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Smart conferring of nuclease resistance to DNA by 3′-end protection using 2′,4′-bridged nucleoside-5′-triphosphates

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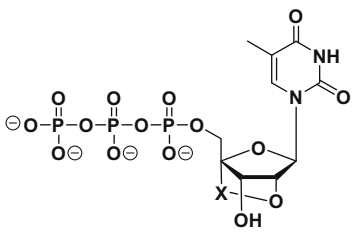
ABSTRACT

Incorporation of 2′,4′-bridged nucleotides into the 3′-end of oligodeoxyribonucleotide (ODN) was examined using terminal deoxynucleotidyl transferase (TdT). The three types of 2′,4′-bridged nucleoside-5′-triphospates with different bridging structures used were incorporated efficiently into the 3′-end of DNA by TdT, although only single nucleotide incorporation was observed. Nuclease resistance was conferred on DNA, depending on the types of bridging nucleotides added.

In the post-genome era, surveys aiming to characterize biological systems have increasingly focused on the spatiotemporal dynamics of bimolecular activity in living cells.[1,2] Functional nucleic acids, such as antisense oligodeoxyribonucleotides (ODNs)[3], decoy ODNs[4], and DNA aptamers[5] are powerful tools for such studies. Nucleic acids are easily degraded by nucleases in both the blood serum and within the cell. Therefore, these nucleic acids are often chemically modified to enhance their biostability.

The 2′-*O*,4′-*C*-methylene bridged/locked nucleic acid (2′,4′-BNA[6,7]/LNA[8]) and its analogs[9–13] are known to possess excellent nuclease resistance and a potential for medical use as a novel class of nucleic acid drugs. We have previously synthesized 2′,4′-bridged nucleoside-5′-triphosphates, that is, **KTP**, **LTP,** and **MTP** from monomer nucleosides of BNA corresponding to 2′,4′-BNA/LNA, 2′,4′-BNA^{COC}, and 2′,4′-BNA^{NC}, respectively (Fig. 1), and examined their substrate properties for thermostable DNA polymerases.[14] Here, we report the substrate properties of these triphosphates for terminal deoxynucleotidyl transferase (TdT) and phosphodiesterase I resistance of ODNs in which a single 2′,4′-bridged nucleotide was enzymatically added to the 3′-end.

First, we investigated the incorporation of the triphosphates (**KTP**, **LTP,** and **MTP**) during the enzyme reaction with TdT (Roche Diagnostics, Basel, Switzerland), using 26-mer single-stranded ODN with 5′-GGC GTT GAG TGA GTG AAT GAG TGA GT-3′ (ODN1) purchased from JBioS (Saitama, Japan). To detect extension products, the 5′-ends of ODN1 were labeled with 6-carboxyfluorescein (6-FAM). The volume of the total reaction mixture was 20 μL, containing 0.4 μM of ODN1, 200 μM of TTP (thymidine-5′-triphosphate), **KTP**, **LTP,** or **MTP**, appropriate concentrations of TdT (0.125 U/μL for TTP, and 0.2 U/μL for the other triphosphates), and the reaction buffer supplied with an enzyme (at 1× concentration). All reaction mixtures were incubated for ~60 min at 37 °C. The reaction products were resolved by denaturing polyacrylamide



KTP : X = 2′–CH_2–4′ *cf.* 2′,4′-BNA/LNA
LTP : X = 2′–CH_2OCH_2–4′ *cf.* 2′,4′-BNA^{COC}
MTP : X = 2′–$NHCH_2$–4′ *cf.* 2′,4′-BNA^{NC}

Figure 1. Chemical structures of the 5′-triphosphate analogues, that is, **KTP**, **LTP**, and **MTP** used in this experiment.

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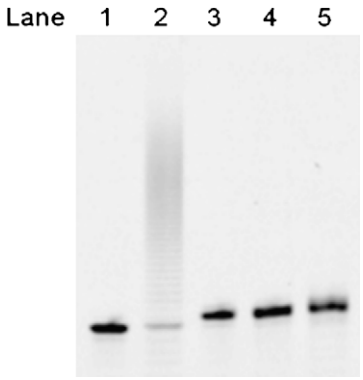

Figure 2. Representative gel images of reactions using the nucleoside triphosphates with TdT. The reaction mixtures for the positive control with TTP (lane 2), those containing **KTP** (lane 3), **LTP** (lane 4), and **MTP** (lane 5). ODN1 migrated only in lane 1.

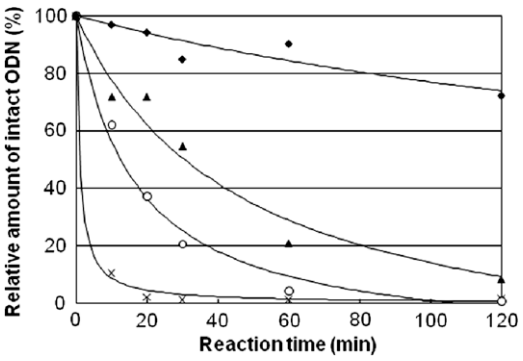

Figure 3. The time course of degradation of ODNs by phosphodiesterase I; reactions using ODN1 (crosses), ODN-K (open circles), ODN-L (closed triangles), and ODN-M (open diamonds). The *x*-axis indicates the reaction time (min), and the *y*-axis represents the relative amount of intact ODNs (%). The total amount of the products was set at 100% in each reaction mixture.

gel electrophoresis (PAGE) and gel images were recorded with excitation of the 5′-labeled fluorophore at 488 nm using Molecular Imager® FX (Bio-Rad, Hercules, CA, USA). The band intensity was quantified using Quantity One® software.

In Figure 2, ladder bands can be seen, which correspond to ~50-mer elongated products, in lane 2 providing a positive control using TTP. If **KTP**, **LTP,** or **MTP** was used, only single nucleotide incorporation was observed as in lanes 3–5. In all three cases, a 60-min incubation period was enough to complete the single nucleotide incorporation. If the enzyme concentration is increased, the use of **KTP** or **MTP** provided the slightly visible band of double nucleotide incorporation in addition to the substantial band of single nucleotide incorporation; however, the use of **LTP** could not produce double nucleotide incorporation within the 60-min incubation period. This is presumably because the bridged ring of **LTP** is larger than that of **KTP** and **MTP**. Double or more incorporation rarely occurred, although single incorporation proceeded smoothly. In addition, only 3–5 nucleotide incorporation was observed when natural ribonucleoside-5′-triphosphates were used (Fig. S1). These results indicate that TdT would be sensitive to the chemical structure adjacent to the 3′-hydroxyl group of the terminus of ODN, and not to that of the nucleoside triphosphate.

Next, we examined nuclease resistance of ODN-K, ODN-L, and ODN-M, which were generated by addition of a single nucleotide to DNA1 using **KTP**, **LTP,** and **MTP**, respectively; ODN1 was used as a positive control. Enzymatically prepared ODN-K, ODN-L, and ODN-M were purified by PAGE and desalted with a C18 reverse-phase cartridge column (Waters Corporation, MA, USA). Reactions were performed in a 5-μL reaction volume, containing 0.4 μM of ODN1, ODN-K, ODN-L, or ODN-M, 50 μU of snake venom phosphodiesterase I (Worthington Biochemical Corporation, NJ, USA) with 3′–5′ exonuclease activity, and reaction buffer supplied with an enzyme (at 1× concentration). All reactions were incubated for ~120 min at 37 °C. The gel images were obtained as described above (Fig. S2), and the decay curves of intact ODNs were fitted from band intensities at appropriate intervals of reaction time.

Compared to natural ODN1, the modified ODNs, that is, ODN-K, ODN-L, and ODN-M showed improved nuclease resistance (Fig. 3). The initial reaction rates estimated from the decay curve were 103%, 7.1%, 2.7%, and 0.32%/min, respectively. It is worth noting that ODN-M shows high resistance against the nuclease; it was about 320-fold more stable than natural ODN1, and 22-fold more stable than ODN-K involving a prototype 2′,4′-BNA/LNA nucleotide, under the reaction conditions. These data seem to partially contradict our previously reported results;[10,12] the order of stability against the nuclease was successively ODN-L, -M, and -K. However, in the ODN used previously, the bridged nucleotide was located at the second residue numbered from the 3′ end. This may cause reversal of the stability between ODN-L and ODN-M. Position of the bridged nucleotide as the obstacle on the side of the 3′–5′ phosphodiester linkage that the 3′–5′ exonuclease firstly encounters would influence the degradation. That is, –L*p*T-$_{OH}$-3′ is less sensitive than –M*p*T-$_{OH}$-3′; however, –T*p*L-$_{OH}$-3′ is more sensitive than –T*p*M-$_{OH}$-3′ against the exonuclease; here, T, L, M, and *p* represent thymidine, 2′,4′-bridged nucleosides of type L and M, and phosphodiester linkage, respectively.

In conclusion, we demonstrated that ODN could gain nuclease resistance via a one-step enzymatic process using **MTP** and TdT, which enhanced resistance by 320-fold that of its level before treatment. Furthermore, this reaction could proceed in a quantitative manner as shown in Figure 2. Unlike the 3′-end of DNA, the 5′-end of DNA is easy to protect because 5′-modified ODN can be used as a primer for the polymerase reaction. This method could provide a method to conveniently confer nuclease resistance onto DNA. We are, therefore, currently investigating the enzymatic addition of BNA nucleotides to long double-stranded DNA fragments in order to apply this to gene-delivery research.[15–18]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.064.

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