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Evaluation of PI polyamide conjugates with eight-base pair recognition and improvement of the aqueous solubility by PEGylation

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ABSTRACT

To investigate the effect of elongating base-pair (bp) recognition sequences, we synthesized *N*-methylpyrrole-*N*-methylimidazole (PI) polyamide conjugates with eight-bp recognition (**3–5**). The DNA alkylating activities of conjugates **3–5** were evaluated by high-resolution denaturing polyacrylamide gel electrophoresis with a 208-bp DNA fragment. Conjugates **3–5** showed high alkylating activities at nanomolar concentrations.

We then addressed the following issue about PI conjugates. Generally, PI polyamide conjugates hardly dissolve in aqueous solution. To improve the aqueous solubility, by the introduction of hydrophilic groups, we synthesized PI polyamide conjugates that were modified with a *seco*-CBI moiety (**6–11**). Conjugates **9–11** that were modified by methoxypolyethylene glycol (PEG) 750 acquired moderate solubility and stability in aqueous solution. In addition, conjugates **10** and **11** had high cytotoxicity against A549 and DU145.

1. Introduction

DNA alkylating agents, which include well-known anticancer agents, such as chlorambucil and cyclophosphamide, are frequently used in chemotherapy.[1,2] However, these agents are toxic, not only for cancer cells but also for normal cells, because of the lack of ability to distinguish between them. The introduction of sequence specificity to the DNA alkylating agents may be a promising method to improve the efficiency as an anticancer agent.

Minor groove-binding polyamides that are composed of *N*-methylpyrrole (P) and *N*-methylimidazole (I) recognize each of the four Watson–Crick base pairs uniquely. Antiparallel pairing of Im opposite Py (I/P) recognizes a G–C base pair, whereas antiparallel pairing of Py/Py (P/P) recognizes A–T or T–A base pairs.[3] PI polyamides have strong binding affinity and sequence specificity for targeting DNA sequences.[4] Using PI polyamides, we developed various sequence-specific alkylating agents by coupling PI polyamides with alkylating moieties.[5–11] As a representative moiety, *seco*-CBI[12,13] was used as a DNA alkylating agent and indole as a linker.[14] It has been reported that PI hairpin polyamide-*seco*-CBI conjugates with six-bp recognition showed high sequence specificity. These conjugates, such as conjugate **1**, efficiently alkylate at the adenine (A) of the matching sequences, 5′-WGGCCA-3′ (W = A or T), in DNA fragments (Fig. 1). Moreover, the introduction of the β-alanine in polyamides allows the crescent-shaped polyamides to fit into the minor groove of DNA, and antiparallel pairings of β/β, β/P recognize T–A or A–T base pairs.[4,15] By introducing β-alanine, we synthesized PI hairpin conjugates with seven-bp recognition,[9] like conjugate **2**. However, further investigations into PI hairpin conjugates with eight-bp recognition have not been conducted yet. Herein, we describe the results of an evaluation of PI hairpin polyamide conjugates with eight-bp recognition (**3–5**) by polyacrylamide gel electrophoresis (PAGE) analysis and cytotoxicity assay.

We also addressed the following issue. Although PI polyamide conjugates have very interesting features, they do present a significant problem—they have low aqueous solubility. This has been one of the barriers to application in animal tests, especially dosage by intravenous administration. To overcome this issue, Saito et al. addressed it by modifying duocarmycins[18,19] and Boger et al. also modified the *seco*-CBI unit and improved aqueous solubility.[20] Taking a new approach, we assumed that modifying the hydroxyl group of the *seco*-CBI unit by hydrophilic groups might improve the aqueous solubility. To confirm this hypothesis, we tried to synthesize PI conjugates with hydrophilic modification (**6–11**) and investigated whether these conjugates would dissolve in aqueous solution. Cytotoxicity potencies for these conjugates were also investigated to check the effect of these modification.

2. Synthesis and evaluation of conjugates 3–5 with eight-base pair recognition

2.1. Molecular design and synthesis of conjugates 3–5

To evaluate the effect of eight-bp recognition, we designed three types of PI conjugates **3–5**, as shown in Figure 2. PI

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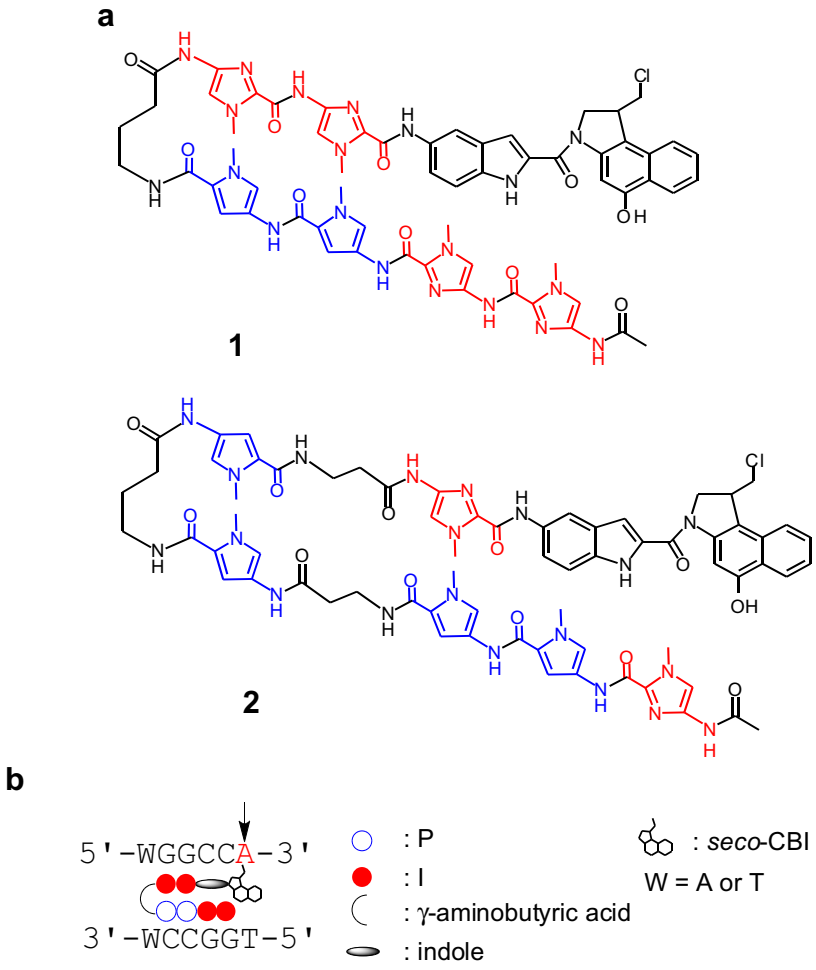

Figure 1. (a) Chemical structures of PI *seco*-CBI conjugates **1** and **2**. (b) Schematic representation of sequence-specific alkylation in the specific DNA sequence by conjugate **1**. An arrow indicates the sequence-specific site of adenine N3 alkylation.

polyamide moieties of **3–5** on oxime resin or CLEAR-Acid Resin were synthesized by using Fmoc solid-phase synthesis and then cleaved with acid or alkali.[16,17] Subsequently, indole *seco*-CBI was coupled with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and *N*,*N*-diisopropylethylamine (DIEA).[7,12] The synthesized PI polyamide conjugates **3–5** were purified by reverse HPLC and confirmed by ESI–TOFMS.

2.2. DNA alkylating evaluation of conjugates 3–5

We investigated the DNA alkylation ability of conjugates **3–5** on a 5′-Texas Red-labeled 208-bp DNA fragment, including each of one targeting site. DNA alkylating activities of conjugates **3–5** were evaluated using PAGE analysis. DNA alkylation was carried out at 20 °C for 18 h, followed by quenching with calf thymus DNA. The samples were heated at 95 °C for 5 min. Under these heating conditions, all purine N3 alkylated sites in DNA produced cleavage bands on the gel. Figure 3 shows the DNA alkylation by conjugates 3–**5**. It showed that conjugates **3–5** efficiently alkylated at the A of target sequences 5′-ATTCACTA-3′ (site 1) and 5′-ATTTGTCA-3′ (site 2) and 5′-TCCATCCA-3′ (site 3), respectively, at nanomolar concentrations. Faint band near site a was found to be A alkylation at the match sequence of 5′-AATACTCAA-3 by **3**. However, conjugates **4** and **5** alkylated at the A of mismatch sequences 5′-AATACTCA-3′ (site a, by **4**, 54%), 5′-TGCATCCA-3′ (site b, by **5**, 47%), 5′-*GT*CATCCA-3′ (site c, by **5**, 11%). Since conjugate **3** showed the best sequence selectivity among **3–5**, it was used for following hydrophilic modification.

3. Solubility and chemical stability of conjugates 6–11 in aqueous solution

3.1. Molecular design and synthesis of conjugates 6–11

To check the aqueous solubility and chemical stability, we synthesized conjugates **6–9** from conjugate **3**. To examine the effect of the bp recognition length, conjugates **10** and **11** were also synthesized from conjugates **1** and **2**, respectively. The chemical structures of conjugates **6–11** are shown in Figure 4 (synthetic procedures are shown in the experimental section). Conjugate **3** was modified by lysine, *N*,*N*-dimethyl-1,3-propanediamine (Dp) and 2-methoxyethylamine (PEG, $n = 0$) for **6–8**, and methoxypolyethylene glycol amine (PEG) 750 for **9–11**, respectively. Corresponding hydrophilic groups were coupled with *N*,*N*-dicyclohyxylcarbodiimide (DCC) and *N*,*N*-dimethyl-4-aminopyridine (DMAP) for **6**, triethylamine and 4-nitrophenyl chloroformate for **7–11**. Conjugates **6–11** were also purified by reverse HPLC and confirmed by ESI–TOFMS.

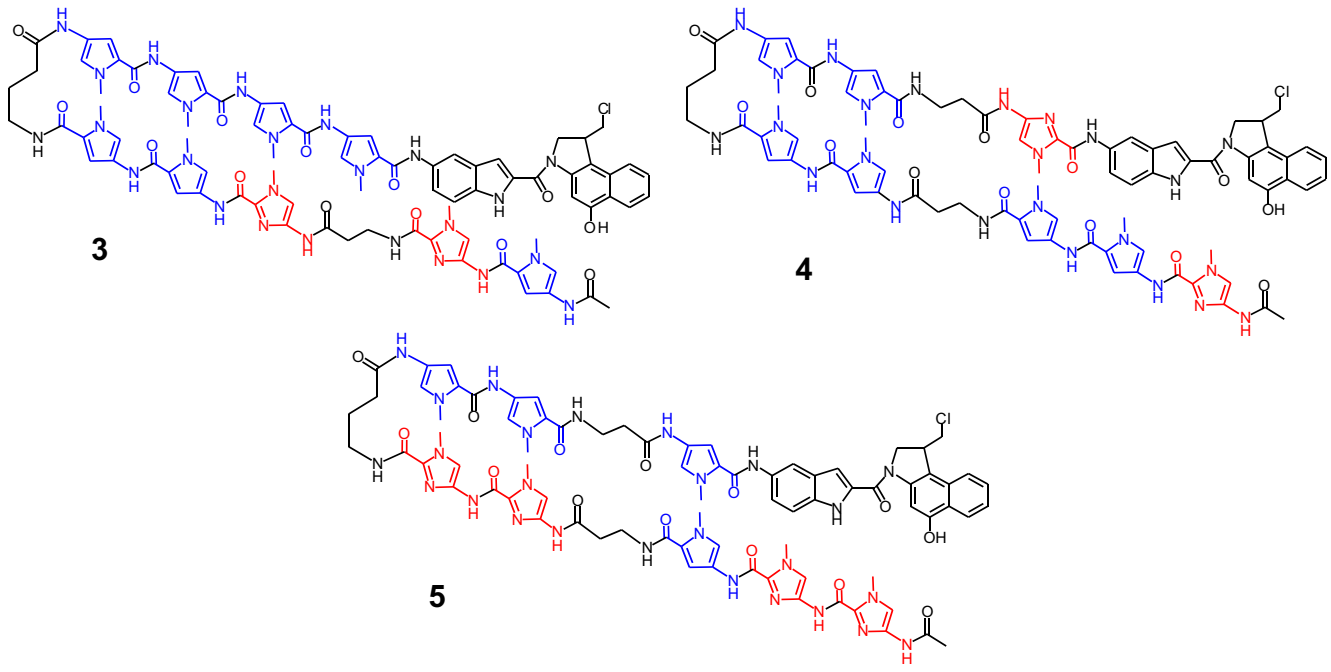

Figure 2. Chemical structures of PI polyamide conjugates **3**–**5** with eight-bp recognition.

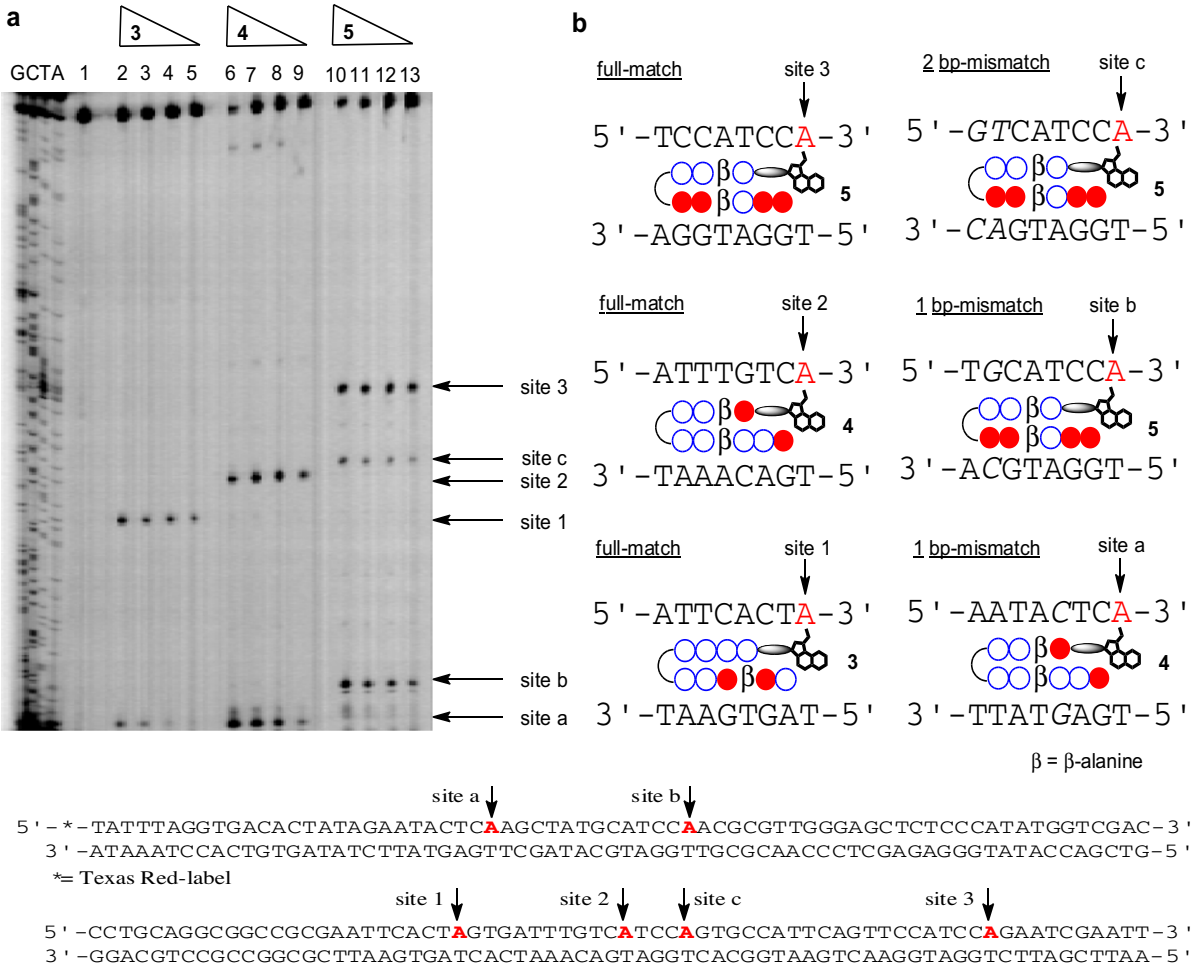

Figure 3. (a) Thermally induced strand cleavages of the 5′-Texas Red-labeled 208-bp DNA fragment (6 nM) by conjugates **3**–**5** incubated for 18 h at 20 °C: lane 1, DNA control; lanes 2–5: 200, 100, 50, and 25 nM of **3**; lanes 6–9: 200, 100, 50, and 25 nM of **4**; lanes 10–13: 200, 100, 50, and 25 nM of **5**. (b) A schematic representation of sequence-specific alkylation by conjugates **3**–**5**. Arrows indicate the sites of adenine N3 alkylation. The alkylating base is shown in red, and the mismatch binding is shown in italics.

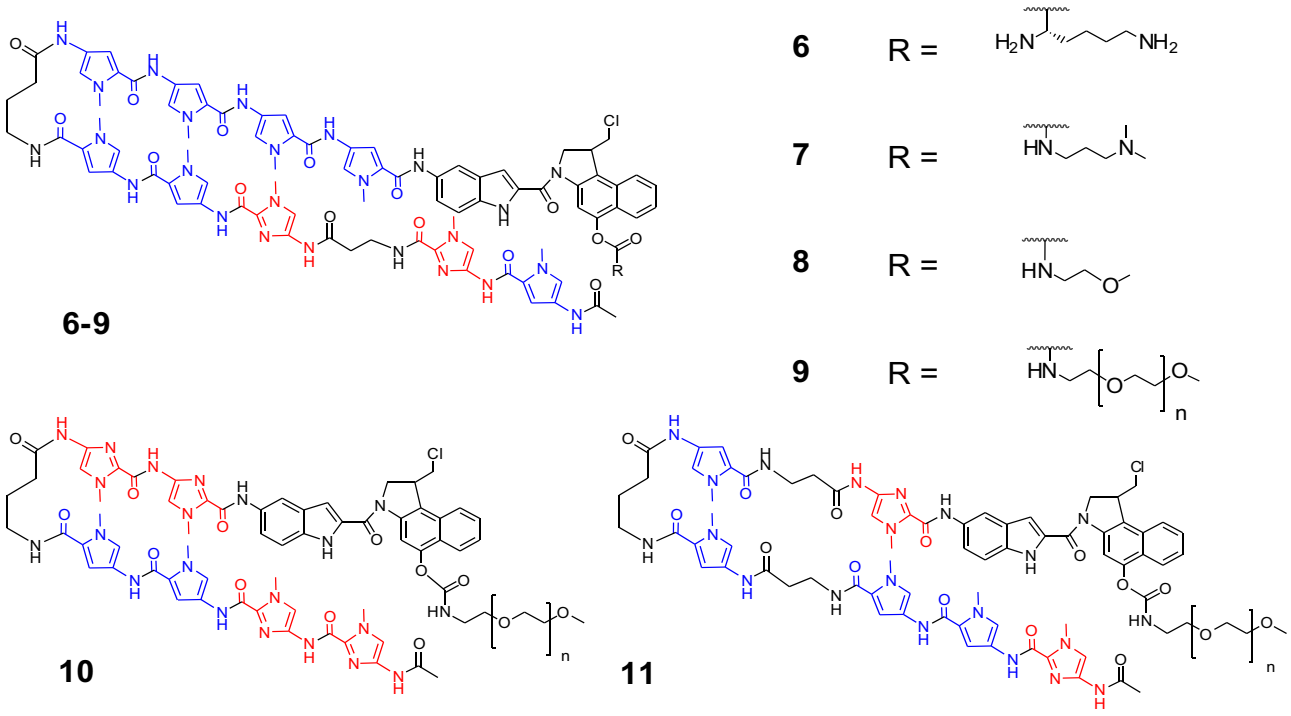

Figure 4. Chemical structures of PI polyamide conjugates 6–11.

3.2. Evaluating the aqueous solubility and chemical stability

We investigated the aqueous solubility of conjugates **1–3** and **6–11**. The results are shown in Table 1. As expected, while conjugates **1–3** hardly dissolved in Milli-Q water at 25 °C, the aqueous solubility of conjugates **6–11** that were modified by a corresponding hydrophilic group had dramatically improved.

The chemical stability of conjugates **6–11** was also investigated. The results are shown in Table 1. The stability of conjugates **6–11** was measured in 5.0 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 37 °C by HPLC analysis. Conjugates **6** and **7** easily hydrolyzed into conjugate **3** within a few hours. Only conjugates **9–11** were very stable over 48 h without hydrolysis. However, conjugate **8**, which was also modified by PEG ($n = 0$), easily hydrolyzed into conjugate **3** group.

These results lead two conclusions. One is that PI conjugates with relatively small molecular size show higher water solubility and the other is that the stability of PI polyamide conjugates with hydrophilic modification depends on the bulkiness of the hydrophilic group.

Table 1
Aqueous solubility and chemical stability of conjugates **1–3** and **6–11**

conjugate	Water solubility[a] (mM)	Stability $t1/2$[b] (h)
1	N.D.[c]	–
2	N.D.[c]	–
3	N.D.[c]	–
6	1.2	<1[d]
7	0.23	1.1[d]
8	0.18	3.5[d]
9	0.59	>48
10	4.4	>48
11	5.2	>48

[a] Against Milli-Q water at 25 °C.
[b] A half-life in 5.0 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 37 °C.
[c] ND, not determined.
[d] Conjugate **2** and cyclopropane derivatives were detected.

4. Cytotoxicity

To evaluate the cytotoxic potencies, we investigated the 50% cell growth inhibition (IC_{50}) concentrations of conjugates **1–5** and **9–11** by using two human cancer cell lines (A549, DU145). See Table 2.

First, we investigated the cytotoxicity of conjugates **3–5** with eight-bp recognition. Although conjugates **3** and **5** showed slightly lower cytotoxicity than conjugates **1** and **2**, these conjugates showed IC_{50} values at nanomolar concentrations. On the other hand, conjugate **4** showed higher cytotoxicity than conjugates **1** and **2**. These results suggest that elongating bp recognition sequences has almost no effect on the cytotoxicity.

Subsequently, to evaluate the effect of PEGylation, we compared the cytotoxicity of both conjugates with and without PEGylation. Contrary to our expectations, conjugate **9** dramatically decreased their cytotoxic potency compared with conjugate **3**, due to a decline in cell permeability. To investigate the size-dependency of PI moiety, we then compared the cytotoxicity of conjugates **10** and **11**, with that of conjugates **1** and **2**. Results showed that conjugates **10** and **11** had almost the same, or slightly higher cytotoxicity than conjugates **1** and **2**, which were clear contrast to the results of conjugate **3** and **9**. These results indicated that

Table 2
Cytotoxicity of conjugates **1–5**, **10** and **11** ($n = 3$) against A549 and DU145

Conjugate	IC_{50}	
	A549 (nM)	DU145 (nM)
1	135	47
2	51	28
3	240	62
4	29	7.9
5	240	45
9	1900	590
10	110	37
11	35	33

PEGylation for conjugates **10** and **11** improved the cell permeability compared with conjugates **1** and **2**. It is not clear why cell permeability of **9** decreased; one possible reason is the large molecular size of the PI moiety. We have already reported it appeared that larger molecular size of PI polyamides decreased their nuclear membrane permiability.[21] It can be assumed that PEG 750 is not enough conjugate **9** to enhance its cell permeability. In fact, relatively small improvement of solubility of **9** by PEGylation was observed. These results also suggest that PEGylation should be used considering the PI molecular size.

We have confirmed that conjugates with PEGylation did not show any DNA alkylation as determined by PAGE (data not shown). Therefore, it can be speculated that modification of PEGylation is somewhat contributed to during introducing into cell lines and deprotection of PEG moiety occurs in target cell lines, then conjugates show high cytotoxic potencies.

5. Conclusion

We synthesized conjugates **3–5** with eight-bp recognition and measured their alkylating ability and cytotoxicity against A549 and DU145. These conjugates showed high DNA alkylating ability and cytotoxicity at nanomolar concentrations. These results illustrate the potential of PI polyamide conjugates with eight-bp recognition. We also synthesized conjugates **6–11**, which were modified by hydrophilic groups. Conjugates **9–11**, which were modified by PEG 750, showed moderate aqueous solubility and good chemical stability. In particular, conjugates **10** and **11** had high cytotoxicity, similar to conjugates **1** and **2**. These conjugates, with both high cytotoxicity and water solubility, offer new possibilities in terms of ease of administration in vivo, directly, without the use of liposomes. Applying these conjugates to animal testing is a future challenge.

6. Experiments

6.1. General

Reagents and solvents were purchased from standard suppliers and used without further purification. 4-Nitrophenyl chloroformate, 2-methoxyethylamine and methoxypolyethylene glycol amine 750 were purchased from Aldrich. ^{1}H NMR spectra were recorded with a JEOL JNMX-FX 400 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as an internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), m (multiplet). High-performance liquid chromatography (HPLC) analysis was performed with both a JASCO PU-2080 Plus HPLC pump, a JASCO UV2075 HPLC UV/VIS detector and a Chemcobond 5-ODS-H reversed phase column (4.6 × 150 mm) in 0.1% TFA in water with CH_3CN as eluent at a flow rate of 1.0 mL/min, and a linear gradient elution of 0–100% CH_3CN over 20 min with detection at 254 nm. HPLC purification was performed with a JASCO CCPS HPLC pump, a JASCO UV8020 HPLC UV/VIS detector and a Chemcobond 5-ODS-H reversed phase column (10 × 150 mm) in 0.1% TFA in water with CH_3CN as eluent at a flow rate of 3.0 mL/min, and a linear gradient elution of 40–60% CH_3CN over 40 min with detection at 254 nm. Electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) were produced on a BioTOF II (Bruker Daltonics) mass spectrometry using a positive ionization mode. UV spectra were measured on a Nanodrop ND-1000 spectrophotometer. All DNA fragments, 5′-Texas Red-labeled primers and cold primers were purchased from Sigma–Aldrich. Thermo sequence core sequencing kit was purchased from GE Healthcare. Polymerase chain reaction (PCR) was performed on an iCyclear (BIO-RAD). Loading dye was composed of formamide 10 mL, H_2O 200 mL, 0.5 M aqueous solution of di-sodium dihydrogen ethylenediaminetetraacetate dehydrate (nacalai tesque Inc.) 300 mL, and New fuchsin (Merck) 2.5 mg. 50% Long Ranger™ gel solution was purchased from Lonza Rockland Inc. Polyacrylamide gel electrophoresis was performed on a HITACHI 5500-S DNA sequencer, and data were analyzed by FLAGLYS version 2 software (HITACHI).Machine-assisted PI polyamide syntheses were performed on a peptide synthesizer, PSSM-8 (SHIMADZU) in a stepwise reaction by Fmoc solid phase protocol.

6.2. Solid-phase synthesis of PI polyamides

All the PI polyamides were prepared in a stepwise reaction by Fmoc solid-phase protocol according to reported procedures.[15–17] PI polyamides supported by oxime resin (for the synthesis of conjugates **3**, **5**) were cleaved with alkali conditions (2.0 M NaOH aq, 1,4-dioxane, 55 °C, 3 h) and PI polyamides supported by CLEAR-Acid resin (for the synthesis of conjugates **1**, **2**, and **4**) were cleaved with acidic conditions (TFA/H_2O/triisopropylsilane = 95/2.5/2.5, rt, 0.5 h). HPLC purification (0.1% TFA-CH_3CN 40–60% linear gradient, 0–40 min, 254 nm) was used to obtain the desired polyamides.

6.2.1. AcIIPP-g-II-indole-*seco*-CBI (1)

A solution of AcIIPP-γ-II-OH (8.8 mg, 0.010 mmol), NH_2-indole-*seco*-CBI·HCl (8.1 mg, 0.015 mmol) and PyBOP (13.2 mg, 0.020 mmol) in DMF (150 mL) and DIEA (12.3 mL, 0.090 mmol) was stirred overnight at rt. After consumption of activated ester was confirmed by HPLC analysis, Et_2O was added to the mixture and resultant was collected by centrifugation, and washed by Et_2O and CH_2Cl_2. A weight of the product was 1.5 mg (0.0018 mmol, 18% yield) after HPLC purification. Analytical HPLC: t_R = 13.7 min. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.70 (s, 1H, NH), 10.42 (s, 1H, OH), 10.35 (s, 1H, NH), 10.31 (s, 1H, NH), 10.28 (s, 1H, NH), 10.10 (s, 1H, NH), 9.91 (s, 1H, NH), 9.42 (s, 1H, NH), 9.32 (s, 1H, NH), 8.15 (d, J = 0.4 Hz, 1H, CH), 8.18 (d, J = 4.0 Hz, 1H, CH), 8.03 (s, 1H, CH), 7.96 (s, 1H, CH), 7.85 (d, J = 9.6 Hz, 1H, CH), 7.61–7.44 (m, 7H), 7.36 (m, 1H, CH), 7.27 (s, 1H, CH), 7.17 (s, 1H, CH), 7.15 (s, 1H, CH), 6.90 (s, 1H, CH), 4.03 (s, 3H, CH_3), 4.00 (s, 3H, CH_3), 3.99 (s, 3H, CH_3), 3.97 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 3.80 (s, 3H, CH_3), 3.40–3.16 (m, 6H), 2.54–2.32 (m, 2H, CH_2) 2.03 (s, 3H, CH_3), 1.80 (t, J = 8.0 Hz, 1H, CH), 1.08 (m, 2H, CH_2); ESI-TOFMS m/e: calcd $C_{60}H_{59}ClN_{20}O_{10}$ $[M+2H]^{2+}$ 628.23, found 628.25.

6.2.2. AcIPP-b-P-g-I-b-I-indole-*seco*-CBI (2)

A synthetic procedure similar to that used for the preparation of conjugate **1** provided conjugate **2**. Analytical HPLC: t_R = 14.2 min; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.70 (s, 1H, NH), 10.44 (s, 1H, OH), 10.42 (s, 1H, NH), 10.22 (s, 1H, NH), 9.95 (s, 1H, NH), 9.90 (s, 1H, NH), 9.83 (s, 1H, NH), 9.78 (s, 1H, NH), 9.76 (s, 1H, NH), 8.12 (d, J = 8.0 Hz, 1H, CH), 8.05–7.98 (m, 3H), 7.97 (s, 1H, CH), 7.85 (d, J = 8.0 Hz, 1H, CH), 7.55–7.35 (m, 8H), 7.27–7.12 (m, 6H), 6.84 (s, 1H, CH), 6.69 (s, 1H, CH), 6.66 (s, 1H, CH), 4.81 (t, J = 10.0 Hz, 1H, CH), 4.56 (d, J = 11.6, 1H, CH), 3.99 (s, 3H, CH_3), 3.94 (s, 3H, CH_3), 3.84 (s, 3H, CH_3), 3.81 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 3.77 (s, 3H, CH_3), 3.48–3.36 (m, 4H), 3.18 (d, J = 7.2 Hz, 2H, CH_2), 2.67–2.57 (m, 2H), 2.50–2.33 (m, 2H), 2.24 (t, J = 7.6 Hz, 1H, CH_3), 2.02 (s, 3H), 1.77–1.74 (m, 2H); ESI-TOFMS m/e: calcd $C_{68}H_{71}ClN_{20}O_{12}$ $[M+2H]^{2+}$ 698.26, found 698.27.

6.2.3. AcPI-b-IPP-g-PPPP-indole-*seco*-CBI (3)

A synthetic procedure similar to that used for the preparation of conjugate **1** provided conjugate **3**. Analytical HPLC: t_R = 14.6 min.

ESI-TOFMS *m*/*e*: calcd $C_{83}H_{84}ClN_{25}O_{14}$ $[M+2H]^{2+}$ 845.83, found 845.84.

6.2.4. AcIPP-b-PP-g-PP-b-I-indole-*seco*-CBI (4)

A synthetic procedure similar to that used for the preparation of conjugate **1** provided conjugate **4**. Analytical HPLC: t_R = 14.1 min. ESI-TOFMS *m*/*e*: calcd $C_{80}H_{83}ClN_{24}O_{14}$ $[M+2H]^{2+}$ 820.32, found 820.29.

6.2.5. AcIIP-b-II-g-PP-b-P-indole-*seco*-CBI (5)

A synthetic procedure similar to that used for the preparation of conjugate **1** provided conjugate **5**. Analytical HPLC: t_R = 14.0 min. ESI-TOFMS *m*/*e*: calcd $C_{78}H_{81}ClN_{26}O_{14}$ $[M+2H]^{2+}$ 821.32, found 821.30.

6.2.6. 3-Lys (6)

A solution of conjugate **3** (3.8 mg, 2.2 mmol), Boc-Lys(Boc)-H·DCHA (4.9 mg, 9.3 mmol), *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.8 mg, 8.7 mmol) and *N*,*N*-dimethyl-4-aminopyridine (DMAP) (1.1 mg, 9.0 mmol) in DMF (50 mL) was stirred for 4 h at rt. After consumption of starting material was confirmed by HPLC analysis, Et_2O was added to the mixture. The solid was collected by centrifugation, and washed by Et_2O and CH_2Cl_2. A suspension of resulting powder in 1.0 mol/L HCl in AcOEt (200 mL) and CH_2Cl_2 (200 mL) was stirred for 1 h at rt. Et_2O was added to the mixture and the solid was collected by centrifugation, and washed by Et_2O and CH_2Cl_2. A weight of the product was 1.5 mg (0.89 mmol, 10% yield) after HPLC purification. Analytical HPLC: t_R = 10.9 min. ESI-TOFMS *m*/*e*: calcd $C_{89}H_{96}ClN_{27}O_{15}$ $[M+2H]^{2+}$ 909.88, found 909.89.

6.2.7. 3-Dp (7)

To a solution of conjugate **3** (2.9 mg, 1.7 mmol) in dehydrated DMF (100 mL) and Et_3N (0.50 mL, 4 mmol) at -68 °C ($CHCl_3$/dry ice), 4-nitrophenyl chloroformate (0.70 mg, 3.5 mmol) was added. Then, the resulting mixture was stirred for 0.5 h at −68 °C. 50% *N*,*N*-dimethyl-1,3-propanediamine (Dp) aqueous solution (4.6 mL, 18 mmol) was added to the solution, and the mixture was stirred for 1 h at 0 °C. Et_2O was added to the mixture and the solid was collected by centrifugation, and washed by Et_2O and CH_2Cl_2. A weight of the product was 0.50 mg (0.30 mmol, 20% yield) after HPLC purification. Analytical HPLC: t_R = 12.9 min. ESI-TOFMS *m*/*e*: calcd $C_{89}H_{96}ClN_{27}O_{15}$ $[M+2H]^{2+}$ 909.88, found 909.94.

6.2.8. 3-PEG (*n* = 0) (8)

To a solution of conjugate **3** (3.8 mg, 2.2 mmol) in dehydrated DMF (100 mL) and Et_3N (1.6 mL, 11 mmol) at −68 °C, 4-nitrophenyl chloroformate (2.2 mg, 11 mmol) was added. Then, the resulting mixture was stirred for 1 h at −68 °C. 2-methoxyethylamine (2.8 mL, 32 mmol) was added to the solution, and the mixture was stirred for 1 h at 0 °C. Et_2O was added to the mixture and the solid was collected by centrifugation, and washed by Et_2O and CH_2Cl_2. A weight of the product was 0.40 mg (0.22 mmol, 10% yield) after HPLC purification. Analytical HPLC: t_R = 13.6 min. ESI-TOFMS *m*/*e*: calcd $C_{87}H_{91}ClN_{26}O_{16}$ $[M+2H]^{2+}$ 896.35, found 896.39.

6.2.9. 3-peg (9)

To a solution of conjugate **3** (1.5 mg, 0.89 mmol) in dehydrated DMF (100 mL) and Et_3N (0.62 mL, 4.5 mmol) at −68 °C, 4-nitrophenyl chloroformate (0.90 mg, 5 mmol) was added. Then, the resulting mixture was stirred for 1 h at −68 °C. A solution of methoxypolyethylene glycol amine 750 (6.8 mg, 9.1 mmol) and Et_3N (1.2 mL, 8.6 mmol) in dehydrated DMF (10 mL) was added to the solution, and the mixture was stirred for 1 h at 0 °C. Et_2O was added to the mixture and the solid was collected by centrifugation, and washed by Et_2O and CH_2Cl_2. A weight of the product was 0.50 mg (0.20 mmol, 20% yield) after HPLC purification. Analytical HPLC: t_R = 13.3 min. ESI-TOFMS *m*/*e*: calcd $C_{87+2n}H_{91+4n}ClN_{26}O_{16+n}$ $[M+3H]^{3+}$ 774.01 (*n* = 12), 788.68 (*n* = 13), 803.36 (*n* = 14), 818.03 (*n* = 15), 832.71 (*n* = 16), 847.38 (*n* = 17), 862.06 (*n* = 18), 876.73 (*n* = 19), 891.41 (*n* = 20) found 774.07 (*n* = 12), 788.72 (*n* = 13), 803.40 (*n* = 14), 818.07 (*n* = 15), 832.75 (*n* = 16), 847.41 (*n* = 17), 862.09 (*n* = 18), 876.78 (*n* = 19), 891.46 (*n* = 20), found 774.07 (*n* = 12), 788.72 (*n* = 13), 803.40 (*n* = 14), 818.07 (*n* = 15), 832.75 (*n* = 16), 847.41 (*n* = 17), 862.09 (*n* = 18), 876.78 (*n* = 19), 891.46 (*n* = 20).

6.2.10. 1-peg (10)

A synthetic procedure similar to that used for the preparation of conjugate **9** provided conjugate **10**. Analytical HPLC: t_R = 13.3 min. ESI-TOFMS *m*/*e*: calcd $C_{64+2n}H_{66+4n}ClN_{21}O_{12+n}$ $[M+2H]^{2+}$ 920.90 (*n* = 11), 942.92 (*n* = 12), 964.93 (*n* = 13), 986.94 (*n* = 14), 1008.95 (*n* = 15), 1030.97 (*n* = 16), 1052.98 (*n* = 17), 1074.99 (*n* = 18), 1097.01 (*n* = 19), 1192.02 (*n* = 20), 1141.03 (*n* = 21), found 920.91 (*n* = 11), 942.95 (*n* = 12), 964.96 (*n* = 13), 986.97 (*n* = 14), 1008.99 (*n* = 15), 1031.00 (*n* = 16), 1053.01 (*n* = 17), 1075.03 (*n* = 18), 1097.05 (*n* = 19), 1119.06 (*n* = 20), 1141.07 (*n* = 21) found 920.91 (*n* = 11), 942.95 (*n* = 12), 964.96 (*n* = 13), 986.97 (*n* = 14), 1008.99 (*n* = 15), 1031.00 (*n* = 16), 1053.01 (*n* = 17), 1075.03 (*n* = 18), 1097.05 (*n* = 19), 1192.06 (*n* = 20), 1141.07 (*n* = 21).

6.2.11. 2-peg (11)

A synthetic procedure similar to that used for the preparation of conjugate **9** provided conjugate **11**. Analytical HPLC: t_R = 13.4 min. ESI-TOFMS *m*/*e*: calcd $C_{72+2n}H_{78+4n}ClN_{21}O_{14+n}$ $[M+2H]^{2+}$ 690.32 (*n* = 13), 704.99 (*n* = 14), 719.67 (*n* = 15), 734.34 (*n* = 16), 749.61 (*n* = 17), 763.69 (*n* = 18), 778.36 (*n* = 19), 793.03 (*n* = 20), found 690.31 (*n* = 13), 704.98 (*n* = 14), 719.67 (*n* = 15), 734.34 (*n* = 16), 749.68 (*n* = 17), 763.70 (*n* = 18), 778.37 (*n* = 19), 793.06 (*n* = 20),

6.3. Cloning of 208 bp DNA fragments

DNA fragments were annealed in a final volume of 20 mL containing 50 mM of each strand (5′-TGTCATCCAGTGCCATTCAGTTCCATCCAGA -3′ and 3′-CTGGATGGAACTGAATGGCACTGGATGACAA-5′), and ligated into pGEM-T Easy vector (Promega). *Escherichia coli* DH5a competent cells (TOYOBO) were transformed and cultured on a LB plate with 100 mg/mL ampicillin and 20 mg/mL, 25 mL X-gal 100 mM, 25 mL IPTG overnight at 37 °C. White colonies were identified by colonyPCR in 20 nM of each primer (T7 primer: 5′-TAATACGACTCACTATAGGG-3′, SP6 primer: 5′-TATTTAGGTGACACTATAG-3′), 200 μM of dNTPs (Sigma–Aldrich), 2 units Taq polymerase and 1 ThermoPol reaction buffer (New England Bio Labs). Amplification cycles were carried out with an iCycler (BIO-RAD). The reaction mix was incubated at 95 °C for 5 min, followed by 35 cycles of 95 °C for 35 s, 50 °C for 35 s, 72 °C for 30 s, with a final extension step of 72 °C for 7 min. The appropriate colony was selected for transfer to 5 mL of LB medium with 100 mg/mL ampicillin and cultured overnight at 37 °C. The plasmids were extracted using GenElute™ Plasmid Miniprep Kit (Sigma–Aldrich) and identified by PCR (program and reaction mixtures were the same as above).

6.4. High-resolution gel electrophoresis

The 5′-Texas Red labeled 208 bp DNA fragments (6.0 nM) were alkylated by various concentration of conjugates **3–5** in 10 mL of 5.0 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 20 °C. After incubation for 18 h, the reaction mixture was quenched by calf thymus DNA and heating for 5 min at 95 °C. The solution was concentrated by vacuum centrifugation. The pellet was dissolved with 6 mL loading dye (formamide with fuschin red), heated at 95 °C for 20 min, and then immediately cooled to

0 °C. A 2.0 mL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi DNA sequencer.

6.5. Water solubility

Water solubility of conjugates **1–5** and **9–11** was examined by nanodrop. A saturated solution of a test compound in Milli-Q was prepared. The supernatant solution (1.0 mL) was diluted in DMF (99 mL). The absorbance of the peak around 310 nm of each sample was measured and concentration of each conjugate was calculated.

6.6. Chemical stability in aqueous solution

The stability of conjugates **1–5** and **9–11** under aqueous conditions was examined by HPLC analysis. A solution of a test compound in DMF (1.0 mM) was prepared. This solution (1 mL) was diluted in aqueous solution (9 mL). Aqueous solution was composed of 5.6 mM sodium phosphate buffer (pH 7.0). This resulting solution was incubated at 37 °C. Samples were injected at intervals directly into HPLC injection port and were investigated a half life by measuring HPLC chart area.

6.7. Cytotoxicity assay

Human lung adenocarcinoma epithelial A549 cells were obtained from Health Science Research Resources Bank and human prostate carcinoma epithelial-like DU145 cells were obtained from RIKEN. Each cell line (5×10^5 cell/dish) was precultured in the culture medium in Cellstar™ 60 × 15 mm cell culture dish (Greiner Bio-One) for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. To 96-well cell culture plate (Greiner Bio-One), each cell line (5×10^3 cell/well) in the culture medium (100 mL) and each conjugate were added. After incubation for 48 h at 37 °C in a humidified atmosphere of 5% CO_2, cell count reagent SF (nacalai tesque, Inc) was added to the medium. After further incubation for a few hours, absorbance of wells at 450 nm were determined by microplate reader MPR-A4i II (Tosho). The IC_{50} values (drug concentration required for 50% inhibition of the cell growth) were determined.

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