Bioorganic & Medicinal Chemistry 18 (2010) 2887–2893

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Alkylation of a human telomere sequence by heterotrimeric chlorambucil PI polyamide conjugates

Gengo Kashiwazaki [a], Toshikazu Bando [a,*], Ken-ichi Shinohara [a], Masafumi Minoshima [a], Hana Kumamoto [a], Shigeki Nishijima [a], Hiroshi Sugiyama [a,b,*]

[a] Department of Chemistry, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo, Kyoto 606-8502, Japan
[b] Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

ARTICLE INFO

Article history:
Received 27 January 2010
Revised 4 March 2010
Accepted 5 March 2010
Available online 10 March 2010

Keywords:
PI polyamide
Alkylating agent
Telomere
Chlorambucil
Antitumor agent

ABSTRACT

We designed and synthesized human telomere alkylating *N*-methylpyrrole-*N*-methylimidazole (PI) polyamide conjugates (**1**–**6**). The C-type conjugates **1**–**3** possessed a chlorambucil moiety at the C terminus, whereas the N-type conjugates **4**–**6** had one of these moieties at the N terminus. The DNA alkylating activity of these conjugates was evaluated by high-resolution denaturing polyacrylamide gel electrophoresis using a 220 bp DNA fragment containing the human telomere repeat sequence 5′-$(GGGTTA)_4$-3′/5′-$(TAACCC)_4$-3′. C-type conjugates are designed to alkylate the G-rich-strand-containing 5′-GGGTTA-3′ and N-type conjugates were designed to alkylate the complementary C-rich strand-containing 5′-TAACCC-3′ sequence. The difference between conjugates **1**–**3** and **4**–**6** lies in the linker region between the polyamide moiety and chlorambucil. Conjugates **1** and **4** efficiently alkylated the 5′-GGTTAGGGTTA-3′ and 5′-CCCTAACCCTAA-3′ sequences, respectively, by recognizing 11 bp in the presence of distamycin A (Dist), in a heterotrimeric manner: one long alkylating polyamide conjugate (**1**–**6**) and two short partners (Dist).

1. Introduction

Minor groove-binding *N*-methylpyrrole-*N*-methylimidazole (PI) polyamides recognize each of the four Watson–Crick base pairs uniquely. The binding rule of PI polyamides is the recognition of a GC base pair by antiparallel pairing of I opposite P (I/P), whereas a P/P pair recognizes AT or TA base pairs.[1,2] This has attracted considerable attention from scientists involved in DNA chemistry,[3–6] medical sciences,[7,8] and nanostructure.[9,10] Among these applications of PI polyamides, we vigorously developed various sequence specific alkylating agents by conjugating PI polyamides with alkylating moieties.[3,11–16] The human telomere is one of the targets of the alkylation mediated by PI polyamide conjugates. Telomeres[17] exist at the ends of eukaryotic chromosomes and protect them from DNA repair and degradation. The sequence of human telomere consists of repeats of 5′-$(GGGTTA)_n$-3′/5′-$(TAACCC)_n$-3′. Telomeres are a priority research area because of their association with aging[18] and various diseases, including cancer.[19] Because of end replication problems, human and mouse telomeres lose terminal sequences at a rate of 50–150 bp/end/cell division.[20] However, in most malignant cells (85–90%), the maintenance of telomeres is achieved via the upregulation of the expression of telomerase, which extends telomeres using their 3′ single-stranded overhangs.[21] Telomerase is essential for the proliferation of cancer cells; thus, agents such as telomestatin[22] and TMPyP4[23] that target telomeres and telomerase have attracted interest for the development of anticancer drugs. We tried to achieve this goal by alkylating telomeres.[14–16]

Recently, a heterotrimeric design led to the alkylation of the adenines in the telomeric sequence 5′-GGTTAGGGTT**A**-3′[14] (Fig. 1). *seco*-CBI was used as an alkylating moiety and indole was used as a linker. Although high reactivity and site specificity were observed, we obtained low yields in the reaction of the final coupling of polyamide with the indole-*seco*-CBI unit. In contrast, chlorambucil PI polyamide conjugates are easier to synthesize, as (i) chlorambucil is commercially available, (ii) coupling reactions are completed within 1 h, (iii) there are few byproducts, and (iv) reagents are removed by washing with ethyl acetate. Herein, we describe the results of the evaluation of the alkylating ability of a chlorambucil-PI polyamide conjugate and of the optimal linker for C-terminal chlorambucil and for N-terminal chlorambucil. These investigations unraveled the relationships between the linker and alkylating profiles successfully.

Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; HCTU, 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

* Corresponding author. Tel.: +81 75 753 4002; fax: +81 75 753 3670.
E-mail address: hs@kuchem.kyoto-u.ac.jp (H. Sugiyama).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.03.011

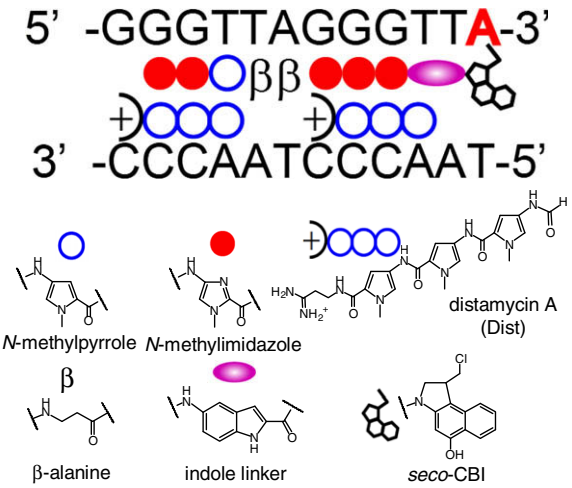

Figure 1. Heterotrimeric design used for the alkylation of human telomere repeat sequences reported previously.[14]

2. Results and discussion

2.1. Molecular design and syntheses

PI polyamide moieties of **1–6** on CLEAR-Acid Resin were synthesized using Fmoc solid-phase synthesis and were cleaved with 3,3′-diamino-*N*-methyl-dipropylamine, 1,7-diaminoheptane, and 1,3-diaminopropoane, for **1**, **2**, and **3**, respectively, and *N*,*N*-dimethylamino-1,3-propanediamine, for **4–6**. Subsequently, chlorambucil was coupled with amino groups using PyBOP and DIEA, which was used as a base. The target products were used for the evaluation of alkylating activity after HPLC purification. The structures of the PI polyamide conjugates **1**–**6** are shown in Figure 2.

2.2. DNA alkylating activity of conjugates 1–6 in the presence and absence of Dist

2.2.1. Alkylation of the G-rich strand

The DNA alkylating activity of conjugates **1–6** was evaluated in the presence and absence of Dist as a partner, using polyacrylamide gel electrophoresis (PAGE) analysis. Alkylation was carried out at rt for 24 h, followed by quenching by addition of calf thymus DNA. The samples were heated at 95 °C under neutral conditions for 5 min. Under these heating conditions, all the N3 purines at the alkylated sites in the DNA fragment produced cleavage bands quantitatively on the gel.

Figure 3 shows the alkylation of the G-rich strand. Conjugate **1** efficiently alkylated 5′-GGTTAGGGTT**A**-3′ (Fig. 3, lanes 2 and 3, sites 1–3), which is three bases away from the C terminus of the polyamide moiety. Actually, alkylation bands of G-rich strand did not show the same mobility as the corresponding sequencing bands. For example, the band at site 1 (Fig. 3) seems to indicate 5′-AGTGAGGGTT**A**-3′. This shift (~6 bp) might be explained as following: the sample DNA folds into a higher-order conformation presumably involving this GC-rich, palindromic sequence 5′-CGGCCGCCATGGCGGCCG-3′ at the upstream of the alkylation sites (see Section 4.4. for the whole sequence), which leads to higher mobility. In contrast, sequencing reaction used 7-deaza dGTP in stead of dGTP for circumventing compressions. This structural difference of G unit could induce that kind of mobility discordance, and this observation is not the first example.[14,16]

Conjugate **2**, which did not alkylate (Fig. 3, lanes 4 and 5), was an analog that contains a linker of almost the same length as that of conjugate **1**, but in which the *N*-methyl of the linker of conjugate **1** was replaced by methylene to evaluate the electrostatic effect of the protonated amine. The difference between conjugates **1** and **2** indicates that this positive charge contributes to the DNA binding. Conjugate **3** was another analog with a linker (propylene) that was half the size of that of conjugate **1**. Conjugate **3** showed weak alkylation bands in the telomeric region (Fig. 3, lane 6). In addition, the ethylene and butylene linker analogs yielded a similar weak alkylation of telomere sequences (data not shown). These observations suggest one possible rule regarding linker size and the disposition of the protonated amine, that is, the introduction of a positive charge is a factor that is more influential than the length of the linker when designing polyamide conjugates with chlorambucil at the C terminus. This little dependency of the length of the linker might be attributed to its flexibility derived from a hydrocarbon chain.

Conjugate **5** also alkylated 5′-GGGTT**A**-3′ weakly. Unexpectedly, conjugate **5** alkylated site 4 more efficiently than it did those in the telomere repeat sequence. We propose a tentative binding mode, in which alkylation by chlorambucil occurs at a guanine (this alkylated guanine is cleaved thermally[24]); the N-terminal region (N-Chl-β-I-I-P-β-C) lies in the opposite orientation (normally, the N to C direction is parallel to 5′→3′), (which is energetically possible[25]); the other C-terminal polyamide moiety binds the DNA minor groove monomerically (this binding mode is also possible[26]). The other conjugates (**4** and **6**) did not alkylate any sites significantly. In addition, no significant bands of alkylation were observed in the absence of Dist (Fig. 3, lanes 14–19).

2.2.2. Alkylation of the C-rich strand

Next, the alkylating patterns of the complementary C-rich strand were investigated using PAGE analysis (Fig. 4). Conjugates that carried a chlorambucil moiety at the N terminus (**4–6**) alkylated 5′-CCCTAACCCT**AA**-3′. Specifically, conjugate **4** exhibited the highest alkylating activity. The alkylating sites were in the strand opposite to that bound closely by conjugates **4–6** and were located three or four bases away from the N-terminus of the polyamide moiety. These results indicate that N-type conjugates accept a broader range of linker lengths for DNA alkylation compared with C-type conjugates. Another difference between C-type and N-type conjugates was alkylation by the latter conjugate at site 4, presumably through partial monomeric binding at the C-terminal end (Fig. 3, conjugate **5**), and site 5 (Fig. 4, conjugates **4–6**). These results suggest that N-type conjugates have a lower sequence specificity, as they do not accompany the partner in the whole binding region.

C-type conjugates **1–3** did not alkylate the C-rich strand, which was in contrast with what was observed for the G-rich strand alkylation mediated by N-type conjugates (especially conjugate **5**). These results suggest that C-type conjugates are superior to N-type conjugates regarding sequence specificity. However, it is noteworthy that chlorambucil can be designed to alkylate the C-rich strand or the opposite polyamide-bound strand, which had not been possible using *seco*-CBI conjugates based on a heterotrimeric design.[14]

2.2.3. Alkylation of the G-/C-rich strand by optimized chlorambucil conjugates 1 and 4

Finally, we examined the dependence of the intensity of alkylation on the concentration of conjugates **1** and **4** (Fig. 5). Conjugates **1** and **4** alkylated the telomere sequence of the G- and C-rich strands, respectively. In addition, the alkylation reaction was detected at a concentration that was as low as 10 nM. However, it should be noted that conjugate **1** exhibited a sequence specificity that was lower than that of the conjugate of PI polyam-

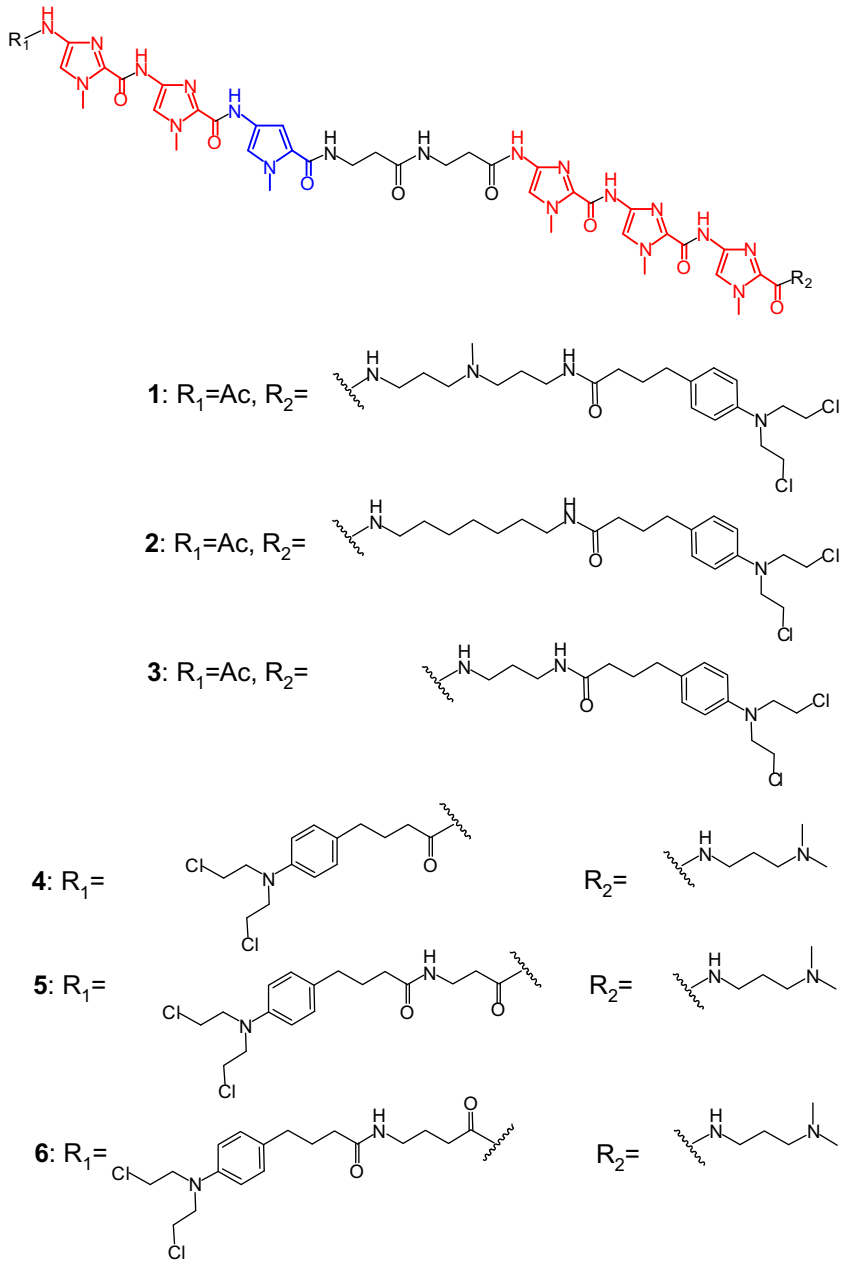

Figure 2. Structures of the PI polyamide conjugates **1**–**6**.

ide and indole-*seco*-CBI,[14] and that miscellaneous bands were observed at concentrations <500 nM (Fig. 5, lane 2).

3. Conclusions

We synthesized chlorambucil PI conjugate **1**–**6** and used high-resolution denaturing polyacrylamide gel electrophoresis to evaluate their alkylating activity toward the human telomere sequence, 5′-(GGGTTA)$_4$-3′/5′-(TAACCC)$_4$-3′, which were genetically inserted in 220 bp DNA fragment. PAGE analyses revealed that C-type and N-type conjugates alkylated both strands of telomere sequence efficiently. In addition, the linkers at the N terminus were variably acceptable. Sequence specificity was enhanced in C-type conjugates compared with N-type conjugates. Although the sequence specificity of PI-indole-*seco*-CBI conjugates was the best, chlorambucil conjugates were easier to synthesize and allowed the targeting of both strands (the polyamide-bound side by C-type conjugates and the opposite side by N-type conjugates). These findings will contribute to the development of alkylating PI-polyamide chlorambucil conjugates.

4. Experimental

4.1. General

Reagents and solvents were purchased from standard suppliers and used without further purification.

^{1}H 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. An API 150 (PESCIEX) and BioTOF II (Bruker Daltonics) mass spectrometer were used for analyses by Electrospray ionization mass spectrometry (ESIMS) and electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS), respectively. PCR amplification was carried out with an iCycler (BIO-RAD). Taq DNA polymerase

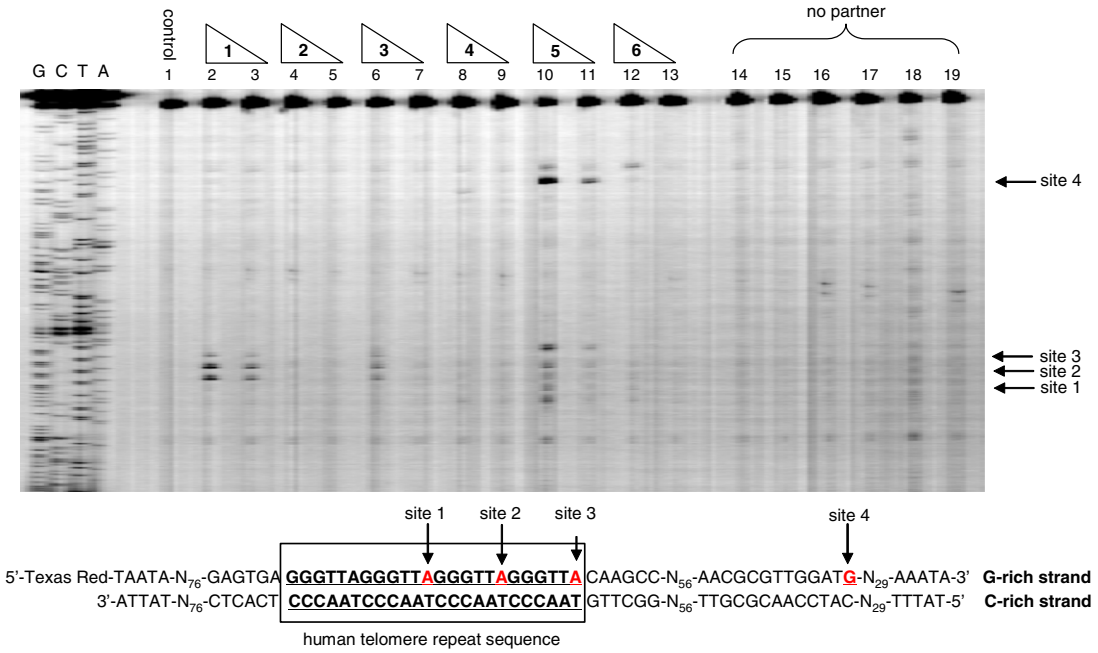

Figure 3. Thermally induced strand cleavage of the 5′ Texas Red-labeled 220 bp DNA fragment (6 nM) containing 5′-$(GGGTTA)_4$-3′ by conjugates **1**–**6** in the presence and absence of Dist at 23 °C for 24 h. Lane 1, DNA control; lanes 2–13, 100 nM of Dist and 200 or 50 nM of **1** (lanes 2 and 3), **2** (lanes 4 and 5), **3** (lanes 6 and 7), **4** (lanes 8 and 9), **5** (lanes 10 and 11), and **6** (lanes 12 and 13); lanes 14–19, 200 nM of **1**, **2**, **3**, **4**, **5,** or **6**.

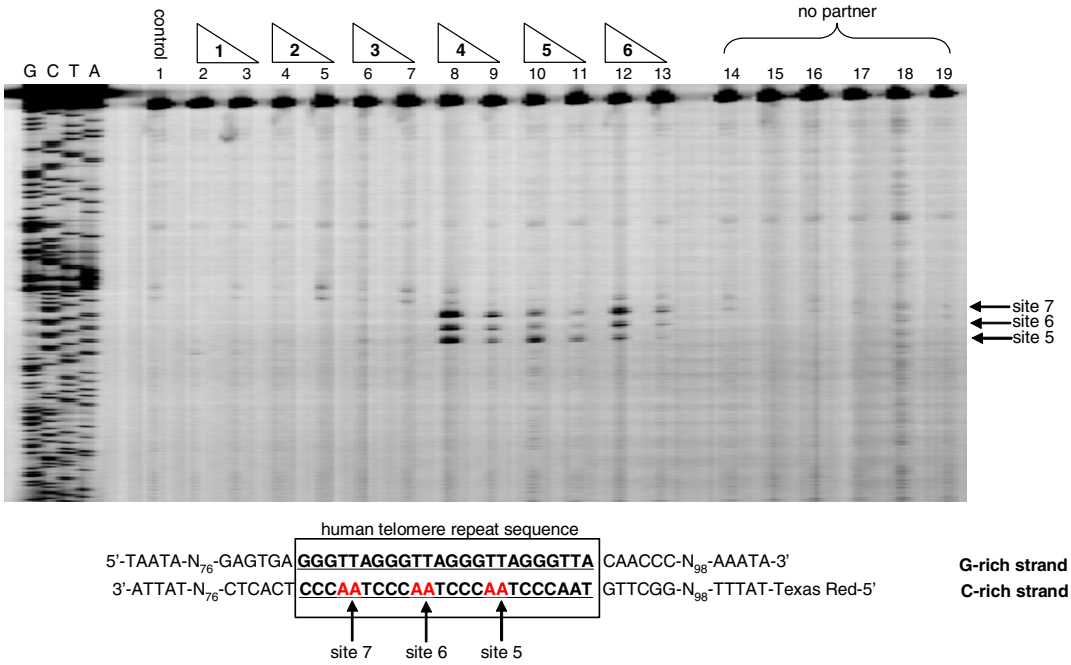

Figure 4. Thermally induced strand cleavage of the 5′ Texas Red-labeled 220 bp DNA fragment (6 nM) containing 5′-$(TAACCC)_4$-3′ by conjugates **1–6** in the presence and absence of Dist at 23 °C for 24 h. Lane 1, DNA control; lanes 2–13, 100 nM of Dist and 200 or 50 nM of **1** (lanes 2 and 3), **2** (lanes 4 and 5), **3** (lanes 6 and 7), **4** (lanes 8 and 9), **5** (lanes 10 and 11), and **6** (lanes 12 and 13); lanes 14–19, 200 nM of **1**, **2**, **3**, **4**, **5,** or **6**.

was purchased from Takara Co.; the Thermo Sequence core sequencing kit and loading dye (formamide with New fuchsin) were from Amersham Co. Ltd; 50% Long Ranger gel solution was from FMC bioproducts. CLEAR-Acid Resin (100–200 mesh) were purchased from PEPTIDES international. Distamycin A hydrochloride form *Streptomyces distallicus* was purchased from Sigma–Aldrich.

4.2. Solid-phase synthesis of PI polyamides

4.2.1. Preparation of Fmoc-*N*-methylimidazole-loading CLEAR-Acid Resin

Fmoc-*N*-methylimidazole-CLEAR-Acid Resin was prepared. CLEAR-Acid Resin (0.38 mmol/g, 3 g) was swelled in DMF 10 mL for 30 min. The following compounds were mixed in another tube:

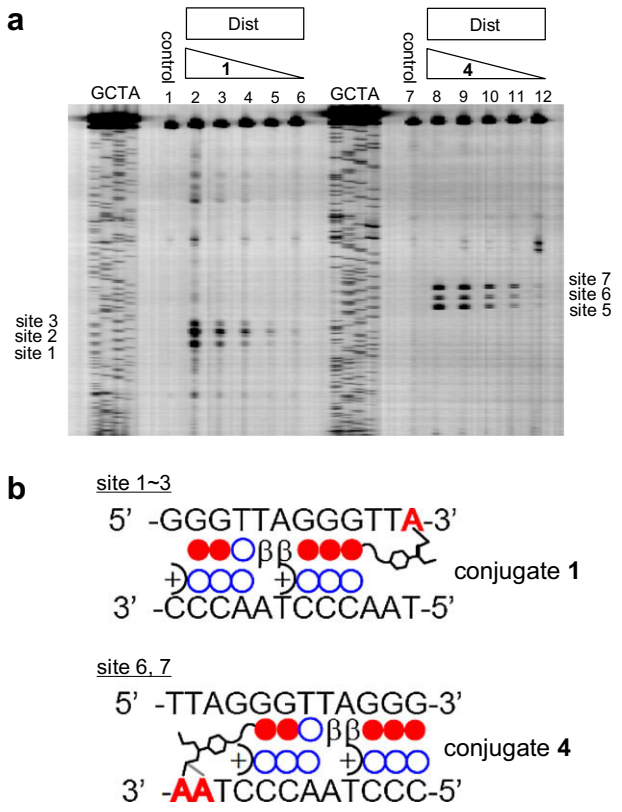

Figure 5. (a) Thermally induced strand cleavage of the 5 Texas Red-labeled 220 bp DNA fragment (6 nM) containing 5-$(GGGTTA)_4$-3 by conjugate **1** (lanes 1–6) and containing 5-$(TAACCC)_4$-3 by conjugate **4** (lanes 7–12) at 23 °C for 24 h. Lane 1, DNA control; lanes 2–6, 500, 200, 100, 50, or 10 nM of **1** in the presence of 100 nM of Dist; lanes 7–12, 500, 200, 100, 50, or 10 nM of **4** in the presence of 100 nM of Dist. (b) Schematic representation of the sequence specific alkylation of the telomeric sequence by conjugate **1** in the presence of Dist. The letter A in red indicates the sites of adenine N3 alkylation.

FmocNH-imidazole-CO_2H (1.22 g, 3.4 mmol, 3.0 equiv), MSNT (1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole) (993 mg, 3.4 mmol, 3.0 equiv) and *N*-methylimidazole (712 μL, 8.9 mmol, 8.0 equiv), CH_2Cl_2 3 mL, DMF 2 mL. The cocktail was added to the tube containing the previously swelled resin. DMF and *N*-methylimidazole can be added as occasion demands but it is not necessary to get transparent solution. These coupling agents with resin were shaken overnight (~12 h). The resultant suspended solution was removed and the resin was washed with DMF, MeOH and CH_2Cl_2, and dried. The non-coupled sites on the resin was capped with Ac_2O (472 μL, 5.0 mmol, 5.0 equiv) and DMAP (*N*,*N*-dimethylaminopyridine) (13.2 mg, 0.10 mmol, 0.10 equiv), and incubated at rt for 1 h. Subsequently, the resin was washed with DMF and CH_2Cl_2, and dried.

4.2.2. Quantification of the Fmoc-*N*-methylimidazole on CLEAR-Acid Resin

To the loading resin (ca. 5 mg) was added piperidine (500 μL) and MeOH (500 μL), and 10 min after, the absorbance of the peak around 310 nm was measured using Nanodrop. The value of loaded Fmoc-*N*-methylimidazole was estimated by calculating the concentration of the leaving 9-methylene-9*H*-fluorene. The value 7800 cm^{-1} M^{-1} was used as its molar extinction coefficient.

4.2.3. Fmoc solid-phase protocol

Fmoc-*N*-methylimidazole-loading CLEAR-Acid Resin (equal to 100 μmol of *N*-methylimidzole) was swelled with DMF (3 mL) and 20% piperidine solution (2 mL) in DMF to deprotect Fmoc group for 20 min.

(i) Coupling: The coupling agent was prepared beforehand: 4.0 equiv (400 μmol) of HCTU and 4.0 equiv (400 μmol) of each monomer unit. To the resin in DMF (2 mL) was added coupling agent dissolved in DMF (800 μL) and DIEA (70 μL). The vessel was shaken normally for 1 h. The mixture was filtered and washed meticulously. (ii) Capping (optional): To the resin was added DMF (3 mL) and Ac_2O (60 μL) (and DMAP). 30 min after, the resin was washed with DMF and CH_2Cl_2. (iii) Deprotection: To the resin was added DMF (3 mL) and 20% piperidine solution (2 mL) in DMF, and occasionally the UV–vis spectrum was measured after 10 min to confirm successful coupling of the new monomer unit. This cycle was repeated until the final unit was coupled. For compounds **1**–**3**, the last unit was deprotected and acetylated. For compounds **4**–**6**, the last unit was deprotected and the amino group was exposed.

4.3. Cleavage and coupling reaction after solid-phase synthesis

4.3.1. AcIIP-β-β-III-NH$(CH_2)_3$-NMe-$(CH_2)_3$-NH-Chl (1)

AcIIP-β-β-III-CLEAR-Acid Resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol (vide ante). Subsequently, the resin (80 mg) was treated with 3,3′-diamino-*N*-methyl-dipropylamine (500 μL) at 55 °C for 9 h. The solution was evaporated and dissolved in CH_2Cl_2, and the target product was deposited by addition of the solution into Et_2O. White powder (AcIIP-β-β-III-NH$(CH_2)_3$-NMe-$(CH_2)_3$-NH_2) was obtained (43.5 mg, 41 μmol). ESI-TOFMS *m/z* calcd for $C_{46}H_{63}N_{22}O_9$ $[M+H]^+$ 1067.51, found 1067.56. For the next coupling, 10 mg of the white powder was dissolved in DMF (100 μL), to which PyBOP (20 mg, 38 μmol, 4.0 equiv), DIEA (6.6 μL, 38 μmol, 4.0 equiv) and chlorambucil (12 mg, 38 μmol, 4.0 equiv) was added, and stirred at rt for 1 h. After washing with EtOAc and subsequent vacuum evaporation, the resultant was purified with HPLC and a white powder was obtained (2.3 mg, 1.7 μmol, 18%). ESI-TOFMS *m/z* calcd for $C_{60}H_{80}Cl_2N_{23}O_{10}$ $[M+H]^+$ 1352.58, found 1352.63. ^1H NMR (400 MHz, DMSO-d_6) 10.39 (s, 1H; I/P-amide), 10.28 (s, 1H; I/P-amide), 10.25 (s, 1H; I/P-amide), 9.59 (s, 1H; I/P-amide), 9.55 (s, 1H; I/P-amide), 9.31 (s, 1H; I/P-amide), 8.47 (t, *J* = 5.2 Hz, 1H; amide), 8.01 (m, 2H; amide), 7.92 (t, *J* = 5.2 Hz, 1H; amide), 7.64 (s, 1H; I-H), 7.55 (s, 1H; I-H), 7.53 (s, 1H; I-H), 7.52 (s, 1H; I-H), 7.50 (s, 1H; I-H), 7.22 (s, 1H; P-α-H), 6.99 (d, *J* = 8.8 Hz, 2H; Ph-H), 6.94 (s, 1H; P-β-H), 6.64 (d, *J* = 8.8 Hz, 2H; Ph-H), 4.01 (s, 3H; I-NMe), 3.99 (s, 3H; I-NMe), 3.97 (s, 3H; I-NMe), 3.97 (s, 3H; I-NMe), 3.96 (s, 3H; I-NMe), 3.80 (s, 3H; P-NMe), 3.68 (s, 8H; chl-methylene), 3.32 (s, 3H; diamine-NMe), 3.32 (m, 2H; methylene), 3.09 (m, 4H; methylene), 2.75 (m, 4H; methylene), 2.50 (m, 2H; methylene), 2.42 (m, 2H; methylene), 2.33 (m, 2H; methylene), 2.06 (m, 2H; methylene), 2.01 (s, 3H; Ac), 1.87 (m, 2H; methylene), 1.74 (m, 4H; methylene)

4.3.2. AcIIP-β-β-III-NH$(CH_2)_7$NH-Chl (2)

AcIIP-β-β-III-CLEAR-Acid Resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol (vide ante). Subsequently, the resin (80 mg) was treated with 1,7-diaminoheptane (500 μL) at 55 °C for 9 h. The solution was evaporated and dissolved in CH_2Cl_2, and the target product was deposited by addition of the solution into Et_2O. White powder was obtained. See Section 4.3.1. for the procedure of the next coupling of this amine and chlorambucil. ESI-TOFMS *m/z* calcd for $C_{60}H_{79}Cl_2N_{22}O_{10}$ $[M+H]^+$ 1337.57, found 1337.50.

4.3.3. AcIIP-β-β-III-NH$(CH_2)_3$NH-Chl (3)

AcIIP-β-β-III-CLEAR-Acid Resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol (vide ante). Subsequently,

the resin (80 mg) was treated with 1,3-diaminopropoane (500 μL) at 55 °C for 9 h. The solution was evaporated and dissolved in CH_2Cl_2, and the target product was deposited by addition of the solution into Et_2O. White powder was obtained. See Section 4.3.1. for the procedure of the next coupling of this amine and chlorambucil. ESI-TOFMS *m/z* calcd for $C_{56}H_{71}Cl_2N_{22}O_{10}$ $[M+H]^+$ 1281.51, found 1281.51.

4.3.4. Chl-IIP-β-β-III-NH(CH_2)$_3$NMe$_2$ (4)

H_2N-IIP-β-β-III-CLEAR-Acid Resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol (vide ante). Subsequently, the resin (80 mg) was treated with *N*,*N*-dimethyl-1,3-propanediamine (500 μL) at 55 °C for 9 h. The solution was evaporated and dissolved in CH_2Cl_2, and the target product was deposited by addition of the solution into Et_2O. White powder (24.2 mg, 67%) was obtained. ESI-TOFMS *m/z* calcd for $C_{42}H_{56}N_{21}O_8$ $[M+H]^+$ 982.46, found 982.50. See Section 4.3.1. for the procedure of the next coupling of this amine and chlorambucil. 5.0 mg of the starting material was converted to compound **4** (1.6 mg, 24% after HPLC purification). ESI-TOFMS *m/z* calcd for $C_{56}H_{73}Cl_2N_{22}O_9$ $[M+H]^+$ 1267.53, found 1267.63. ^{1}H NMR (600 MHz, DMSO-d_6) 10.41 (s, 1H; I/P-amide), 10.25 (s, 1H; I/P-amide), 10.25 (s, 1H; I/P-amide),9.60 (s, 2H; I/P-amide), 9.32 (s, 1H; I/P-amide), 8.36 (t, *J* = 6.0 Hz, 1H; β-alanine-amide), 8.01 (t, *J* = 6.0 Hz, 1H; β-alanine-amide), 8.01 (t, *J* = 6.0 Hz, 1H; Dp-amide), 7.63 (s, 1H; I-H), 7.55 (s, 1H; I-H), 7.53 (s, 1H; I-H), 7.51 (s, 2H; I-H), 7.22 (s, 1H; P-α-H), 7.04 (d, *J* = 8.2 Hz, 2H; Ph-H), 6.94 (s, 1H; P-β-H), 6.67 (d, *J* = 8.2 Hz, 2H; Ph-H), 4.00 (s, 3H; I-NMe), 3.99 (s, 3H; I-NMe), 3.97 (s, 3H; I-NMe), 3.97 (s, 3H; I-NMe), 3.95 (s, 3H, I-NMe), 3.80 (s, 3H; P-NMe), 3.69 (s, 8H; chl-methylene), 3.36 (s, 6H; Dp-NMe), 3.35 (m, 2H; methylene), 3.32 (m, 2H; methylene), 3.25 (m, 2H; methylene), 2.50 (m, 2H; methylene), 2.50 (m, 2H; methylene), 2.33 (m, 4H; methylene), 2.29 (br, 2H; methylene), 1.81 (m, 2H; methylene), 1.68 (m, 2H; methylene).

4.3.5. Chl-β-IIP-β-β-III-NH(CH_2)$_3$NMe$_2$ (5)

H_2N-β-IIP-β-β-III-CLEAR-Acid Resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol (vide ante). Subsequently, the resin (80 mg) was treated with *N*,*N*-dimethyl-1,3-propanediamine (500 μL) at 55 °C for 9 h. The solution was evaporated and dissolved in CH_2Cl_2, and the target product was deposited by addition of the solution into Et_2O. White powder was obtained. See Section 4.3.1. for the procedure of the next coupling of this amine and chlorambucil. ESI-TOFMS *m/z* calcd for $C_{59}H_{78}Cl_2N_{23}O_{10}$ $[M+H]^+$ 1338.57, found 1338.59.

4.3.6. Chl-γ-IIP-β-β-III-NH(CH_2)$_3$NMe$_2$ (6)

H_2N-γ-IIP-β-β-III-CLEAR-Acid Resin was synthesized in a stepwise reaction by Fmoc solid-phase protocol (vide ante). Subsequently, the resin (80 mg) was treated with *N*,*N*-dimethyl-1,3-propanediamine (500 μL) at 55 °C for 9 h. The solution was evaporated and dissolved in CH_2Cl_2, and the target product was deposited by addition of the solution into Et_2O. White powder was obtained. See Section 4.3.1. for the procedure of the next coupling of this amine and chlorambucil. ESI-TOFMS *m/z* calcd for $C_{60}H_{80}Cl_2N_{23}O_{10}$ $[M+H]^+$ 1352.58, found 1352.67.

4.4. Preparation of 220 bp DNA fragment[16] and its sequence

All DNA fragments and primers were purchased from Proligo. Two DNA fragments were annealed in a final volume of 20 μL containing 50 μM of each strand (5′-GCAGAGTGAGGGTTAGGGTTAGGGTTAGGGTTACAAGCCCTCA-3′ and 5′-GAGGGCTTGTAACCCTAACCCTAACCCTAACCCTCACTCTGCA-3′), and ligated into pGEM-T Easy vector (Promega). *Escherichia coli* DH5α competent cells (TOYOBO) were transformed and cultured on a LB plate with 100 μg/mL ampicillin and 32 μg X-gal /400 μg IPTG overnight at 37 °C. White colonies were identified by colony PCR in 20 μL of the reaction mixtures containing 250 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 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 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 μg/mL ampicillin and cultured overnight at 37 °C. The plasmids were extracted using GenElutePlasmid miniprep kit (Sigma Aldrich). The sequence of 220 bp DNA fragment containing 4 repeats of 5′-GGGTTA-3′ region: 5′-TAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGCAGAGTGAGGGTTAGGGTTAGGGTTAGGGTTACAAGCCCTCAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAA ATA-3′.

4.5. Preparation of 5′-Texas Red-modified DNA fragment and polyacrylamide 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 SP6 promoter primer (5′-TATTTAGGTGACACTATAG-3′) and T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′) or that of T7 and 5′-Texas Red-labeled SP6 promoter primer and 1 ng of the telomere fragment inserted pGEM-T Easy vector. The fragment was purified by GenElute PCR cleanup kit (Sigma Aldrich). The 5′-Texas Red-labeled DNA fragments were alkylated by various concentrations alkylating polyamides in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at rt for 24 h. The reaction was quenched by the addition of calf thymus DNA (10 mg/mL, 1 μL) and heating at 95 °C for 5 min. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 5 μL of loading dye (formamide with fuchsin), heated at 95 °C for 20 min and the 1.0 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi SQ5500-E DNA Sequencer.

Acknowledgments

This work was supported by a Grant-in-Aid for Priority Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The authors acknowledge the support by the Global COE Program 'Integrated Materials Science' (#B-09).

References and notes

1. Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1996**, *382*, 559.
2. Dervan, P. B.; Edelson, B. S. *Curr. Opin. Struct. Biol.* **2003**, *13*, 284.
3. Bando, T.; Sugiyama, H. *Acc. Chem. Res.* **2006**, *39*, 935.
4. Harki, D. A.; Satyamurthy, N.; Stout, D. B.; Phelps, M. E.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 13039.
5. Chenoweth, D. M.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 13175.
6. Westrate, L.; Mackay, H.; Brown, T.; Nguyen, B.; Kluza, J.; Wilson, W. D.; Lee, M.; Hartley, J. A. *Biochemistry* **2009**, *48*, 5679.
7. Chen, M.; Matsuda, H.; Wang, L.; Watanabe, T.; Kimura, M. T.; Igarashi, J.; Wang, X.; Sakimoto, T.; Fukuda, N.; Sawa, M.; Nagase, H. *Mol. Ther.* **2010**, *18*, 519.
8. Fukuda, N.; Tahira, Y.; Matsuda, H.; Matsumoto, K. *J. Nephrol.* **2009**, *22*, 708.
9. Schmidt, T. L.; Heckel, A. *Small* **2009**, *5*, 1517.
10. Cohen, J. D.; Sadowski, J. P.; Dervan, P. B. *Angew. Chem., Int. Ed.* **2007**, *46*, 7956.
11. Minoshima, M.; Chou, J. C.; Lefebvre, S.; Bando, T.; Shinohara, K.; Gottesfeld, J. M.; Sugiyama, H. *Bioorg. Med. Chem.* **2010**, *18*, 168.
12. Minoshima, M.; Bando, T.; Shinohara, K.; Kashiwazaki, G.; Nishijima, S.; Sugiyama, H. *Bioorg. Med. Chem.* **2010**, *18*, 1236.
13. Sasaki, S.; Bando, T.; Minoshima, M.; Shinohara, K.; Sugiyama, H. *Chem. Eur. J.* **2008**, *14*, 864.

14. Kashiwazaki, G.; Bando, T.; Shinohara, K.; Minoshima, M.; Nishijima, S.; Sugiyama, H. *Bioorg. Med. Chem.* **2009**, *17*, 1393.
15. Takahashi, R.; Bando, T.; Sugiyama, H. *Bioorg. Med. Chem.* **2003**, *11*, 2503.
16. Sasaki, S.; Bando, T.; Minoshima, M.; Shimizu, T.; Shinohara, K.; Takaoka, T.; Sugiyama, H. *J. Am. Chem. Soc.* **2006**, *128*, 12162.
17. Blackburn, E. H. *Cell* **2001**, *106*, 661.
18. Aubert, G.; Lansdorp, P. M. *Physiol. Rev.* **2008**, *88*, 557.
19. Lansdorp, P. M. *EMBO J.* **2009**, *28*, 2532.
20. Smogorzewska, A.; Lange, T. d. *Annu. Rev. Biochem.* **2004**, *73*, 177.
21. Olaussen, K. A.; Dubrana, K.; Domont, J.; Spano, J.-P.; Sabatier, L.; Soria, J.-C. *Crit. Rev. Oncol. Hematol.* **2005**, *57*, 191.
22. Shin-ya, K.; Wierzba, K.; Matsuo, K.; Ohtani, T.; Yamada, Y.; Furihata, K.; Hayakawa, Y.; Seto, H. *J. Am. Chem. Soc.* **2001**, *123*, 1262.
23. Wheelhouse, R. T.; Sun, D.; Han, H.; Han, F. X.; Hurley, L. H. *J. Am. Chem. Soc.* **1998**, *120*, 3261.
24. Wurtz, N. R.; Dervan, P. B. *Chem. Biol.* **2000**, *7*, 153.
25. White, S.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 8756.
26. Kopka, M. L.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1376.