOSPHONATE)S ACROSS ORGANIC LIQUID MEMBRANE+

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The stereodependent transport of a P-stereoregular oligonucleotide through a model organic liquid membrane is described. The electroneutral tetra(thymidine methylphosphonate) was used as oligonucleotide. The transportability increased in the order: all-R_p > random distribution of P-diastereomers > all-S_p. These findings extend our knowledge of the physicochemical properties of single-stranded methylphosphonate oligonucleotides in solution, and might facilitate cellular uptake of future antisense oligonucleotide drugs.

Keywords: Antisense oligonucleotides; Methylphosphonate analogs; Backbone modified oligonucleotides; Transport; Stereoselectivity; Liquid membrane; Cell membrane models.

Antisense DNA oligomers have significant potential for genome-targeted therapy[1–5]. A major issue in the development of antisense oligonucleotides as future biopharmaceuticals is at present their selectivity[3] and effective transport across cellular membranes[4]. Several approaches have been used to enhance cellular uptake of oligonucleotides including coupling with lipophilic or membrane-destabilizing chemical moieties, liposomal entrapment and binding to polymeric nanoparticles[4,5]. Using lipophilic prooligonucleotides and non-ionic oligonucleotide analogues is another ap-

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proach to improve oligonucleotide transportation across the cell membrane[1,6].

In order to optimize physicochemical, biological and pharmacological characteristics of antisense oligonucleotides a variety of DNA derivatives containing electroneutral internucleotide linkages[1–5] have been synthesized. Methylphosphonates[1–5,7,8], dephosphooligonucleotides[9] and S-acylthioethyl (SATE) groups bearing oligomers[10] are among the most extensively studied non-ionic or chimeric ionic/non-ionic sugar phosphate backbone modifications[11]. Oligomers of 10–20 nucleotide units in length are usually used as antisense agents. Shorter modified oligonucleotides are often utilized as segments of "chimeric" modified-unmodified sequences or as a tandem complex immediately adjacent each to other on the complementary target sequence in a "tandem antisense oligonucleotide" approach[12].

Replacement of one of the anionic oxygen atoms of internucleotide phosphodiester linkage with a methyl group or another substituent creates a new center of chirality at phosphorus. It has been known for more than two decades that the configuration at phosphorus in P-chiral oligonucleotide analogues can affect their physicochemical and biological properties[13]. We and others demonstrated that R_P methylphosphonate substitution in DNA results in more stable complementary binding as evidenced by an increase in melting temperature (T_m), than S_P diastereomer substitution[14–16].

Recently it was hypothesized that the transport of P-chiral oligomers through lipophilic cellular membranes is possibly also stereodependent[13,17] and, consequently, that the stereochemistry might affect oligonucleotide cellular uptake. In this communication we show, using a model dichloromethane membrane, that the transport of short methylphosphonate oligonucleotides across a lipophilic liquid barrier is indeed stereoselective.

EXPERIMENTAL

Materials

Analytical grade dichloromethane was purchased from POCh (Lublin, Poland), distilled and saturated with water before use. A glass U-tube of 11.5 mm i.d. with a contact surface (inner area) of 1.04 cm^2 was used as transport system. The transport was monitored by measuring the absorbances at 260 nm on a Pharmacia Biotech Ultrospec 2000 UV-VIS spectrometer (Cambridge, England).

Methods

All-R_P and all-S_P tetra(thymidine methylphosphonate) were synthesized as described previously[18]. Random tetra(thymidine methylphosphonate) was synthesized as descibed[19]. Natural tetrathymidylic acid was obtained by solid-phase automatic synthesis using a standard 2-cyanoethyl cycle[20].

Partition coefficient measurement[21,22]. Oligonucleotide (1.0 A_{260} optical density units, ODU) was dissolved in deionized H_2O (0.6 ml) in a 1.5 ml Eppendorf tube, then organic solvent (CH_2Cl_2 or octanol, 0.6 ml) was added. The resulting mixture was shaken vigorously at room temperature (20–22 °C) for 2 h to ensure that the oligonucleotide transfer between the two phases was at equilibrium and then the mixture was left standing for 24 h to separate the phases. Each sample was subsequently centrifuged for a few seconds then 0.1 ml of H_2O or organic solution was diluted with 0.4 ml ethyl alcohol and the UV absorption at λ = 260 nm of the resulting solution was measured. The partition coefficient is defined as the ratio of the amount of oligonucleotide present in the organic phase to that present in the aqueous phase.

Oligonucleotide transport through CH_2Cl_2 liquid membrane[23]. A glass U-tube (11.5 mm i.d.) was charged with CH_2Cl_2 (7.0 ml) followed by H_2O (2.5 ml) in one arm ("acceptor" arm), CH_2Cl_2 and H_2O were equilibrated by vigorous mixing for 15 min and then left overnight to allow water and organic phases to separate. At time zero an oligonucleotide stock solution was added into the other arm containing H_2O ("donor" arm) to a final concentration of 1.0 mmol l^{-1} and final volume of 2.5 ml. The CH_2Cl_2 layer was stirred using a "flea" stirbar (2 × 7 mm) and an electromagnetic stirrer capable of delivering approximately 300 rpm to all experiments. This stirring rate was insufficient to cause mechanical transfer of aqueous solution from one arm to the other. All experiments were performed at room temperature, 20–22 °C. At predetermined intervals, a 0.5 ml sample was withdrawn from the acceptor arm and the concentration of oligonucleotide and UV absorption at 260 nm was measured. The following molar absorption coefficients were used: all-S_P tetra(thymidine methylphosphonate) ε_{265} = 34 000, all-R_P tetra(thymidine methylphosphonate) ε_{265} = 33 200 and random tetra(thymidine methylphosphonate) ε_{265} = 33 600 (an average value for all-S_P and all-R_P oligomers), unmodified tetra(thymidine phosphate) ε_{265} = 34 800 (ref.[18]). The sample was then returned to the acceptor arm and the whole water layer was gently mixed to avoid CH_2Cl_2/H_2O mechanical transfer. Otherwise the water solutions in donor and acceptor arms were undisturbed. The pH of H_2O solutions in both arms was 7 as measured at the beginning and at the end of the experiment. A control experiment with no oligonucleotide added to the donor arm was performed simultaneously.

Molecular modeling. The molecular structure of all-S_P and all-R_P tetra(thymidine methylphosphonate), and unmodified tetra(thymidine phosphate) were constructed and optimized using the AMBER force field with HyperChem MolecularMechanics software (Hypercube, Inc.). The structure of both stereoregular tetra(thymidine methylphosphonate)s and tetra(thymidine phosphate) was energy-minimized in isolation and in water solution to determine the effects of the solvent on the optimal structure. The structure was refined until the energy gradient with respect to atomic coordinates was lower than 0.1 kcal mol^{-1} $Å^{-1}$.

RESULTS AND DISCUSSION

P-Chiral, P-tactic oligonucleotide transport through a CH_2Cl_2 membrane was studied in a standard downhill U-tube system[23], using $R_PR_PR_P$ (all-R_P) and $S_PS_PS_P$ (all-S_P) tetra(thymidine methylphosphonate) as a model P-stereoregular oligonucleotide (Fig. 1). Tetra(thymidine methylphosphonate) with a random distribution of diastereomers and unmodified tetra(thymidine phosphate) was used as references. A control experiment with no oligonucleotide added was also performed.

A typical set of kinetic results is presented in Fig. 2. An induction period was observed in all experiments. The relative rates for transport of all-R_P and all-S_P oligomers were estimated after an induction period of 40 h. It was found that the transport of all-R_P tetra(thymidine methylphosphonate) is *ca* five-fold faster than that of all-S_P tetra(thymidine methylphosphonate) and *ca* three-fold faster than that of random tetranucleotide. The transport of unmodified tetrathymidylic acid was least efficient. The higher transport rate observed for the all-R_P oligomer is consistent with a higher partition coefficient (K) in CH_2Cl_2–H_2O and octanol–H_2O systems found for the all-R_P oligomer, as compared to the all-S_P counterpart (Table I). Interest-

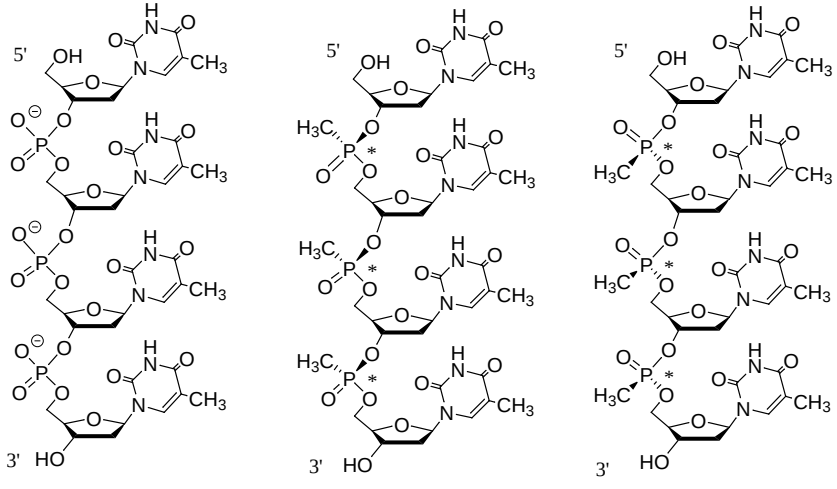

FIG. 1
Chemical structure of stereoregular tetra(thymidine methylphosphonates) and tetra(thymidine phosphate)

ingly, the water solubility of the stereoregular all-R_P oligomer is much higher than that of the all-S_P one[17], and the affinity of the all-R_P oligomer to a lipophilic C_{18} RP-HPLC sorbent is lower than that of the opposite diastereomer as judged from the retention time (R_t) comparison (all-R_P oligomer: R_t = 10.26 min, all-S_P oligomer: R_t = 10.50 min)[18]. The conformational flexibility allowing for a different exposure of hydrophilic

TABLE I

Partition coefficient of all-S_P, all-R_P and random tetra(thymidine methylphosphonate) [$(T_{PMe})_3T$], and unmodified tetra(thymidine phosphate) [$(T_P)_3T$]

Oligonucleotide	$K_{CH_2Cl_2} \cdot 10^{-2}$ [a]	$K_{oct.} \cdot 10^{-2}$ [a]
all-$R_P(T_{PMe})_3T$	5.5 ± 0.4	9.6 ± 0.9
all-$S_P(T_{PMe})_3T$	1.2 ± 0.1	3.9 ± 0.8
random $(T_{PMe})_3T$	2.7 ± 0.1	5.7 ± 0.1
$(T_P)_3T$	1.0 ± 0.1	1.5 ± 0.3

[a] The ratio of the amount of oligonucleotide present in the organic phase to this present in the aqueous phase (data from at least two measurements).

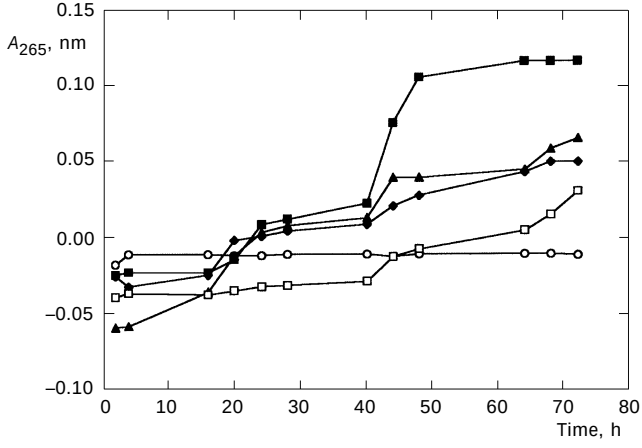

FIG. 2

Time dependence of P-chiral, stereoregular all-S_P and all-R_P tetra(thymidine methylphosphonate) concentrations in the receiving phase. All-R_P tetra(thymidine methylphosphonate) (■), all-S_P tetra(thymidine methylphosphonate) (◆), random tetra(thymidine methylphosphonate) (▲), natural tetrathymidylic acid (❑), control experiment with no oligonucleotide added (○). All runs were measured in duplicate, the calculated standard deviation using all time points measured was not higher than ±5%

and lipophilic segments of tetra(thymidine methylphosphonate)s in water and water–CH_3CN mixture used as HPLC eluting solvent system, could be responsible for the seeming contradiction.

The ability of an oligonucleotide to act as an effective antisense agent depends strongly on its intracellular concentration and, consequently, on its cellular uptake. In spite of the extensive studies on oligonucleotide uptake and pharmacokinetics in cell culture and in animal models[1–5], the relationship between the structure of a single-stranded methylphosphonate oligonucleotide and its physicochemical characteristics has received little attention.

In theory, oligonucleotides can either passively diffuse through the cell membrane or enter cells *via* endocytosis. Several mechanisms have been proposed to account for the cellular uptake of oligonucletides[4,24]. Phosphodiester oligonucleotides were reported to bind to a 80-kDa surface protein and enter cells *via* receptor-mediated endocytosis[25,26]. Mac-1 oligonucleotide-binding protein is another candidate for an oligonucleotide transporting molecule[27]. Methylphosphonates, which have an uncharged backbone, were originally thought to enter the cells through passive diffusion[28]. However, more recent studies with a random mixture of P-diastereomeric methylphosphonate oligonucleotides, indicate that uptake of these compounds occurs by an endocytotic route[29]. It is of interest therefore, that the results described in this communication suggest that a random mixture of methylphosphonate oligonucleotides is not well adapted for passive diffusion, and that only a fraction of the random oligomer pool with proper stereochemical characteristics may have chance to cross the membrane *via* a passive diffusion mechanism.

The rate of passive diffusion across a lipid bilayer depends on two factors. The first is lipophilicity of the compound, which can be quantitatively determined by measuring the partition coefficient (*K*). The other determinant is the ability of the compound to pass through the membrane, expressed as the diffusion coefficient. The parameters contributing to the diffusion coefficient, with the exception of the size of the diffusant, are related only to membrane properties[30]. The experimental evidence for the same or different sizes of stereoregular all-R_P and all-S_P tetra(thymidine methylphosphonate)s is not available at present. If the size of all-R_P and all-S_P oligomers did not differ significantly, the transport of these oligomers should depend primarily upon their lipophilicity. The assumption regarding the same size and shape of these oligomers is based on the same chemical structure and type of modification for both diastereomers. It is supported by the finding that no stacking, which could potentially affect

oligonucleotide conformation and shape, was detected for the single strands of methylphosphonate oligothymidylates with random configuration at the phosphorus[31]. On the other hand, CD measurements of stereoregular methylphosphonate oligonucleotides have shown a strong influence of methylphosphonate internucleotide configuration on CD spectra of all-R_P and all-S_P methylphosphonate oligomers[14,18]. Taking into account that CD spectra are sensitive to the mode of base stacking, the tentative conclusion can be drawn that the conformation, and possibly size and shape of all-R_P and all-S_P methylphosphonate oligomers may be different. Therefore, although unambiguous evidence for the different sizes of stereoregular all-R_P and all-S_P tetra(thymidine methylphosphonate) is not available, such a rationale for the different rates of transport through the CH_2Cl_2 liquid membrane cannot be excluded at this stage of our research. Differing abilities of all-R_P and all-S_P methylphosphonate oligomers to form aggregates in solution is a further possibility that requires consideration.

The effects of oligonucleotide size on diffusion across the CH_2Cl_2 liquid membrane were further supported by the results of modeling experiments. Figure 3 shows the results of molecular modeling of stereoregular all-R_P and all-S_P tetra(thymidine methylphosphonate) and natural tetra(thymidine phosphate). The conformation of stereoregular all-R_P tetra(thymidine methylphosphonate) clearly resembles those of tetra(thymidine phosphate) more than that of all-S_P counterparts.

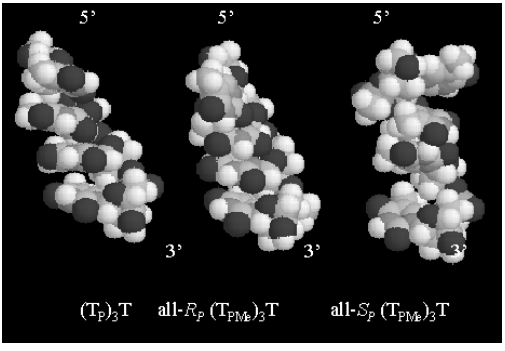

FIG. 3

The energy-minimized structure of stereoregular tetra(thymidine methyl-phosphonates) and tetra(thymidine phosphate) in water solution. The structure was refined until the energy gradient with respect to atomic coordinates was lower than 0.1 kcal mol^{-1} $Å^{-1}$ using the AMBER force field with HyperChem MolecularMechanics software (Hypercube, Inc.)

The results of molecular modeling show that all-R_P tetramers can form a compact hydrophobic core attributed to a larger base-base overlapping.

On the other hand, the all-S_P tetranucleotide forms a bulky hydrophobic core that is assigned to less aligned bases owing to the absolute configuration at the phosphorus atom of the internucleotide methylphosphonate group.

The above results are consistent with the different lipophilicity, as measured by affinity to C_{18} RP-HPLC sorbent, of all-R_P and all-S_P tetra(thymidine methylphosphonate)s (see above), and with their CD characteristics[14,18]. In addition, it was shown recently that the order of elution of diastereomeric oligonucleotides is affected by the degree of hydrophobic interactions between the oligonucleotide and the stationary phase. These interactions, in turn, are affected by the configuration of oligonucleotide chains, resulting in a faster elution (shorter R_t) of more compact molecules, as compared to the counterparts characterized by longer R_t and bulky hydrophobic core[32–34].

The above reasoning is further supported by the order of peak intensities in CD spectra of unmodified and stereoregular methylphosphonate tetranucleotides. The magnitude of molecular ellipticity for these oligomers decreases in the order: unmodified tetra(thymidine phosphate) ≅ all-R_P tetra(thymidine methylphosphonate) >> all-S_P tetra(thymidine methylphosphonate)[14,35]. The profiles of CD spectra are qualitatively similar suggesting the same B-like conformation, however the higher molecular ellipticity value for the all-R_P tetranucleotide compared to the all-S_P oligomer suggests greater base–base stacking in the oligonucleotide with all-R_P configurations of internucleotide methylphosphonate groups, and therefore a more compact conformation than the all-S_P oligonucleotide.

The effect of lipophilicity, which is another factor affecting molecular transport through liquid membranes, is difficult to define for such chemically similar molecules as stereoregular all-R_P and all-S_P methylphosphonate oligomers. Removal of the negative charge of an internucleotide linkage is an effective way of increasing penetration of the oligonucleotide into an organic environment[6,10]. The modification can potentially increase the lipophilicity of the oligonucleotide in two ways: neutralization of the negative charge of the phosphate backbone and addition of a lipophilic moiety. For all-R_P and all-S_P methylphosphonate oligomers, both factors are the same; nevertheless, different degrees of lipophilicity, as judged from the different partition coefficients and rates of diffusion across the CH_2Cl_2 membrane are obtained. We hypothesize that this may be due to different hydration of these oligomers.

Hydration of modified oligonucleotides has received limited attention in contrast to the extensive experimental and molecular dynamics studies on the hydration of unmodified nucleic acids[26–39]. There are, however, several molecular dynamics simulations of methylphosphonate-natural oligonucleotide heteroduplexes, and of the effect of the configuration of methylphosphonate on the stability and hydration of heteroduplexes[40,41]. Six water molecules are associated with two anionic phosphoryl oxygens in native DNA, but only 2.2 water molecules are associated with the single methylphosphonate phosphoryl oxygen[41]. Moreover, on the basis of molecular modeling it is postulated that the hydration of the phosphate backbone in heteroduplex of normal DNA hybridized with R_P- and S_P-substituted DNA is different[40]. It is suggested on the basis of these findings and our observations that hydration of single-stranded, stereoregular methylphosphonate oligomers is possibly also stereodifferentiated. Consequently, hydration may affect transport through a lipophilic CH_2Cl_2 membrane due to modulation of the oligomer penetrating ability by the number of water molecules associated with the oligonucleotide, according to the dehydration-hydration model of passive diffusion across lipophilic membranes.

In summary, we have shown for the first time the stereoselectivity of transport of an electroneutral, P-stereoregular oligonucleotide analogue through an organic liquid membrane. This finding extends our knowledge of the mechanisms of oligonucleotide transport across lipophilic membranes and may help improve cellular uptake of future antisense oligonucleotide drugs.

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