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Cooperative alkylation of double-strand human telomere repeat sequences by PI polyamides with 11-base-pair recognition based on a heterotrimeric design

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ABSTRACT

We designed and synthesized alkylating conjugates **5**–**7** and their partner *N*-methylpyrrole-*N*-methylimidazole (PI) polyamides **8**, **9**. The DNA alkylating activities of conjugates **5**–**7** were evaluated by high-resolution denaturing polyacrylamide gel electrophoresis with a 219 base pair (bp) DNA fragment containing the human telomere repeat sequence. Conjugate **5** efficiently alkylated the sequence, 5′-GGTTAGGGTT**A**-3′, in the presence of partner PI polyamide **8** or distamycin A (Dist). In contrast, the heterodimer system of **5** with **9** showed very weak alkylating activity. Accordingly, this heterotrimeric system of **5** with two short partners is an expedient way to attain improved precision and extension of the recognition of DNA sequences.

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1. Introduction

Telomeres, G-rich DNA arrays and associated proteins, protect eukaryotic chromosome ends from damage and degradation. Because of the end replication problems, human and mouse telomeres loose terminal sequences at a rate of 50–150 bp/end/cell division.[1] Conversely, in most malignant cells (85–90%), the maintenance of telomeres is achieved by upregulating the expression of telomerase, which extends telomeres using their 3′ single-strand overhangs.[2] Telomerase is so essential for the proliferation of cancer cells that agents targeting telomeres and telomerase have attracted interest for the development of anticancer drugs.

Minor groove-binding *N*-methylpyrrole-*N*-methyl imidazole (PI) polyamides uniquely recognize each of the four Watson–Crick base pairs. Antiparallel pairing of I opposite P (I/P) recognizes a G–C base pair, whereas a P/P pair recognizes A–T or T–A base pairs. We have developed various sequence-specific alkylating agents by conjugating PI polyamides and alkylating moieties.[3] In our previous studies, a design using conjugated PI polyamides enables us to target one repeat of the human telomere repeat sequence, 5′-GGGTT**A**-3′,[4,5] using 1,2,9,9a-tetrahydrocyclopropa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI)[6–9] as an alkylating moiety.

Cooperative binding motif provides a promising approach to extending the sequence specificity of PI polyamides, as we can design smaller molecules to recognize longer sequences. We discovered that the alkylating site of duocarmycin A dramatically changes from the A residues to the G residues in G–C rich sequences through formation of a heterodimer with distamycin A (Dist) in the minor groove.[10,11] We also found that PI triamides can modulate the sequence specificity of duocarmycin A in a predetermined manner.[12] Moreover, we recently reported that DNA alkylation with 10-bp recognition could be achieved through heterodimer formation.[13] Inspired by these observations, we addressed heterotrimeric targeting of telomere sequences in this study.

2. Results and discussion

2.1. Molecular design and syntheses

We synthesized PI polyamides **1**, **2**, and **3**, with carboxylic acid termini, and the partner PI polyamides **8** and **9** using Fmoc solid-phase synthesis. The indole linker and DNA alkylating unit, *seco*-CBI, were introduced to **1**, **2**, and **3** by coupling with conjugate **4** using FDPP for **1**and **3,** and HCTU for **2** to produce three alkylating PI polyamide conjugates, **5**–**7**, as previously described (Fig. 1).[4]

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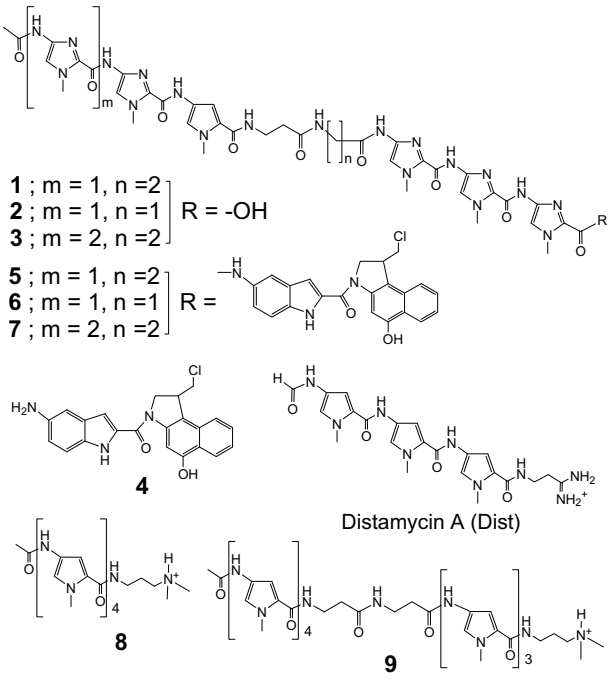

Figure 1. Structures of the synthesized *seco*-CBI conjugates **5**–**7** with an indole linker, the partner PI polyamides **8**, **9,** and distamycin A (Dist).

2.2. DNA alkylating activities of conjugates 5–7 in the presence of partner molecules

We evaluated the DNA alkylating activities of conjugates **5**–**7** in the presence and absence of partner PI polyamides **8**, Dist, or **9** using polyacrylamide gel electrophoresis (PAGE) analysis. Alkylation was carried out at 23 °C for 12 h, followed by quenching by the addition of calf thymus DNA. The samples were heated at 95 °C under neutral conditions for 20 min. The alkylation sites were visualized by thermal cleavage of the DNA strand. Under these heating conditions, all the purine N3 at the alkylated sites in the DNA produced cleavage bands almost quantitatively on the gel. Sequencing gel analysis showed that no alkylation reaction occurred, when the DNA fragment was treated with conjugate **5** alone (Fig. 2a, lanes 2–6). In the presence of partner PI polyamide **9**, conjugate **5** alkylated the target site, indicating that the alkylation occurs through heterodimer formation, which is consistent with a previous report.[13] However, at higher concentrations of **5**, only weak alkylation was observed in the presence of partner **9** (lanes 17 and 18). Therefore, we tried a heterotrimeric design approach, that is, division of the long partner 9 into two short partners, such as 8. In fact, this approach significantly improved the reaction. Although there is no direct evidence of heterotrimeric complex formation, we can enumerate some suggestions and examples to support that (i) one or two pyrroles should be present in an opposite side of the indole linker.[4] (ii) A precise recognition as long as 11 bp could not have been achieved without a whole range binding. Thus, it is reasonable to consider that the partners exist in both termini of the long alkylating agent. (iii) Not a few reports are on ABB heterotrimeric proteins such as the bacteriophage λ terminase, the DNA packaging motor (one gpA and two

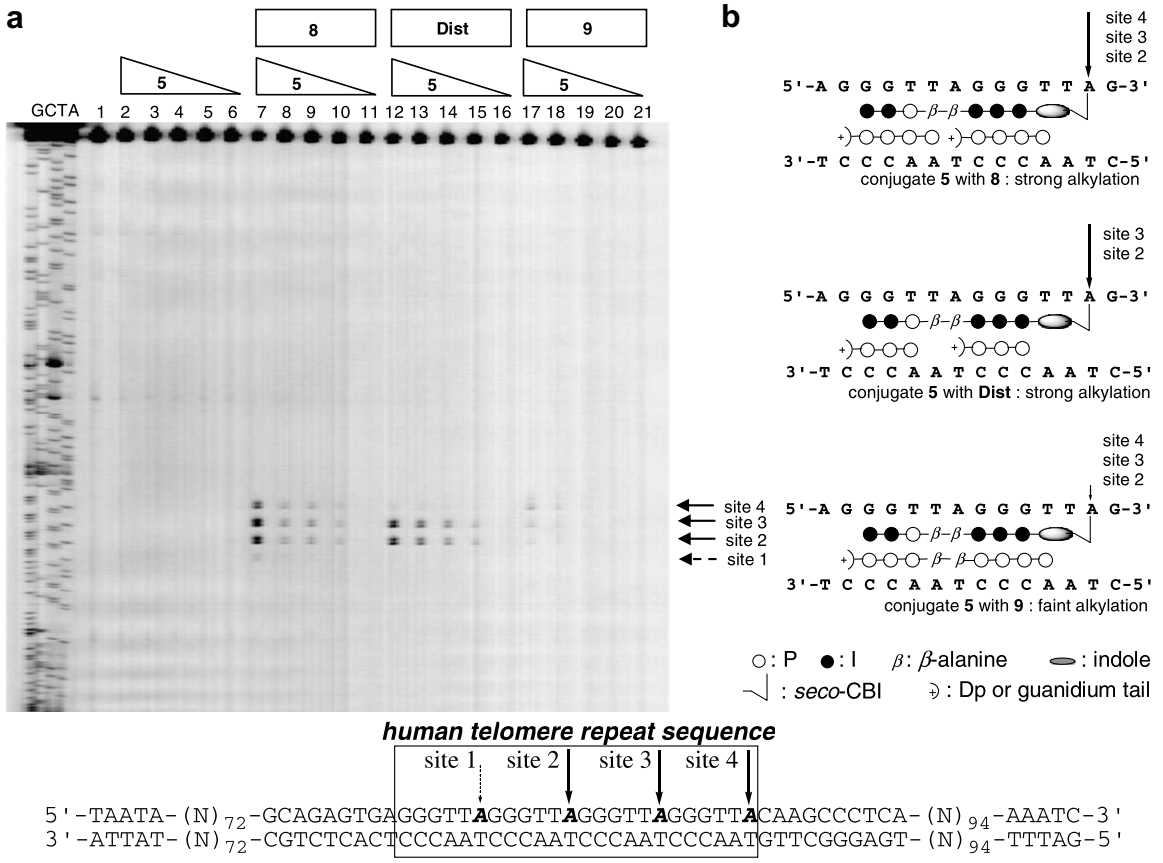

Figure 2. (a) Thermally induced strand cleavage of a 5′-Texas Red-labeled 219 bp DNA fragment (6 nM) incubated for 12 h at 23 °C; Lane 1, DNA control; Lanes 2–6, 500, 200, 100, 50, 20 nM of **5**; Lanes 7–11, 500, 200, 100, 50, 20 nM of **5** with 100 nM of **8**; Lanes 12–16, 500, 200, 100, 50, 20 nM of **5** with 100 nM of Dist; Lanes 17–21, 500, 200, 100, 50, 20 nM of **5** with 100 nM of **9**. (b) Schematic representation of sequence-specific alkylation in the telomere sequence by conjugate **5** with **8** or Dist. Arrows indicate the sites of adenine N3 alkylation.

gpNu1 proteins)[14], and type IV collagen (two α1 fragments and one α2 fragment).[15] Treatment of the DNA fragment with **5** in the presence of **8** (lanes 7–10) or the natural analogue Dist (lanes 12–15), respectively, produced discrete alkylation bands at sites 2, 3, and 4 in 5′-GGTTAGGGTT**A**-3′, with a faint band detected at the 2 bp mismatched site 1. These results clearly indicated that heterotrimeric design could precisely recognize two repeats of the human telomere sequence, and conjugate **5** efficiently alkylated the N3 of adenine (A) through heterotrimer formation with short partner PI polyamides. These results clearly indicate that heterotrimeric approach is better in terms of both DNA alkylation efficiency and sequence specificity. A proposed binding model for conjugate **5** and partner **8** or Dist is shown in Figure 2b. The results also suggest that a cooperative binding of multiple polyamides might have utility for targeting longer specific DNA sequences by PI *seco*-CBI conjugates.

The DNA fragment was treated with a constant concentration of conjugate **5** (200 nM) with different concentrations of the partner **8**, Dist, or **9, and the result of this is shown in** Figure 3a. These results indicated that **8** and Dist efficiently function as heterotrimeric partner molecules with **5** for sequence-specific DNA alkylation. However, the reason for the difference between **8** and Dist at site 4 was unclear. It is speculated that heterotrimeric binding complex formed by **5** with Dist at site 4 might be more unstable than that formed by **5** with **8**.

We next evaluated the DNA alkylating activities of two analogous conjugates **6**, **7** as shown in Figure 3b and c. In sharp contrast, conjugate **6**, which contains glycine (α) instead of one of the β-alanine (β) units, was observed to weakly alkylate this DNA fragment. These results suggest that a difference of one methylene group would have a significant effect on DNA alkylation of a specific sequence. It was assumed based on the recognition rule of PI polyamides that conjugate **7** would show better reactivity and selectivity. However, **7** did not efficiently alkylate sites 2, 3, and 4 in the presence of **8** (30-fold less than conjugate **5**. See Fig. 3b), which suggests that longer PI polyamide recognition units do not necessarily give rise to better sequence-specific alkylating activity presumably due to entropic disadvantage. Maeshima et al. also showed that PIII segment of the PI polyamides is disfavored targeting the human telomere repeat sequence, for instance, compound TH58 did not protect TTAGGG repeats by DNase I footprinting analysis, and they devised an alternative PI polyamide by moving one of the three imidazoles to the opposite side of the hairpin and leaving it unpaired (compound TH59 binds to the telomere).[16] The type of partner, PI polyamide **8** or Dist, did not affect the order of reactivity: conjugate **5** was superior to conjugates **6** and **7**.

2.3. Cytotoxicity of conjugate 5 in the absence and presence of partner 8 or Dist

To evaluate the cytotoxic potencies of conjugate **5**, we investigated the 50% cell growth inhibition (IC_{50}) values of conjugate **5** with and without the partners **8** and Dist (Fig. 4). The concentra-

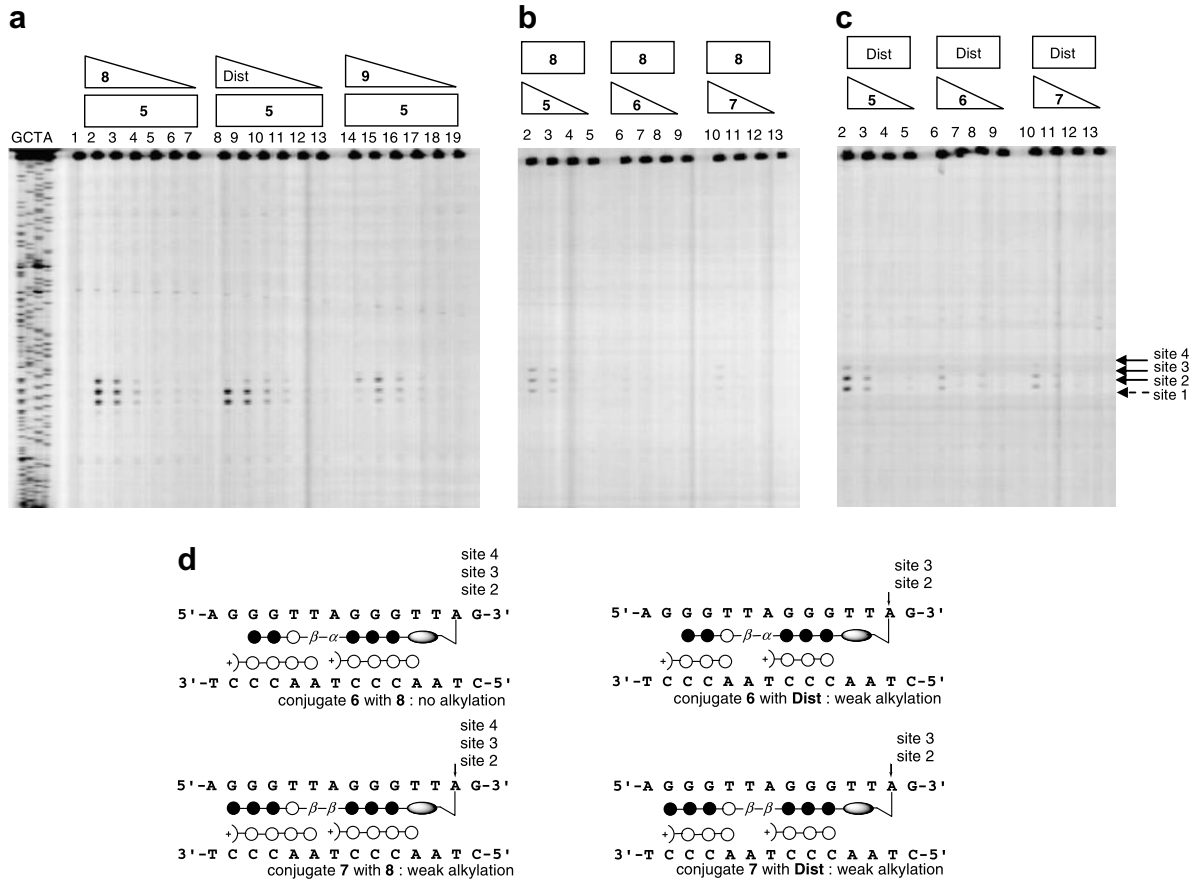

Figure 3. (a) Thermally induced strand cleavages of a 5′-Texas Red-labeled 219 bp DNA fragment (6 nM) incubated for 12 h at 23 °C; Lane 1, DNA control; Lanes 2–7, 200 nM of **5** with 200, 100, 50, 20, 10, 5 nM of **8**; Lanes 8–13, 200 nM of **5** with 200, 100, 50, 20, 10, 5 nM of Dist; Lanes 14–19, 200 nM of **5** with 200, 100, 50, 20, 10, 5 nM of **9**. (b) Thermally induced strand cleavages of a 5′-Texas Red-labeled 219 bp DNA fragment (6 nM) incubated for 12 h at 23 °C; Lanes 2–5, 500, 200, 100, 50 nM of **5** with 100 nM of **8**; Lanes 6–9, 500, 200, 100, 50 nM of **6** with 100 nM of **8**; Lanes 10–13, 500, 200, 100, 50 nM of **7** with 100 nM of **8**. (c) Thermally induced strand cleavages of a 5′-Texas Red-labeled 219 bp DNA fragment (6 nM) incubated for 12 h at 23 °C; Lanes 2–5, 500, 200, 100, 50 nM of **5** with 100 nM of Dist; Lanes 6–9, 500, 200, 100, 50 nM of **6** with 100 nM of Dist; Lanes 10–13, 500, 200, 100, 50 nM of **7** with 100 nM of Dist. (d) Schematic representation of sequence-specific alkylation in the telomere sequence by conjugates **6** and **7** with **8** or Dist.

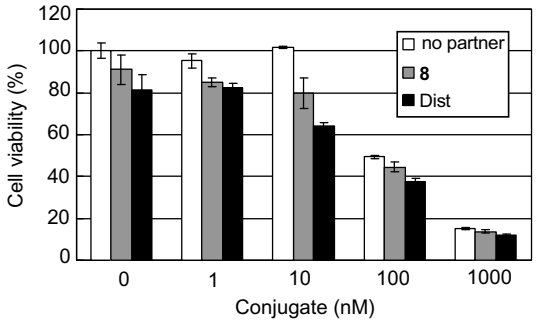

Figure 4. Growth inhibition activities of conjugate **5** with and without 10 μM partner (**8** or Dist) on Jurkat cells. Cells were pretreated with partners for 24 h, then treated with conjugate **5** for 48 h. White, gray, and black columns indicate pretreatment and no partner, and 10 μM **8** and Dist, respectively.

tions of both **8** and Dist were 10 μM (IC_{50} of Dist ~30 μM[17]). The IC_{50} values of conjugate **5** estimated from the data shown in Figure 4 were 100, 79, 32 nM without a partner, with **8,** and with Dist, respectively. These results indicate that conjugate **5** itself has high cytotoxicity against Jurkat cells, presumably due to non-specific alkylation-related side effects. Only a small enhancement of cytotoxicity was observed in the presence of the partner molecules, suggesting that further improvement is necessary in designing heterotrimeric alkylation for use in living cell systems.

3. Conclusions

We designed and synthesized conjugate **5**, which targets human telomere duplex repeats. PAGE showed that conjugate **5** selectively recognized and alkylated at target telomere repeat sequence 5′-GGTTAGGGTT**A**-3′ at nanomolar concentrations only when combined with a partner molecule, **8** or Dist, whereas a combination of **5** and the heterodimeric partner **9** showed low alkylation reactivity. These results illustrate the potential of combination of long PI polyamide conjugates and short PI partner polyamides for targeting specific DNA sequences. In particular, the heterotrimeric system of **5** with two short partners would be an expedient way to extend the recognition of specific DNA sequences in future.

4. Experimental

4.1. General

Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: Fmoc, fluorenylmethoxycarbonyl; Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; FDPP, pentafluorophenyl diphenylphosphinate; HCTU, 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxidehexafluorophosphate.

NMR spectra were recorded with a JEOL JNM-FX 400 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as the 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), m (multiplet). Electrospray ionization mass spectrometry (ESIMS) and electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) were produced on an API 150 (PESCIEX) and BioTOF II (Bruker Daltonics) mass spectrometer. Polyacrylamide gel electrophoresis was performed on a HITACHI SQ5500-S and HITACHI SQ500-E DNA sequencer. PCR amplification was carried out with an iCycler (BIO-RAD). Ex Taq DNA polymerase was purchased from Takara Co.; the Thermo Sequence core sequencing kit and loading dye (formamide with New fuchsin) were from Amersham Co. Ltd.; 5′-Texas Red-modified DNA oligomer (20-mer) was from Proligo Co. Ltd.; and 50% Long Ranger gel solution was from FMC bioproducts. Oxime resin (200–400 mesh) and CLEAR-acid resin (100–200 mesh) were purchased from Novabiochem and PEPTIDES international, respectively.

4.2. Solid-phase synthesis of PI polyamides

4.2.1. AcIIP-β-β-III-CO_2H (1)

AcIIP-β-β-III-CLEAR-acid resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol.[4] The polyamide moiety was cleaved from the resin with 3 mL (TFA 95%, triisopropylsilane 2.5%, water 2.5%) for 30 min at room temperature. The solvent was removed and the oil was washed with Et_2O to yield a yellow powder (34.0 mg, 36.2 μmol, 36%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H; NH), 10.28 (s, 1H; NH), 10.24 (s, 1H; NH), 9.91 (s, 1H; NH), 9.48 (s, 1H; NH), 9.31 (s, 1H; NH), 7.99 (s, 2H; NH), 7.61 (s, 2H; I-H), 7.54 (s, 1H; I-H), 7.50 (s, 1H; I-H), 7.49 (s, 1H; I-H), 7.23 (s, 1H; P-H), 6.93 (s, 1H; P-H), 3.99 (s, 6H; NCH_3), 3.97 (s, 6H; NCH_3), 3.93 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.35 (m, 6H; CH_2), 2.33 (m, 2H; CH_2), 2.03 (s, 3H; $COCH_3$). ESI-TOFMS *m*/*z* calcd for $C_{39}H_{46}N_{19}O_{10}$ $[M+H]^+$ 940.36, found 940.72.

4.2.2. AcIIP-β-α-III-CO_2H (2)

A synthetic protocol similar to that used for the preparation of compound **1** was followed to prepare compound **2** (64.1 mg, 69.2 μmol, 69%), which was confirmed by HPLC and ESI-TOFMS analyses. ESI-TOFMS *m*/*z* calcd for $C_{38}H_{44}N_{19}O_{10}$ $[M+H]^+$ 926.34, found 926.46.

4.2.3. AcIIIP-β-β-III-CO_2H (3)

A synthetic protocol similar to that used for the preparation of compound **1** was followed to prepare compound **3** (48.6 mg, 45.7 μmol, 46%), which was confirmed by HPLC and ESI-TOFMS analyses. ESI-TOFMS *m*/*z* calcd for $C_{44}H_{51}N_{22}O_{11}$ $[M+H]^+$ 1063.40, found 1063.57.

4.2.4. H_2N-indole-*seco*-CBI (4)

Compound **4** was prepared as previously reported.[13]

4.2.5. AcIIP-β-β-III-indole-*seco*-CBI (5)

To a solution of compound **1** (5.0 mg, 5.3 μmol) in DMF (50 μL) were added DIEA (5.6 μL, 32 μmol) and FDPP (4.2 mg, 11 μmol). The reaction mixture was incubated for 3 h at room temperature. After conversion from **1** to activated ester was confirmed by HPLC and ESIMS analyses, DIEA (5.6 μL, 32 μmol) and **4** (2.5 mg, 5.8 μmol) were added to the reaction vessel. The reaction mixture was shaken for 2 h at room temperature. Evaporation and subsequent washing with Et_2O, and sonication gave a gray powder. This powder was purified using HPLC to produce gray powder (1.1 mg, 0.84 μmol, 16%). It was used in the DNA alkylation. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.72 (s, 1H; NH), 10.44 (s, 1H; OH), 10.41 (s, 1H; NH), 10.27 (s, 1H; NH), 10.24 (s, 1H; NH), 10.09 (s, 1H; NH), 9.73 (s, 1H; NH), 9.64 (s, 1H; NH), 9.30 (s, 1H; NH), 8.31 (s, 1H; NH), 8.18 (m, 1H; CH), 8.13 (d, J = 8.0 Hz, 1H; CH), 8.02 (m, 1H; CH), 8.00 (s, 1H; NH), 7.98 (m, 1H; CH), 7.86 (d, J = 8.0 Hz, 1H; CH), 7.64 (s, 1H; I-H), 7.61 (s, 1H; I-H), 7.60 (m, 1H; CH), 7.53 (s, 1H; I-H), 7.51 (s, 1H; I-H), 7.49 (s, 1H; I-H), 7.48 (m, 1H; CH), 7.37 (m, 1H; CH), 7.22 (d, J = 2.0 Hz, 1H; P-H), 7.19 (m, 1H; CH), 6.93 (d, J = 2.0 Hz, 1H; P-H), 4.81 (m, 2H; CH_2), 4.58 (m, 1H; CH_2), 4.56 (m, 1H; CH_2), 4.23 (m, 1H; CH), 4.04 (s, 3H; NCH_3), 4.03 (s, 3H; NCH_3), 3.974 (s, 3H; NCH_3), 3.970 (s, 3H; NCH_3), 3.965 (s, 3H; NCH_3), 3.92 (m, 2H; CH_2), 3.79 (s, 3H; NCH_3), 3.35 (m, 2H; CH_2), 3.29 (m, 2H; CH_2), 2.33 (m, 2H; CH_2)

2.02 (s, 3H; $COCH_3$). ESI-TOFMS *m/z* calcd for $C_{61}H_{62}ClN_{22}O_{11}$ $[M+H]^+$ 1313.48, found 1314.02.

4.2.6. AcIIP-β-α-III-indole-*seco*-CBI (6)

To a solution of compound **2** (5.0 mg, 5.4 μmol) in DMF (50 μL) were added DIEA (5.6 μL, 32 μmol) and HCTU (4.6 mg, 11 μmol). The reaction mixture was incubated for 1 h at room temperature. After conversion from **2** to activated ester was confirmed by HPLC and ESIMS analyses, DIEA (5.6 μL, 32 μmol) and **4** (2.5 mg, 5.8 μmol) were added to the reaction vessel. The reaction mixture was shaken overnight at room temperature. Evaporation and subsequent washing with Et_2O, and sonication gave a gray powder. This powder was purified using HPLC to produce gray powder (0.3 mg, 0.23 μmol, 4%). It was used in the DNA alkylation. ESI-TOFMS *m/z* calcd for $C_{60}H_{60}ClN_{22}O_{11}$ $[M+H]^+$ 1299.46, found 1299.94.

4.2.7. AcIIIP-β-β-III-indole-*seco*-CBI (7)

A synthetic protocol similar to that used for the preparation of compound **5** was followed to prepare compound **7** (0.6 mg, 0.4 μmol, 9%), which was confirmed by HPLC and ESI-TOFMS analyses. ESI-TOFMS *m/z* calcd for $C_{66}H_{67}ClN_{25}O_{12}$ $[M+H]^+$ 1436.52, found 1436.82.

4.2.8. AcPPPP-Dp (8)

AcPPPP-oxime resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol. A sample of resin was cleaved with *N*,*N*-dimethyl-1,3-propanediamine for 10 h at 55 °C, filtered, washed with CH_2Cl_2, evaporated, and again washed with Et_2O to yield a white yellow powder (53.0 mg, 83.8 μmol, 84%). This was used in the DNA alkylation reaction without purification. ESI-TOFMS *m/z* calcd for $C_{31}H_{41}N_{10}O_5$ $[M+H]^+$ 633.33, found 633.30.

4.2.9. AcPPPP-β-β-PPP -Dp (9)

AcPPPP-β-β-PPP-oxime resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol. The polyamide moiety was cleaved from the resin with 5 mL of (1 M NaOH aq 3 mL, DMF 2 mL) for 1 h at 55 °C. Neutralization with 5% HCl followed by vacuum drying and washing with water and Et_2O yielded a yellow powder (28.3 mg, 26.8 μmol, 26%). To a solution of 2.5 mg of that powder (2.4 μmol) and FDPP (5.2 mg, 14 μmol) in 60 μL of DMF was added 2.5 μL of DIEA (14 μmol). One h after the addition of *N*,*N*-dimethyl-1,3-propanediamine (8.5 μL), the solution was vacuum-dried and washed with Et_2O to yield a yellow powder (4.0 mg, quant). ESI-TOFMS *m/z* calcd for $C_{55}H_{69}N_{18}O_{10}$ $[M+H]^+$ 1141.54, found 1141.60.

4.3. Preparation of DNA fragments containing four repeats of the human telomere sequence

All DNA fragments and primers for cloning of DNA amplification were prepared as previously reported.[4]

4.4. Preparation of 5′-Texas Red-modified DNA fragment and high-resolution gel electrophoresis

The 5′-Texas Red-modified DNA fragments containing telomere sequence was prepared by PCR using a primer set of 5′-Texas Red-labeled T7 and sp6 promoter primer or that of T7 and 5′-Texas Red-labeled sp6 promoter primer; 1 ng of the telomere fragment inserted pGEM-T easy vector (program and other reagents same as above). The fragment was purified by GenElute PCR cleanup kit (Sigma Aldrich). The 5′-Texas Red-labeled DNA fragments were alkylated by various concentrations of alkylation polyamides in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23 °C for 12 h. The reaction was quenched by the addition of calf thymus DNA (10 mg/mL, 1 μL) and by heating the mixture for 5 min at 95 °C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 9 μL of loading dye (formamide with New fuchsin), heated at 95 °C for 20 min for thermal cleavage at the alkylated sites,[18–20] and then immediately placed on ice. The 1.2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi SQ5500-S DNA Sequencer or Hitachi SQ5500-E DNA Sequencer.[21–23]

4.5. Cytotoxicity assay[24]

Colorimetric assays using WST-8 (Dojindo) were performed on 96-well plates. Jurkat cells were treated with no partner, **8** or Dist, for 24 h in advance and plated on the well in 50 μL of culture medium. Subsequently added were 50 μL of the medium containing 2×10^{-9}, 2×10^{-8}, 2×10^{-7}, 2×10^{-6} M of **5** and no partner, 10 μM of **8** or 10 μM of Dist which were prepared from DMF solution and thus also contained 0.2% DMF. After treatment for 48 h, 10 μL of WST-8 reagent was added into each well and incubated at 37 °C. Absorbance was then measured at 450 and 600 nm using an MPR-A4*i* microplate reader (Tosoh).

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