# Stability, Specific Binding Activity, and Plasma Concentration in Mice of an Oligodeoxynucleotide Modified at 5'-Terminal with Poly(ethylene glycol)

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**Oligodeoxynucleotide (ODN) composed of 15 nucleotides was modified at 5'-terminal phosphate with hexylamine linker and chemically activated poly(ethylene glycol). This derivative showed improved characteristics in terms of enzymatic stability, binding activity, and *in vivo* retention in mouse. The data are discussed in comparison with those of corresponding unmodified and phosphorothioate ODNs.**

**Key words** antisense; oligodeoxynucleotide; poly(ethylene glycol); *in vivo*; modification; stability

Synthetic oligodeoxynucleotides (ODNs) have been recognized as antisense agents for the sequence-specific inhibition of cellular and viral gene expression.[1] Since phosphodiester internucleotide linkages of natural ODNs are labile to ubiquitous nucleases,[2-4] chemical modifications on the phosphodiester backbone have been designed and investigated.[5-7] Some of these modifications can provide enzymatic stability, however, problems have arisen in stereochemistry,[8] binding specificity,[9] and distribution.[10]

As part of our program on the design and synthesis of antisense ODNs bearing enzymatic stability, high binding affinity, and appropriate pharmacokinetic characteristics, 5'-terminal modification with poly(ethylene glycol) (PEG) molecules is now being investigated. Covalent linkage from the 5' end of ODN is attractive because it can be carried out in the solid-phase synthesis.[11,12] PEG modification is reported to be effective in prolonging retention of liposomes in plasma.[13] We report herein data on 1) enzymatic stability, 2) binding specificity to the corresponding sense ODN, and 3) plasma concentration in mice after i.v. administration of 5'-modified 15-mer ODN.

## MATERIALS AND METHODS

**Analytical Instrumentation** A high-performance liquid chromatography system, consisting of a pump with a linear-gradient system (LC-10A, Shimadzu, Kyoto), a programmable multiwavelength detector (Model 490, Waters, Milford, MA), and a 20-$\mu$l fixed loop injector (Model 7125, Rheodyne, Cotati, CA), was used. Ultraviolet absorption spectra were taken on a spectrophotometer (UV-265, Shimadzu).

Chromatography was performed on an anion-exchange resin column (10 × 50 mm, LiChrospher 4000 DMAE, Merck, Darmstadt, Germany) in a water jacket (Superformance, Merck). The column was eluted first with 20 mM Tris–HCl buffer (pH 8.0) and then with a 1.0 mM NaCl linear gradient from 10 to 100% in 60 min at 1.0 ml/min.

**Synthesis and Purification of ODNs** A 15-base sequence of the chloramphenicol acetyltransferase (CAT) gene (ATG GAG AAA AAA ATC) was selected as the target sense. ODNs, both phosphodiester (PO) and phosphorothioate (SO), were synthesized on a 1.0 $\mu$mol scale by $\beta$-cyanoethyl phosphoramidite chemistry using an automated synthesizer (Cyclon Plus, Millipore, Burlington, MA). For ODNs containing SO internucleotide linkages, the iodine oxidation step was replaced by oxidation with Beaucage's reagent.[14] The ODNs were obtained as a dimethyltrityl (DMT)-on form by treating them for 90 min with 28% aqueous ammonia, and then were purified by an Oligo-Pack (Millipore) cartridge. Elution was performed with 2% trifluoroacetic acid. The fractions containing desired ODN were combined and evaporated to dryness in microtubes. Samples were stored at −40 °C until use.

For modification at the 5'-terminal, first, hexylamine linker was introduced with *N*-monomethoxytritylamino-hexa-6-oxy-$\beta$-cyanoethyl-*N*,*N*-diisopropyl-aminophosphoramidite during the automated synthesis process. After the purification as described above, the solution of the ODN (1.5 units, A260) in buffer (300 $\mu$l, pH 10.0) was added to 2,4-bis-(*O*-methoxypolyethylene glycol)-6-chloro-*S*-triazine (M.W. *ca.* 10000, 5 mg) and stirred at room temperature for 18 h. The reaction mixture was applied to a column of Sephadex G-100 (Pharmacia, Uppsala, Sweden), and eluted with 20% ethanol. The fractions containing the desired derivative were combined and evaporated. The residue was further purified by the anion exchange column described above, and then desalted by Oligo-Pack EX (Millipore).

The purity of the samples was estimated to be more than 98% from a single peak in HPLC analysis.

**Enzymatic Degradation of ODNs** Degradation rates of ODNs (15 $\mu$M) in the presence of S1 nuclease (10 units/ml) were measured in an acetate buffer (50 mM) containing 300 mM NaCl and 2 mM $ZnSO_4$ at 37 °C. Stability in human plasma (40%) and mouse plasma (40%) was measured at a concentration of 15 $\mu$M ODN in 70 mM phosphate buffer (pH 7.4) containing 70 mM NaCl at 37 °C. The changes in concentration were followed by HPLC analysis of samples taken periodically from the mixture.

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**Measurement of Melting Temperature ($T_m$)** Equimolar amounts (7.5 μM) of modified or unmodified antisense and complementary unmodified sense were hybridized in 70 mM phosphate buffer (pH 7.4) containing 70 mM NaCl. Melting experiments were carried out in a thermostatically controlled quartz cell (10 mm in thickness). The temperature of the cell was increased from 15 to 60 °C at a rate of 1 °C/min. Dissociation (melting) of the hybridized ODNs was monitored by simultaneous measurements of the absorbance at 260 nm and the cell temperature.

**Plasma Concentration in Mice after i.v. Administration of ODNs** Male ddY mice 10 weeks old and weighing 48—49 g, were fasted for 16 h. Five units (A260) of ODN dissolved in 200 μl of 70 mM phosphate buffer were administered *via* jugular vein. Blood samples (150 μl) were collected periodically from the other side of the jugular vein into heparinized tubes. The samples were centrifuged at 9000 × *g* for 2 min. Fifty μl of the resulting plasma was mixed with the same volume of acetonitrile for deproteinization. Concentrations in the samples were determined by HPLC analysis.

## RESULTS AND DISCUSSION

Table I shows enzymatic stability of the ODNs in S1 nuclease, human and mouse plasma. Concentrations of the ODNs were exponentially decreased against time in all enzyme systems. Half-life of the ODNs did not increase even when the substrate concentration was doubled from 15 to 30 μM. Half-life of unmodified ODN (PO) was extended in both S1 and plasma systems by the 5′-modification. Though the enzymatic stability of PEG-PO in S1 nuclease was one sixth of the corresponding SO, the difference was decreased to about one third and one-half in mouse and human plasma, respectively. The relatively higher stability of PEG-PO in plasma than that in S1, a single-strand-specific endonuclease, may be attributable to the dominant activity of exonucleases in the systems, though this modification can expect minimal effect on the degradation by 3′-exonucleases.

Table II shows the $T_m$ values of double-stranded complexes, which represent the binding affinity of the antisense ODNs to natural sense. As reported previously,[15] lower $T_m$ for SO was observed. This decrease in specific binding ability may be attributable to the introduction of a substituent on the phosphorus, *i.e.*, generation of a new center of chirality or stereoisomers. Modification at the 5′-terminal with PEG, however, intensified the binding affinity, increasing the $T_m$ value from 50.7 to 53.1 °C. Since this terminal modification cannot directly affect hydrogen bonds between the nucleotide bases, the increase in the $T_m$ value may be caused by stabilization of the single-stranded antisense molecules or reinforced internucleotide stacking effect.[16]

Plasma concentration–time profiles of the ODNs after intravenous administration in mice are shown in Fig. 2. Unmodified ODN (PO) rapidly disappeared from the plasma with a half-life of less than 0.2 min. Retention in the plasma was prolonged about 5 times by the 5′-modification (PEG-PO) to about 1 min, though its enzymatic stability (half-life) in mouse plasma was only 1.6 times that of corresponding unmodified ODN. Since

Table I. Half-Life[a] of ODNs in the Presence of S1 Nuclease or Human Plasma

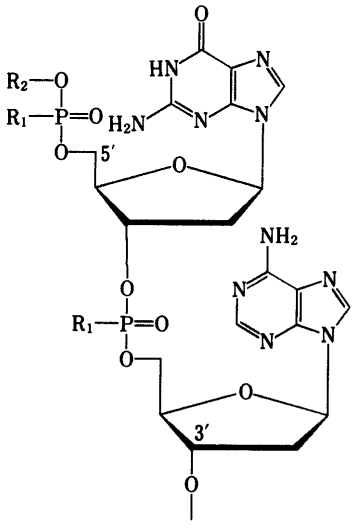
| ODN | S1 nuclease[b] | Human plasma[c] | Mouse plasma[d] |
|---|---|---|---|
| PO | 1.05±0.22 | 1.01±0.12 | 0.64±0.08 |
| SO | 10.06±0.65 | 5.32±0.73 | 3.78±0.33 |
| 5′-PEG (PEG-PO) | 1.67±0.15 | 2.37±0.24 | 1.05±0.18 |

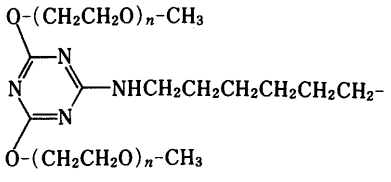
*a*) h, the mean±S.D. of three experiments. *b*) 10 units/ml, at 37 °C. *c*) 40% at 37 °C. *d*) 40% at 37 °C.

Table II. Specific Binding Ability of ODNs; $T_m$ Values of Double-Stranded Complexes of Antisense and Unmodified Sense ODNs

| Antisense | $T_m$[a] (°C) |
|---|---|
| Unmodified PO | 50.7±0.5 |
| SO | 42.1±0.7** |
| 5′-PEG-phosphodiester (PEG-PO) | 53.1±0.9* |

*a*) Mean±S.D. of three experiments. * Statistically significant ($p<0.05$). ** Statistically significant ($p<0.01$).




PEG

| $R_1$ | $R_2$ | |
|---|---|---|
| O | H | phosphodiester (PO) |
| S | H | phosphorothioate (SO) |
| O | PEG | (PEG-PO) |

Fig. 1. Chemical Structures of ODNs

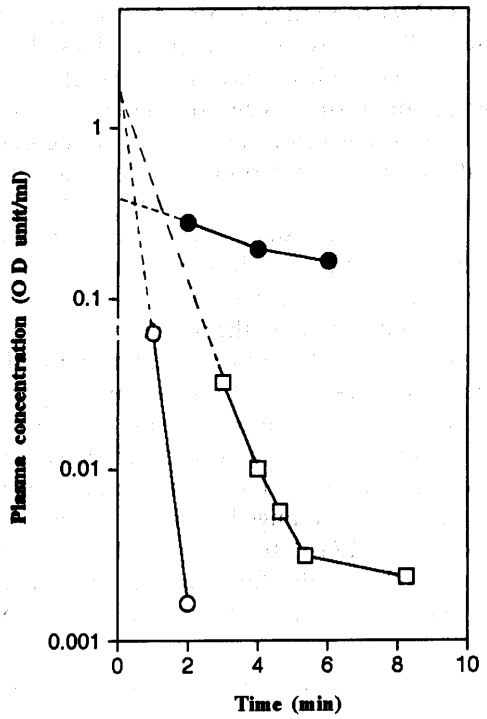



Fig. 2. Plasma Concentration–Time Profiles of ODNs after Intravenous Injection in Mice

Points are means of two experiments, —○—, PO; —●—, SO; —□—, PEG-PO.

distribution volumes ($V_d$) of natural ODN (PO) and PEG-PO in the mice were almost the same: 2.0 and 2.3 ml, respectively, this prolonged retention of PEG-PO in mouse may partly account for its stability against enzymatic degradation in other tissues such as liver and kidney. SO showed the longest half-life of 2.3 min in the ODNs, though its profile was quite different from those of PO and PEG-PO because of its remarkably larger $V_d$ value of 10.5 ml. Since the high protein binding property of SO has been reported,[10] a rapid distribution/binding to serum proteins and slow release from the compartment may explain in the above observation.

The 5′-terminal modification using PEG improves the stability against nucleases, the activity for specific binding, and retention in plasma of antisense ODN. Antisense effects of the above derivatives on inhibition of CAT protein synthesis are now being investigated and will be reported in the near future.

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