



Supporting Information

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Intracellular Ability of the Inducible Alkylation System to Exhibit Antisense Effects with Greater Potency and Selectivity

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Synthesis of PEG-conjugates of the oligonucleotides.

General procedure. 3'-Amino DNA (**5**) in phosphate buffer (Na-phosphate 20 mM, NaCl 150 mM, EDTA 1 mM, pH7.5) was treated with 20 equivalents of SPDP for 1 hr at room temperature. Then 25 equivalents of DTT was added and incubated at room temperature for additional 1 hr. The desired thiol-containing ODNs (**7a-d**) were isolated in about 70% yields by HPLC purification. To the solution of **7** in a phosphate buffer (100 mM Na- phosphate, 300 μ M EDTA, pH 8.0) was added 30 equivalents of an aqueous solution of acetal-PEG-acrylate, and the reaction mixture was incubated for 3 h. Examples of the HPLC profiles are shown in Figure 5, and the MALDI-TOF MS data of the isolated ODN are summarized in Table 1.

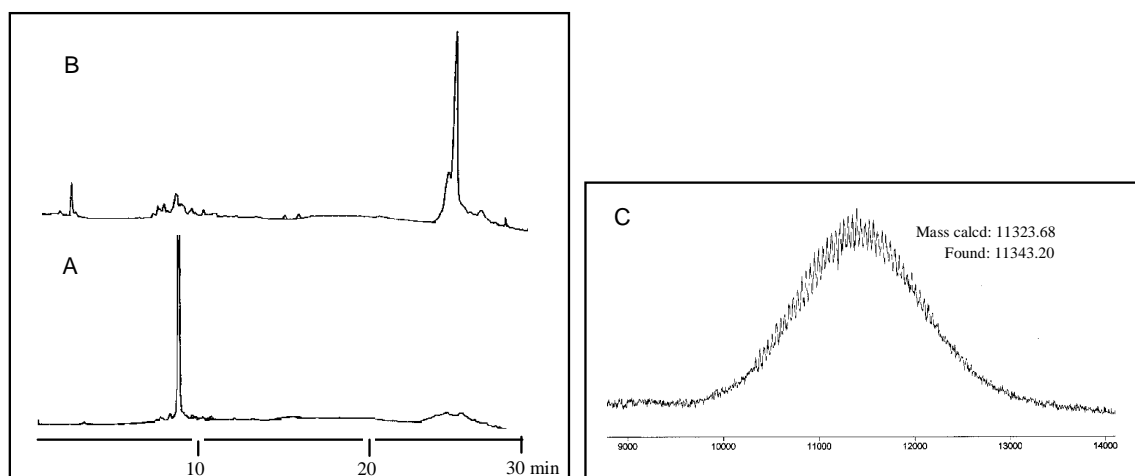


Figure 8. Examples of the HPLC profiles and MS Spectra of the conjugate of **7c** with acetal-PEG-acrylate. **A:** Only **7A(c)** was observed at t_R = 9 min in the reaction mixture at 0 h. **B:** Reaction mixture at 3 h indicated the formation of adducts at t_R = 25 min. **C:** MALDI-TOF MS of the isolated PEG-ODN conjugate **8A(c)** indicates desired MS spectra. HPLC conditions: Solvents, A= 0.1 M TEAA, B= CH₃CN, % of B: 10 to 30%/20min, 30 to 100%/30min. Flow rate 1.0 mL/min, UV 254nm, Column: Nacalai Tesque Cosmosil Packed 5C18-AR-II.

Table 1. MS data of **7A** and **8A**.

ODN ($R^2=$)	calcd	found	ODN ($R^2=$)	calcd (Mn)	found (Mn)
7A(a)	6756.67	6756.99	8A(a)	11216.67	11239.90
7A(b)	6822.33	6824.95	8A(b)	11280.33	11282.90
7A(c)	6863.68	6864.36	8A(c)	11323.68	11343.20
7A(d)	6863.76	6865.48	8A(d)	11323.76	11365.70
7A(e)	6805.33	6810.78	8A(e)	11265.33	11261.00
7B(b)	6843.34	6847.04	8B(b)	11303.34	11295.30
7C(b)	6822.33	6826.36	8C(b)	11282.33	11282.20

A mixture of **5A** and biotin 4-nitrophenyl ester (20 equivalents to **5A**) in saturated NaHCO_3 was kept at room temperature for 12 hours, and the corresponding conjugate was purified by HPLC.

Biotin-DNA (SPh) calcd 6958.44, found 6958.73

Biotin-DNA (G) calcd 6838.08, found 6834.16

Analysis of the reaction mixture of the ODN **6A** in a cell lysate.

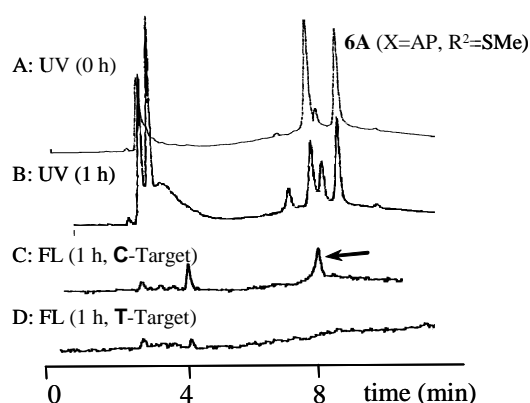


Figure 9. HPLC Profiles of the Reaction in a Cell Lysate Showing Regeneration of the Vinylpurine Derivative. A: 0 hr, B: 1 h, C: 1h in the presence of the C-target, D: 1 h in the presence of the T-target. A and B were monitored by UV at 254 nm and C and D were monitored by fluorescence with emission at 427 nm and excitation at 349 nm. The peak indicated by an arrow in C corresponds to that of the ODN with 2-amino-6-vinylpurine derivative. The target ODN is complementary to the sequence **A**.

C-Target:3' TACGGGTATGA-C-AACTCGTTA

T-Target:3' TACGGGTATGA-T-AACTCGTTA

Inhibition of Translation of Firefly Luciferase mRNA by the ODN-PEG Conjugate.

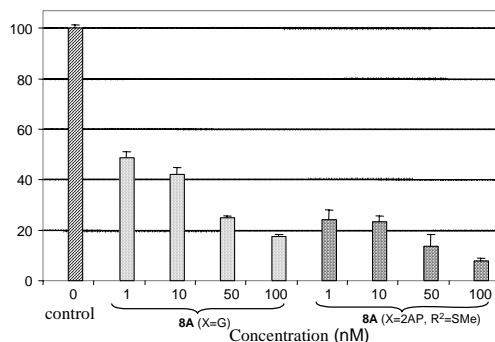


Figure 10. Inhibition of the ODN-PEG Conjugate in Translation of Firefly Luciferase mRNA. The ODN-PEG conjugate was incubated with mRNA of firefly luciferase for 30 minutes in a buffer, then transcription was performed by the addition of amino acids mixture in a cell lysate for 90 minutes at 30 °C.

Non-cell luciferase transcription and translation assay (Figure 3 and 5). The non-cell transcription/translation was performed for 1 h with TNT Luciferase SP6 Control DNA coupled Wheat Germ Extract systems and FluoroTect™ GreenLys in vitro translation labeling system (Promega) using 0.3 µg DNA in the absence and the presence of the antisense ODNs (6). The fluorescent translation products were subjected to gel-electrophoresis with SDS-PAGE, then visualized and quantified by Typhoon TM 8600. The ratios of the produced luciferase in the presence of the antisense ODN (6) to that of the control in the absence of the antisense are shown in Figure 3. The non-cell translation was started first by keeping the mixture of the luciferase control mRNA (25ng, Promega)) and the ODNs indicated concentration in Figure 6 in a buffer at room temperature for 2.0 h. In the meantime, a master mixture of the remaining components except amino acids was made in a separate vessel on ice bath. An equal aliquot of this master mixture was transferred to each mRNA and the ODN mixture. Finally, the amino acids mixture was added to the reaction mixture and incubated at 30 °C for 90min. The translation was stopped by keeping the reaction vessels on ice. 3uL of this reaction mixture was mixed with a solution of luciferin and luciferase activity was assayed with the luminometer.

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361 gtgttgAgcg cggtatttat cggagttgca gttgcgccccg cgaacgacat ttataatgaa
421 cgtgaCattgc tcaacagtat gggcatttcg cagcctaccg tgggtttcgt ttcaaaaaag
481 gggttgcaaaa aaattttgaa cgtgcaaaaa aagctcccaa tcatcaaaaa aattattatc

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541 atggattcta aaacggatta ccagggattt cagtcgatgt acacgttcgt cacatctcat

A: the site for upstream primer, B: the site for down stream primer, C: the target site for the antisense.

Cell culture. HuH-7 human cancer cells, derived from a hepatocarcinoma cell line, were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Dual luciferase reporter assay (Figure 8 and 9). The PEG-ODN conjugates were mixed with PLL (poly-L-Lysine, DP=100, Mw=20,900) with an equal unit molar ratio of the phosphate group in the PEG-ODN conjugate and the amino group in PLL (N/P=1) to form the PIC micelles. HuH-7 cells were plated in a 24-well plate (5 x 10⁴ cells/well) to reach about 50% confluence at transfection. The cells were grown for 24 h and the culture medium was changed to OPIMEM I. The cells were co-transfected with two luciferase plasmids (firefly luciferase, pGL3-control and renilla luciferase, pRL-TK) in the presence of LipofectAMINE (Invitrogen). For each well, 0.0835 µg of pGL3 and 0.75 µg of pRL were applied; the final volume was 250 µL/well. The cells were incubated for 4 h, and then the transfection medium was changed to DMEM with 10% FBS (225 µL/well). The PIC micelles (N/P=1) (25 µL/well) were added to make a prescribed concentration. After 24h incubation, the transfection medium was changed to fresh DMEM with 10% FBS, and further, the cells were incubated for 24 h. The luciferase expression was monitored with the dual luciferase assay kit (Promega) and ARVOSX-1 (PerkinElmer). Normalized ratios (%) between the firefly luciferase and the renilla luciferase activity are shown in Figure 4.

Affinity purification of the RNA/ODN cross-linked product following the streptavidin-biotin technique, then RT-PCR amplification (Figure 7).

The mixture of the components (wheat germ extract, TNT buffer, RNase OUT, RNA and 5'-Biotin-ODN) was incubated at 30 °C for 90min, followed by the addition of HEPES buffer (100mM, HEPES, 1.5 M NaCl, pH 7.5). Pre-washed streptavidin-agarose beads were added to this mixture, and the mixture was kept at room temperature for 1hr. The supernatant lysate was removed with a pipette carefully. The beads were washed with buffer (10mM, HEPES, 150 mM NaCl, pH 7.5) in the absence or in the presence of 10 equivalents of the sense ODN with heating at 65 °C for 5 min, washed again with water (RNase free) twice with heating at 65 °C for 5 min. The beads obtained above were mixed with the RT-PCR components (RT-PCR buffer, dNTP mixture, a reverse primer (5'-fluorescein-ACGAACGTGTACATCGACTG

(590-570)), a forward primer (5' GCGCGTTATTTATCGGAGTT (367-387)), MgSO₄, AMV reverse transcriptase and Tfl Polymerase) in an ice bath, and subjected to the RT-PCR reactions. RT was done at 45 °C for 45 min, and terminated by heating at 94 °C for 2 min, then PCR amplification was done with 40 cycles of the amplification procedure including denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 68 °C for 2 min, and final extension at 68 °C for 7 min. The reaction vessel was cooled at 4 °C, diluted with a loading buffered, denatured at 90 °C for 5 min, and analyzed by 8% polyacrylamide denature gel.

Cell Uptake of FITC-Labeled DNA-PEG-PIC Micelles in NIH3T3 Cells. The PIC-micelles of the FITC-labeled ODN-PEG were prepared using 2 μM DNA and poly-L-lysine (PLL, Mw 75 900, DP=460) with N/P ratio = 1. NIH3T3 cells were incubated in the presence of the above PIC micelles for 3 hrs at 37 °C in a new serum-free DMEM medium. The medium was then removed and cells were washed 3 times with new serum-free DMEM medium followed by the addition of 2 mL serum-free DMEM medium. Cells were incubated for 10 min at room temperature, and cells were washed three times with new serum-free DMEM medium followed by the addition of 2 mL serum-free DMEM medium. Finally cells were observed using confocal laser microscope. Although quantitative discussion with microscope image is difficult, FITC-labeled DNA-PEG PIC micelles penetrate into cells effectively.



Figure 11. Cell Uptake of FITC-Labeled DNA-PEG-PIC Micelles in NIH3T3 Cells. A: confocal fluorescence microscopy at 1 h, B: conventional microscopy at 1 h. The fluorescence microscopy image indicates rapid intracellular distribution of the ODN.

Cytotoxicity of PIC Micelles. Cytotoxicity was determined by measuring the total protein concentration in the cell lysates using the micro BCA assay kit (Pierce) and normalizing protein levels from untreated cells.

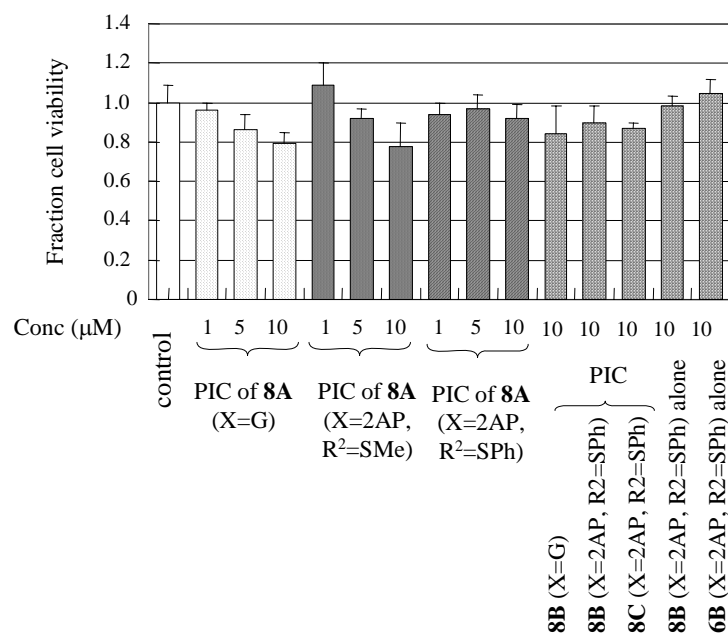


Figure 12. Cytotoxicity of PIC Micelles. Cytotoxicity was determined by measuring the total protein concentration in the cell lysates using the micro BCA assay kit (Pierce) and normalizing protein levels from untreated cells.