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Sequence-specific DNA alkylation and transcriptional inhibition by long-chain hairpin pyrrole–imidazole polyamide–chlorambucil conjugates targeting CAG/CTG trinucleotide repeats

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Sequence-specific DNA alkylation and transcriptional inhibition by long-chain hairpin pyrrole–imidazole polyamide–chlorambucil conjugates targeting CAG/CTG trinucleotide repeats

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\textbf{ABSTRACT}

Introducing novel building blocks to solid-phase peptide synthesis, we readily synthesized long-chain hairpin pyrrole-imidazole (PI) polyamides–chlorambucil conjugates \textsuperscript{3} and \textsuperscript{4} via the introduction of an amino group into a GABA (γ-turn) contained in 3, to target CAG/CTG repeat sequences, which are associated with various hereditary disorders. A high-resolution denaturing polyacrylamide sequencing gel revealed sequence-specific alkylation both strands at the N3 of adenines or guanines in CAG/CTG repeats by conjugates \textsuperscript{3} and \textsuperscript{4}, with 11 bp recognition. \textit{In vitro} transcription assays using conjugate \textsuperscript{4} revealed that specific alkylation inhibited the progression of RNA polymerase at the alkylation sites. Chiral substitution of the γ-turn with an amino group resulted in higher binding affinity observed in SPR assays. These assays suggest that conjugates \textsuperscript{4} with 11 bp recognition has the potential to cause specific DNA damage and transcriptional inhibition at the alkylation sites.

1. Introduction

Pyrrole–imidazole polyamides (PI polyamides) are ligands that bind to the DNA minor groove with high affinity and sequence specificity. These contain N-methylpyrrole (P) and N-methylimidazole (I), and recognize each of the four Watson–Crick base pairs uniquely.\textsuperscript{1,2} The binding rule of PI polyamides, termed Dervan’s rule, is the recognition of a G/C base pair by antiparallel pairing of an I/P, whereas a P/P pair recognizes an A/T or a T/A base pair. A β-alanine/β-alanine (β/β) pair reads an A/T or a T/A base pair in the same way as a P/P pair does. Recently, the introduction of PI polyamides into DNA-alkylating agents has attracted attention because of their sequence-specific alkylation at target sites.\textsuperscript{4}

Trinucleotide repeat sequences exist in some regions of genomic DNA, and the expansion of the repeat sequences often causes neurological disorders.\textsuperscript{5,6} For example, the expansion of CAG trinucleotide repeats within the first exon of the Huntington (HTT) gene (from 36 to >100 repeats) yields a full-length HTT protein harboring expanded polyglutamine tracts, which gain some functions, lead to the selective disruption of neuronal protein function, and ultimately, cause Huntington’s disease.\textsuperscript{7,8} In myotonic dystrophy type 1 (DM1) and Huntington’s disease-like 2, which are associated with CTG repeat sequences located in the 3′ UTR of genes, transcriptional products containing excess CUG repeats accumulate within the nucleus and form a 1 bp mismatch-containing hairpin structure, bind to proteins such as splicing regulators, cause inactivation of their alternative pre-mRNA splicing, and lead to the pathogenesis of each of these diseases.\textsuperscript{9–11} Surprisingly, these trinucleotide repeat sequences have a tendency to expand as a result of the formation of a hairpin-like structure containing a d(CAG/CAG) or d(CTG/CTG) 1 bp mismatch internal loop motif, by reannealing during DNA replication, repair, and recombination. The expansion leads to a decreased age of onset and increased severity of the diseases in successive generations.\textsuperscript{12} Several mechanisms to explain this characteristic inheritance have been proposed; however, a genuine one remains unknown. Recently, studies on antisense oligonucleotides that target the internal loop motifs located in the hairpin structure have been reported.\textsuperscript{13} Moreover, Disney and coworkers reported previously that covalent adduct formation

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sequences, and their length of sequence recognition was elongated by introducing novel building blocks used in Fmoc solid-phase peptide synthesis (SPPS).15 The activities of the compounds obtained were investigated in terms of DNA sequence-specific alkylation, transcriptional inhibition, and dynamics of the interaction with the target sequence.

2. Results and discussion

2.1. Molecular design and synthesis

We rationally designed three types of PI polyamide–Chl conjugates (1–4) to target CAG/CTG repeat sequences with different lengths or structures (Figure 1).26,27 Chl is a nitrogen mustard alkylating agent that belongs to the group of anticancer drugs and has been used widely as a first line of treatment for chronic lymphocytic leukemia. Its efficacy has been confirmed at the clinical level.26,29 The potency of hairpin PI polyamide–Chl conjugates has been investigated in terms of chemical and biological activity.30,31 Hartley and colleagues previously demonstrated preferential alkylation at the N3 of guanine or adenine on 5′–TTTTGG–3′ or 5′–TTTTGA–3′ sequences, respectively, by tripyrrole conjugated with BAM,32 which is one of the nitrogen mustards. In addition, it has been reported that Chl is more cytotoxic than BAM in the human colon cancer LS174T and leukemic K562 cell lines.33

From these reports, we inferred that hairpin PI polyamide–Chl conjugates, the specific alkylation of which at the N3 of guanines has never been investigated, were appropriate for targeting AG-rich sequences as alkylation agents. Moreover, the flexibility of Chl within the structure may allow reaching the opposite strand.34 To promote this function and the alkylation at guanines or adenines of both strands by Chl moieties, β-alanine and subsequently 3,3′-diamino-N-methylidipropylamine (DMMDPA; as a relatively long linker, to obtain additional flexibility) were added to the C terminus. We also designed PI polyamides to allow them to have symmetry and therefore the polyamides binds the match sequence in two orientations.35 This leads to an increase in the number of alkylation sites, which are placed on either side of the binding site. The appropriate positions of PI polyamide moieties were linked with β-alanine, which allows for optimal H-bond donor/acceptor interactions while retaining flexibility, resulting in an increase in overall affinity and specificity.35-38

Regarding the synthesis of long-chain hairpin PI polyamides, only monomeric or/dimeric units have been used in Fmoc SPPS to produce relatively long hairpin polyamides,39 therefore, their yields were worse because of the increase in the number of reaction steps. In particular, Fmoc SPPS had difficulty in coupling with a P after the introduction of a GABA (γ-turn), which functions to transform polyamides into hairpin structures that allow high affinity and specificity.35-40 To overcome these difficulties, we plan to synthesize FmocHN-Pγ-CO2H (5), Boc-P-Dab (FmocHN-P)-CO2H (6), and FmocHN-Pβ-L-CO2H (10) as building blocks of Fmoc SPPS in solution phase,39,41,42 in which time-consuming purifications are never required in any of the reaction steps (Scheme 1). All of them are washed and dried to obtain high yields were worse because of the increase in the number of reaction steps. In particular, Fmoc SPPS had difficulty in coupling with a P after the introduction of a GABA (γ-turn), which functions to transform polyamides into hairpin structures that allow high affinity and specificity.35-40 To overcome these difficulties, we plan to synthesize FmocHN-Pγ-CO2H (5), Boc-P-Dab (FmocHN-P)-CO2H (6), and FmocHN-Pβ-L-CO2H (10) as building blocks of Fmoc SPPS in solution phase,39,41,42 in which time-consuming purifications are never required in any of the reaction steps (Scheme 1). All of them are washed and dried to produce powder states that can then be subjected to Fmoc SPPS without further purification. Compound 5 and 6, which were used in Fmoc SPPS of polyamide 13 and 14, respectively (Scheme 2), played an important role in preventing the P-lacking products and could be prepared using only one step. As compound 10 was used to construct a core moiety of the parent polyamide in this study, its introduction into Fmoc SPPS, in addition to that of compound 5 or 6, enabled a great reduction in reaction steps (from 17 to eight steps). In the present study, we synthesized PI polyamides conjugated to a DNA-alkylating agent, chlorambucil (Chl), to target CAG/CTG repeat

Based on these reports, we hypothesized that the inhibition of transcripts at CAG/CTG repeat sequences that form Watson–Crick base pairs, via specific covalent addition of an alkylating agent possessing polyamides that recognizes CAG/CTG repeat sequences may prevent the subsequent occurrence of expanded polyglutamine tracts and the sequestration of expanded rCUG transcripts.18 Moreover, Sinden and coworkers reported that DNA damaging agents, such as alkylating agents, UV irradiation, and oxidation, increase the rate of triplet repeat deletion.19,20 These are effective approaches for genetic disorders, but have a very limited biological activity.35

Figure 1. A (a) Chemical structure of PI polyamide–chlorambucil (Chl) conjugates (1–4) targeting the CAG/CTG repeat sequences (b) Schematic representation of the binding models of conjugates 1–4 to the CAG/CTG repeat region via a dimer (conjugate 1) or hairpin (conjugate 2–4) conformation.

between expanded rCUG transcripts in the hairpin structure and moieties that target the r(CUG/CUG) mismatch internal loop motif improved DM1-associated pre-mRNA splicing defects.14 In addition, Nakatani et al. developed a small molecule that binds a hairpin d(CAG/CAG) region and flips cytidine nucleotides out.15 Despite those studies, to our knowledge, there are few reports of the targeting of Watson–Crick base pairs in CAG/CTG repeat regions, which are present even in disease-associated expanded CAG/CTG repeats. Our laboratory has developed polyamides with fluorescence dyes that detect CAG repeated sequences as a diagnostic tool.16,17

The activities of the compounds obtained were investigated in terms of DNA sequence-specific alkylation, transcriptional inhibition, and dynamics of the interaction with the target sequence.
anticipated that it was less difficult for such a long-chain polyamide in a hairpin structure to be formed into the open state, we performed a chiral substitution of the γ-turn by an amino group (to produce polyamide 15 and conjugate 4). The products obtained by programmable Fmoc SPPS introducing the building blocks were cleaved with DMDPA, to produce the amino PI polyamides 11–14 (Scheme 2). Conjugates 1–3 were produced via the PyBOP coupling with Chl. By conversion from 14, the PI polyamide–Chl conjugate 4 was synthesized via the deprotection of the Boc group by TFA-DCM in the last step. The diamino PI polyamide 15 was also prepared by acid deprotection for SPR assays. Conjugates 1–4 were purified by reversed-phase HPLC, and confirmed by ESI-TOF-MS.

2.2. DNA-alkylating activity of conjugates 1–4

First, The DNA-alkylating activities of conjugates 1–3 were evaluated using polyacrylamide gel electrophoresis (PAGE), to investigate the effects of the structure or recognition length of PI polyamides on specific alkylation within CAG/CTG repeat sequences.5–5′ Texas Red Red-labeled 214 bp DNA fragments including a (CAG/CTG) repeat sequence were prepared by transformation into pGEM-T Easy vectors, and subsequently by PCR amplification and purification (Figure S1). Alkylation was carried out at 23°C for 18 h, followed by quenching by the addition of calf thymus DNA. The samples were heated at 95°C under neutral conditions for 10 min. Under these conditions, all the N3 of purines at the alkylated sites in the DNA fragment produced cleavage bands quantitatively on the gel.

The results of the sequencing gel analysis of the DNA fragment treated with conjugates 1–3 are shown in Figure 2. Regarding the alkylation of the (CAG) repeat sequence (Figure 2a), no alkylation by conjugates 1 and 2 occurred at the sites of the CAG repeat, and alkylation by conjugate 1 occurred at site a, which can be explained by recognition of expanded state with 1 bp mismatch, instead of dsDNA recognition. Even at 10 times concentrations, conjugate 1 did not specifically alkylate the CAG repeat sequence (data not shown). At around 150 nanomolar concentrations, alkylation by conjugate 2 occurred within the CAG repeat, albeit with a specificity that was much lower than that of conjugate 3 (data not shown). However, we observed specific alkylation by conjugate 3 at sites of the target region with higher alkylation activities and sequence specificity at nanomolar concentrations (lanes 2–5), except alkylation at site b with 3 bp mismatch. Conversely, within the (CTG) repeat sequence (Figure 2b), we observed specific alkylation by both conjugates 2 and 3, except alkylation at site c and d with 3 bp mismatch by conjugate 2 and 3, respectively, and no alkylation by conjugate 1. These results indicate that, although 8 bp recognition through dimer (conjugate 1) or hairpin (conjugate 2) conformation is not sufficient, 11 bp recognition through hairpin conformation (conjugate 3) is required for sequence-specific alkylation in both strands including CAG/CTG repeat sequences.

Next, having established that, among conjugates 1–3, conjugate 3 is the most appropriate for targeting CAG/CTG repeat sequences, we used conjugates 3 and 4 to investigate DNA-alkylating activities for CAG/CTG repeat sequences in detail and the effect of chiral substitution at the α position of the γ-turn by an amino group on DNA-alkylating activities. The results of the sequencing gel analysis using a similar treatment to that mentioned above are shown in Figure 3. The (CAG/CTG) containing DNA fragment prepared in this study comprised the target region (5′–(CAG)3–CAA–3′/5′–TTG(CTG)–3′) for these conjugates (Figure 3c). In fact, specific alkylation by conjugates 3 and 4 was observed within the target region between sites 1 and 13, or sites 1′ and 15′, except non-specific alkylation (site b and site d) as also seen in Figure 2 (Figure 3). For the CTG repeat sequence (Figure 3b), both conjugates alkylated at the N3 position of the guanines in the CTG trinucleotides, particularly from the third CTG (site 6′) to the fifth
CTG trinucleotide (site 8') numbered from the 5' side. These sites are included in ones which can be alkylated by the conjugates through two binding modes (site 6' to 11'), thus showing the relative higher cleavage intensities (Figure 3c). In total, we observed 15 cleavage bands as a result of the N3 adenine or guanine alkylation, which corresponded to prospective alkylating sites within the target region (Figure 3b and c). Conversely, specific alkylation by conjugates 3 and 4 within the CAG repeat occurred similarly and produced 14 cleavage bands. As with the case for the CTG repeat, the cleavage bands with the relatively higher intensities (site 4 to 9) mostly correspond to the sites which can be alkylated by the conjugates through two binding modes (site 4 to 10). In addition, we observed that these cleavage bands on the gel were a little broader than those obtained for the CTG repeat, which suggests that both N3 adenines and their adjacent guanines in CAG trinucleotides were specifically alkylated by conjugates 3 and 4 (Figure 3a and c). We found that, although the effect of a chiral substitution on DNA-alkylating activities was not observed in this experimental system, rationally designed long-chain PI polyamide–Chl conjugates with a relatively long linker had the ability to alkylate both of the strands and increase the number of the alkylating sites by binding in the two orientations.

2.3. The effect of conjugate 4 on transcription

To examine the influence of alkylation by a PI polyamide–Chl conjugate on transcription, non-labelled DNA fragments including (a) a (CAG)$_{12}$ or (b) (CTG)$_{12}$ repeat (8 nM) by conjugates 1, 2, and 3 at 23°C for 18 h. These two DNA strands are complementary. Lane 1: DNA control; lanes 2–5: 2.5, 5, 10, and 20 nM of 3; lanes 6–9: 2.5, 5, 10, and 20 nM of 2; lanes 10–13: 2.5, 5, 10, and 20 nM of 1. (c) Schematic representation of the recognition and alkylating models of these conjugates. The arrows indicate the alkylating sites that were inferred from the sequencing gel analysis. Note that alkylation does not occur at the site indicated by ☆, as this band is also observed in lane 1 (control).
strands by conjugate 4 terminates transcriptional elongation, which is also supported by the fact that other retarded bands on the gels of Figure 4b and c amount to ones indicating non-specific alkylation on the gel of Figure 3b. Conversely, little appearance of retarded bands in lanes 6–8 shows that the parent polyamide 15 dose not inhibit the transcription. This result is consistent with the report that polyamides not conjugated with alkylating reagents dose not inhibit transcriptions by T7 RNA polymerase in the absence of histones.51

2.4. Binding properties of the parent polyamides to target hairpin DNA

To obtain additional information about the interaction of polyamides with the CAG/CTG repeat region, using SPR assays we evaluated and compared the binding affinities and kinetics parameters of the four parent polyamides 11–13 and 15 (which were purified on a reversed-phase flash column and confirmed by 1H-NMR and ESI-TOF-MS) for a biotinylated hairpin DNA that included the target region (5′–AGCAGCAGCA–3′/5′–
TGCTGCTGCT–3') (Figure 5a).

The solution of each polyamide in HBS buffer with 0.1% DMSO flowed on a sensor chip onto which the designed hairpin DNA was immobilized, and the interaction was visualized as a sensogram (Figure 5b–e). The values of $k_a$, $k_d$, and $K_D$ obtained by curve fitting are shown in Table 1. In terms of the influence of the recognition length over binding affinity, the results of this experiment showed that 12 had about 4 times higher binding ability, which means the lower $K_D$ values, than that of 13, mainly owing to the difference in the rates of association ($k_a$). One possible explanation for this observation is that it is more difficult for 13 to penetrate into the DNA minor groove of the target sequence with the closed state (hairpin conformation), because of its flexibility resulting from the long-chain. Another possibility of the stronger binding by 12 is that although this polyamide interacts with the DNA with the 1:1 binding mode, it has two matching positions in the target region. This facilitates the penetration of the polyamide, which is reflected in the $k_a$ value. Concerning the rates of dissociation ($k_d$), we observed that the extension of the recognition length results in a small decrease in the $k_d$ values. This indicates that the increase in the hydrogen bonds from 7 bp recognition does not affect the tendency to localize at the target region in the DNA minor groove, even with the introduction of the cationic amino substituent (polyamide 15). Conversely, comparing the binding affinity of 13 and 15 allowed us to investigate the effect of a chiral substitution on the $\gamma$-turn with an amino group, and revealed that 15 had about 5 times higher binding ability than that of 13. Particularly, we observed the difference in the $k_a$ values, suggesting that 15 is more likely to access the DNA with the cationic amino group positioned in the DNA minor groove to give better electrostatic attraction, retaining the forward hairpin folding of 15. Since Polyamide 11 was designed to bind to the target region with a dimer conformation, the sensogram obtained for 11 was fitted to 2:1 stoichiometry binding model that represents dynamics in forming the complexes with two polyamides bound side by side in the same region (see experimental section). As seen in Table 1, the value of $K_D$ (1.3 × 10$^{-8}$ M) is lower than that of $K_{D1}$ (6.1 × 10$^{-7}$ M), indicating the cooperative binding for the target region. In order to compare the other $K_D$ values that were obtained by fitting to the 1:1 binding model, $K_{D1}$ (1.3 × 10$^{-8}$ M) value was used, which is the value adjusted to a per bound molecule. As a result, the value for 11 (8.9 × 10$^{-8}$ M) was the highest of those in this experiment, which corresponded to lower alkylation activity and specificity.

3. Conclusion

We rationally designed and readily synthesized PI polyamide–Chl conjugates targeting CAG/CTG repeat sequences with different lengths or structures. In particular, by introducing the novel building blocks that were synthesized in solution phase to Fmoc SPPS, we prepared long-chain hairpin PI polyamides which otherwise would be difficult to synthesize. The sequencing gel analysis demonstrated that the long-chain hairpin PI polyamide conjugates showed sequence-specific alkylation at the N3 adenines or guanines in the CAG/CTG repeat sequence. In vitro transcription assays demonstrated that sequence-specific alkylation by a PI polyamide–Chl conjugate terminated the progression of RNA polymerase at the alkalying sites. The chiral substitution on the $\gamma$-turn with an amino group enhanced the binding affinity observed in SPR assays. These in vitro experiments revealed that conjugate 4 was most appropriate for targeting CAG/CTG repeat sequences in terms of chemical activity. The propensity of conjugate 4 to specifically alkylate both strands suggests the possibility of selective DNA damage, which thus may lead to the deletion of expanded CAG/CTG repeat sequences within the nucleus. Based on its property of the transcriptional inhibition by specific alkylation at both the CAG and CTG repeat regions, conjugate 4 may have potency of efficiently preventing the elongation of CAG and CTG repeat sequences and the accumulation of expanded rCUG repeat transcripts and polyglutamine tracts. Moreover, as reported previously, the concept that building blocks are prepared in solution phase to provide relatively long polyamides can be applied not only to this case, but also to various cases of targeting repeated sequences.

4. Experimental section

4.1. General
After the 20.40 mmol of formamide, 200 μL of H₂O, 300 μL of a 0.5 M EDTA 2Na in H₂O (Nacalai Tesque Inc.), and 2.5 mg of new fuchsin (Merck). In vitro transcription assays were carried out by in vitro Transcription T7 Kit (Takara Bio Inc.) and the transcripts were investigated on 10 % TBE-Urea Gels containing 7 M urea (Invitrogen). SPR assays were performed with a Biacore X system (GE Healthcare), and processing of data was carried out by using BIA evaluation, version 4.1. Chlorambucil (Chl) was purchased from Sigma, and BioHN-β-alanine, 3,3′-diamino-N-methyldipropylamine (DMDPA) and 10% Pd/C were from Aldrich. Fmoc-β-Wang resin (0.55 mmol/g) and O-(1H-6-Chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyloxonium hexafluorophosphate (HCTU) were purchased from Peptide International. FmocHN-P-CO₂H, FmocHN-1-CO₂H, O₂N-I-CoCl₂, N,N-dimethylformamide (DMF), 1-methyl-2-pyrrolidone (NMP), and piperidine were purchased from Wako, and Boc-D-Dab-OH was from Watanabe Chemical Ind., Ltd. PyBOP were purchased from Novabiochem. Diisopropylethylamine (DIEA), 4-amino-n-buteric carboxylic acid (GABA) were purchased from Nacalai Tesque, Inc. Trifluoroacetic acid (TFA) was purchased from Kanto Chemical Co., Inc. Dichloromethane (DCM) was purchased from Sasaki chemical co., Ltd. The other reagents and solvents were purchased from standard suppliers and used without further purification.

4.2. Syntheses of compound 8-10

4.2.1. FmocHN-P-γ-CO₂H (5)

Compound 5 was synthesized from FmocHN-P-CO₂H. To a solvent of DMF was added compound 5 (1.50 g, 4.1 mmol), HCTU (1.71 g, 4.1 mmol) and DIEA (1.44 mL, 8.3 mmol), and the mixture was stirred at room temperature for 15 min. The reaction mixture was added slowly to a solution of GABA (858 mg, 8.3 mmol) in 1.4 mL DIEA and 10 mL DMF, and it was stirred at room temperature for 3 h. After concentration, the oil residue was dissolved in the minimum amount of DCM, washed with diethyl ether. After the supernatant was removed, to the residue was added a large amount of water to afford the precipitation. It was filtered and then dried in vacuo to obtain 5 as a brown powder (1.73 g, 94%). The compound was used in the solid-phase synthesis without further purification. 1 H NMR (600 MHz, DMSO-d₆) δ 9.42 (s, 1H, NH), 8.31 (brs, 1H, NH), 7.90 (d, J = 7.1 Hz, 2H, CH), 7.73 (d, J = 7.5 Hz, 2H, CH), 7.42 (t, J = 7.5 Hz, 2H, CH), 7.34 (t, J = 7.6 Hz, 2H, CH), 6.85 (s, 1H, CH), 6.65 (s, 1H, CH), 4.40 (d, J = 6.8 Hz, 2H, CH), 4.27 (t, J = 6.8 Hz, 1H, CH), 3.76 (s, 3H, NCH₃), 3.14 (m, 2H, CH₂), 2.16 (brs, 2H, CH₂), 1.67 (m, 2H, CH₂), ESI-MS m/z calcd for C₂H₂N,O₂ [M+H]⁺ 448.1872; found 448.1869.

4.2.2. Boc-η-Dab (FmocHN-P)-CO₂H (6)

Compound 8 was synthesized from FmocHN-P-CO₂H. To a solvent of 5 mL DMF was added FmocHN-P-CO₂H (300 mg, 0.83 mmol). PyBOP (431 mg, 0.83 mmol) and DIEA (0.40 mL), and the mixture was stirred at room temperature for 1 h. To the reaction mixture was added Boc-D-Dab-OH (190 mg, 0.87 mmol) and 2 mL DMF, and then it was stirred at room temperature for 3 h. After concentration, the oil residue was dissolved in the minimum amount of ethyl acetate, washed with diethyl ether. After the supernatant was removed, to the residue was added a large amount of water to afford the precipitation. It was filtered and then dried in vacuo to obtain 6 as a brown powder (478 mg, 94%). The compound was used in the solid-phase synthesis without further purification. 1 H NMR (600 MHz, CDCl₃), δ 9.77 (d, 2H, J = 7.6 Hz, CH), 7.61 (d, 2H, J = 6.9 Hz, CH), 7.40 (t, 2H, J = 7.2 Hz, CH), 7.31 (t, 2H, J = 7.2 Hz, CH), 6.85 (s, 1H, CH), 6.63 (s, 1H, CH), 6.54 (s, 1H), 5.57 (d, 1H, J = 6.2 Hz, NH), 4.88 (d, 2H, J = 6.2 Hz, CH₂),
Compound 8 was synthesized from O,N-I-COCl. Compound 7 was prepared from O,N-I-COCl by 2 steps using the same method as a previously reported one. To a DMF solvent (12 mL) was added compound 7 (1.37 g, 8.83 mmol), Boc-HN-b-alanine (1.68g, 8.86 mmol), HCTU (3.85 g, 9.30 mmol), DIEA (3.10 mL, 17.7 mmol), and the solution was mixed and stirred at room temperature for 1.5 h. After concentration, a large amount of water was added to the oil residue to produce the precipitation. It was filtered and dried in vacuo to afford 8 as a white powder (2.44 g, 7.46 mmol, 84%). The compound was used in the next reaction step without further purification. 1H NMR (600 MHz, CDCl3) δ 8.45 (s, 1H, NH), 7.67 (d, J = 1.4 Hz, 1H, CH), 7.49 (s, 1H, NH), 4.00 (s, 3H, OCH3), 3.95 (s, 3H, NCH3), 3.49 (q, J = 6.2 Hz, 2H, CH2), 2.63 (t, J = 6.22 Hz, 2H CH2), 1.42 (s, 9H, CH3).

4.3.4. FmocHN-P-β-I-CO2H (10)

TFA (2.4 mL) was used in the deprotection of a Boc group that belongs to compound 8 (1.23 g, 3.75 mmol), and it was then evaporated. To a solution of obtained crude in H2O/MeOH (1:1, total 20 mL) was added NaOH (1.26 g), and the solution was stirred at 40°C for 2 h. After MeOH was removed from the reaction mixture by an evaporator, it was neutralized by 6N HClaq and concentrated in vacuo to afford crude gelatinous 9. To a solution of an intact in DMF (5 mL) was added a solution of FmocHN-P-CO2H (816 mg, 2.25 mmol), HCTU (932 mg, 2.25 mmol) and DIEA (1.3 mL) in DMF (10 mL) which was stirred at room temperature for 1 h, and the reaction mixture was stirred at room temperature for 5 h. After a salt was removed from the reaction mixture by filtration, a large amount of water was added to the oil residue obtained to produce the precipitation. The crude obtained by filtration was dissolved in a minimum amount of DCM and added diethyl ether to be precipitated again. The precipitated product was dissolved in DCM and subsequently was purified by HPLC using a phase flash column chromatography (water with 0.1% TFA as a linear gradient in 5 to 40 min. The peak around 23 min was collected and lyophilized to produce 11 (6.6 mg, 7.9 µmol, 23%) as a yellow solid. 1H NMR (600 MHz, DMSO-d6) δ 10.28 (s, 1H, NH) 10.23 (s, 1H) 9.93 (s, 1H, NH) 9.90 (s, 1H, NH) 8.09-8.05 (m, 3H, NH) 7.45 (s, 1H, CH) 7.41 (s, 1H, CH) 2.72 (d, J = 0.8 Hz, CH2) 2.05 (s, 3H, CH3) 1.78 (s, 3H, NCH3) 3.81 (s, 3H, NCH3) 3.80 (s, 1H, CH3) 2.04 (s, 3H, CH3) 1.77 (s, 3H, CH3) ESI-MS m/z calcd for C21H26N2O4 [M+H]+ 557.2149; found 557.2137.

4.3.3. Syntheses of parent PI polyamides and their chlorambucil conjugate

4.3.3.1. General Procedures of Fmoc Solid-phase Peptide Synthesis

Synthesis of each polyamide was performed on a PSSM (Shimadzu) computer-assisted operation system on a 0.03 mmol scale by using Fmoc Chemistry. An Fmoc building block (0.20 mmol) in each step was set up to solve by NMP on the synthetic line. The synthetic procedure of all PI polyamides was as follows: twice deblocking for 4 min with 20% piperidine/DMF (0.6 mL), activating for 2 min with HCTU (88 mg, 0.21 mmol) in NMP (1 mL) and 10% DIEA/DMF (0.4 mL), coupling for 60 min, and washing with DMF. All coupling were carried out with a single-coupling cycle. Building blocks used in this study are FmocHN-P-CO2H (77 mg), FmocHN-I-CO2H (77 mg), and compound 10 (70 mg) for synthesis of polyamide 11; FmocHN-P-CO2H (77 mg), FmocHN-I-CO2H (77 mg), compound 5 (70 mg), and compound 10 (70 mg) for synthesis of polyamide 12; FmocHN-P-CO2H (77 mg), FmocHN-I-CO2H (77 mg), compound 6 (70 mg), and compound 10 (70 mg) for synthesis of polyamide 14. At the last capping process, the samples were washed with 20% acetic acid in DMF (1 mL). All lines are purged with solution transfers and bubbled by N2 gas for stirring the resin. After the completion of the synthesis, the resin was washed with DMF (2 mL) and methanol (2 mL) and then dried in a desiccator at room temperature in vacuo.

4.3.2. Aclp-β-IP-β-(CH2)2-NCH2-(CH2)3-NH2 (11)

Using a 43 mg Fmoc-β-Wang resin (0.55 mmol/g) and a novel building block 10, compound 11 was synthesized in a stepwise reaction by Fmoc solid-phase protocol described above. After resulting Aclp-β-IP-β-wang resin was cleaved with 200 µL of DMDPA for 3 h at 55°C, the solvent was evaporated. The residue was dissolved in the minimum amount of DCM and subsequently washed with diethyl ether to yield a 15.1 mg white solid, which was used in next coupling reaction without further purification.

4.3.3. Aclp-β-IP-β-(CH2)2-NCH2-(CH2)3-NH2 (11)

To a solution of the crude compound 11 (2.0 mg) in DMF (200 µL), PyBOP (5.1 mg, 10 µmol), DIEA (1.7 µL, 10 µmol) and chlorambucil (3.1 mg, 10 µmol) were added. The reaction mixture was incubated for overnight at room temperature. Evaporation of the solvent gave a yellow crude, which was washed with ether and DCM and subsequently was purified by HPLC using a Chemcobond 5-ODS-H column (water with 0.1% TFA/MeCN 30-75% linear gradient, 0-30 min, detected at 254 nm), to produce 1 (1 mg, 0.91 µmol) as a pale yellow powder. ESI-MS m/z calced for C21H26N2O4 [M+2H]+ 553.2548; found 553.2527.

4.3.4. Aclp-β-IP-γ-IP-β-(CH2)2-NCH2-(CH2)3-NH2 (12)

Using a 45 mg Fmoc-β-Wang resin (0.55 mmol/g) and two novel building blocks 5 and 10, compound 12 was synthesized in a stepwise reaction by Fmoc solid-phase protocol described above. A subsequent synthetic procedure similar to that used for the preparation of compound 11 provided compound 12 (3.6 mg, 2.5 µmol, 10%) as a yellow powder. 1H NMR (600 MHz, DMSO-d6) δ 10.29 (s, 1H, NH) 10.27 (s, 1H, NH) 10.25 (s, 1H, NH) 10.23 (s, 1H, NH) 9.96-9.92 (m, 4H, NH) 8.09-8.06 (m, 5H, NH) 7.45 (s, 2H, CH) 7.43 (s, 1H, CH) 7.40 (s, 1H, CH) 7.22 (s, 1H, CH) 7.21 (s, 1H, CH) 7.20 (s, 2H, CH) 6.95 (s, 1H, CH) 6.93 (s, 3H, CH3) 3.94-3.93 (12H, NCH3) 3.81 (s, 6H, NCH3) 3.80 (s, 6H, NCH3) 3.42 (m, 8H, CH2) 3.19 (t, J = 6.2 Hz, 2H, CH2) 2.86 (s, 3H, NCH3) 2.75-2.73 (m, 6H, CH2) 2.59 (t, J = 7.6 Hz, 6H, CH2) 2.38 (m, 1H, CH2) 2.01 (s, 3H, CH3) 1.78-1.76 (m, 6H, CH2). ESI-MS m/z calcd for C25H28N6O12 [M+2H]+ 733.8567; found 733.8549.

4.3.5. Aclp-β-IP-γ-IP-β-(CH2)3-NCH2-(CH3)-NH2 (12)

To a solution of the crude compound 12 (1.9 mg) in DMF (200 µL), PyBOP (5.9 mg, 12 µmol), DIEA (1.9 µL, 11 µmol) and
chlorambucil (3.3 mg, 11 µmol) were added. A subsequent synthetic procedure similar to that used for the preparation of compound 1 provided 2 (0.5 mg, 0.28 µmol) as a pale yellow powder. ESI-MS m/z calc'd for C_{16}H_{16}Cl_{2}N_{10}O_{4} [M+2H]^{2+} 876.3910; found 876.3892.

4.3.6. AcIb-β-IP-β-IP-γ-IP-β-IP-β-IP-β-(CH_{2})_{3}-NCH_{2}-(CH_{2})_{3}-NH_{2}(13)

Using a 50 mg Fmoc-β-Wang resin (0.60 mmol/g) and two novel building blocks 5 and 10, compound 13 was synthesized in a stepwise reaction by Fmoc solid-phase protocol described above. A subsequent synthetic procedure similar to that used for the preparation of compound 11 provided compound 13 (4.9 mg, 2.4 µmol, 8%) as a yellow solid. 1H NMR (600 MHz, DMSO-d_{6}) δ 10.29 (s, 3H, NH) 10.26 (s, 1H, NH) 10.25 (s, 1H, NH) 10.23 (s, 1H, NH) 9.94 (s, 1H, NH) 9.93 (s, 2H, NH) 9.92 (s, 2H, NH) 9.91 (s, 1H, NH) 8.07 (m, 7H, NH) 7.44 (s, 3H, CH) 7.43 (s, 1H, CH) 7.41 (s, 2H, CH) 7.21-7.19 (m, 6H, CH) 6.95-6.93 (m, 6H, CH) 3.94 (s, 12H, NCH_{3}) 3.93 (s, 6H, NCH_{3}) 3.81 (s, 12H, NCH_{3}) 3.80 (s, 6H, NCH_{3}) 3.41 (m, 14H, CH_{2}) 2.77-2.72 (m, 6H, CH_{2}) 2.58 (t, J = 6.8 Hz, 10H, CH_{3}) 2.37-2.33 (m, 2H, CH_{2}) 2.08 (s, 3H, CH_{3}) 2.02 (s, 3H, CH_{3}) 1.79-1.75 (m, 6H, CH_{3}), ESI-MS m/z calc'd for C_{16}H_{39}N_{9}O_{8} [M+3H]^{3+} 700.3260; found 700.3239.

4.3.7. AcIb-β-IP-β-IP-γ-IP-β-IPy-β-IPy-β-(CH_{2})_{3}-NCH_{2}-(CH_{2})_{3}-NH-Chl (3)

To a solution of the crude compound 13 (2.1 mg) in DMF (200 µL), PyBOP (3.9 mg, 7.6 µmol), DIEA (1.3 µL, 7.5 µmol) and chlorambucil (2.3 mg, 7.6 µmol) were added. A subsequent synthetic procedure similar to that used for the preparation of compound 1 provided 3 (0.6 mg, 0.25 µmol) as a pale yellow powder. ESI-MS m/z calc'd for C_{16}H_{31}N_{10}O_{13} [M+3H]^{3+} 795.3489; found 795.3477.

4.3.8. Boc-n-Dab (AcIb-β-IP-β-IP-IP-β-IP-β-IP-β-(CH_{2})_{3}-NCH_{2}-(CH_{2})_{3}-NH-Chl) (14)

Using a 44 mg Fmoc-β-Wang resin (0.60 mmol/g) and two novel building blocks 6 and 10, compound 14 was synthesized in a stepwise reaction by Fmoc solid-phase protocol described above. Resulting Boc-n-Dab (AcImPy-β-ImPy-β-ImPy) was recovered by vacuum centrifugation and the residue was dissolved in the minimum amount of DCM, washed with diethyl ether to yield a 18.5 mg white solid, which was used in next reactions without further purification.

4.3.9. AcIb-β-IP-β-IP-n-Dab-IP-β-IP-β-IP-β-(CH_{2})_{3}-NCH_{2}-(CH_{2})_{3}-NH-Chl (15)

To a solution of the crude compound 14 (16.3 mg) in DCM (600 µL) was added TFA (400 µL) and the mixture was shook at room temperature for 30 min. The mixture was concentrated in vacuo and the residue was dissolved in the minimum amount of DCM, washed with diethyl ether to afford the oil residue. The residue was purified by reversed-phase flash column as mentioned above to obtain 15 (3.5 mg, 1.7 µmol) as a yellow solid. ESI-MS m/z calc'd for C_{16}H_{31}N_{11}O_{14} [M+3H]^{3+} 705.3262; found 705.3255.

4.3.10. AcIb-β-IP-β-IP-n-Dab-IP-β-IP-β-IP-β-(CH_{2})_{3}-NCH_{2}-(CH_{2})_{3}-NH-Chl (4)

To a solution of the crude compound 14 (16.3 mg) in DCM (1 mL), PyBOP (41.8 mg, 80 µmol), DIEA (15 µL) and chlorambucil (22.6 mg, 74 µmol) were added. The reaction mixture was mixed for 9 h at room temperature. Evaporation of the solvent gave a yellow residue, which was dissolved in the minimum amount of DCM, washed with diethyl ether. It was purified by reverse-phase flash column chromatography as mentioned above and the boc group was then deprotected with TFA-DCM (1:1, total 2.4 mL) to produce the oil residue. It was dissolved in the minimum amount of DCM, washed with diethyl ether, and purified by HPLC as mentioned above to produce 4 (0.7 mg, 0.29 µmol) as a pale yellow powder. ESI-MS m/z calc'd for C_{16}H_{16}Cl_{2}N_{10}O_{2} [M+3H]^{3+} 800.3525; found 800.3488.

4.4. Preparation of plasmid containing (CAG/CTG)_{12} repeat sequences

All DNA fragments and primers for cloning or DNA amplification were purchased from Sigma. The 38 bp DNA fragments were annealed in a final volume of 50 μL containing 10 µM of each strand (5'-CAG-{CAG}_{10}-CA-3' and 5'-G-{CTG}_{10}-A-3') and ligated into pGEM-T Easy vectors (Promega). Escherichia coli JM109 competent cells (Toyobo) were transformed and cultured on LB plates with 50 µg/mL ampicillin and 32 mg X-gal/400 mg IPTG overnight at 37 °C. White colonies were identified by colony direct polymerase chain reaction (PCR) in 15 µL of 1× Go Taq Green Master Mix (Promega) containing 300 nM of primer set (T7: 5'-TAATACGACTCCTATAGG-3', sp6: 5'-ATTATGGTGACTCCTAGAATAC-3'). The reaction mixture was incubated at 95 °C for 3 min then followed by 34 incubation cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min with final extension step of 72 °C for 5 min. The appropriate colony was selected for transfer to 5 mL of LB medium with 50 µg/mL ampicillin and cultured overnight at 37 °C overnight. The plasmids with inserts were extracted using GenElute Plasmid Miniprep Kit (Sigma Aldrich) and their sequences were identified by 310xLc Genetic Analyzer (Applied Biosystems).

4.5. Preparation of 5'-Texas Red-labeled DNA fragments and high resolution gel electrophoresis

The 5'-Texas Red labeled DNA fragments containing the CAG/CTG repeat 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 under the same condition and reagents as above. The PCR products were purified by GenElute PCR cleanup kit (Sigma Aldrich). The 5'-Texas Red-labeled DNA fragments were alkylated by various concentrations of alkylating polyamides in 10 µL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 37 °C, for 18 h. The reaction was quenched by the addition of calf thymus DNA (10 mg/mL, 1 µL) and the mixtures were heated at 95 °C for 10 min to cleave the DNA strands at the alkylating sites. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 7 µL of loading dye (formamide with New fuchsin), heated at 95 °C for 25 min, and then immediately chilled on ice. The 1.8 µL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a HITACHI SQ5500-E DNA Sequencer.

4.6. In vitro transcription assays

DNA fragments including (CAG/CTG)_{12} repeats for RNA transcription was prepared from the plasmids, which is described above, by PCR-amplification using 1X Go Taq Green Master Mix containing T7 promoter primer (5'-TAATACGACTCCTATAGG-3') and sp6 promoter primer (5'-ATTATGGTGACTCCTAGAATAC-3'). The reaction mixture was incubated at 95 °C for 3 min then followed by 30 incubation cycles of 95 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 30 sec. After incubation, the PCR products were purified by QIAquick Gel Extraction Kit (Qiagen) and QIAquick Nucleotide Removal Kit (Qiagen). The obtained DNA fragments (8 nM) were incubated with various concentrations of 4 and 15 in a 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at the room temperature for 18 h. The alkylation reagents were quenched by the addition of DNA oligomer (200 µM, 2 µL). The reaction...
mixtures were purified by illustra MicroSpin G-25 Columns (GE Healthcare) and then lyophilized for 3 h. The DNA fragments were transcribed with in vitro Transcription T7 Kit (Takara Bio Inc.). After transcriptions, the remaining DNA fragments were digested by RNase free DNase I (10 Units) included in the transcription kit for 30 min at 37 °C and the obtained RNAs were purified by RNeasy Mini Kit (Qiagen). Transcription products were analyzed by PAGE at 180 V for 80 min using 10 % TBE-Urea Gels containing 7 M urea (Invitrogen) and detected by SYBR Gold (Applied Biosystems). Before loading, samples were dissolved in TBE Urea Sample Buffer (Invitrogen), heated for 5 min at 95 °C, and then immediately cooled on ice for a few minutes. Low Range ssRNA Ladder (New England Biolabs) was used as a RNA marker and this was heated and then cooled similarly in loading buffer included in the ladder set. The bands were photographed and analyzed with Fluoro Image Analyzer FLA-3000GF (Fujifilm).

4.7. SPR assays

SPR experiments were performed on a Biacore X instrument. A biotinylated hairpin DNA was purchased from Sigma and the hairpin DNA is shown in Figure 5a. A streptavidin-functionalized SA sensor chip was purchased from Biacore. The biotinylated DNA is immobilized to the chip to obtain the desired immobilization level (approximately 1200 RU rise). SPR assays were carried out using HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% Surfactant P20) with 0.1 % DMSO at 25 °C. A series of sample solutions with various concentrations were prepared in the buffer with 0.1 % DMSO and injected at a flow rate of 20 µl/min. To measure the values of binding affinity and kinetics parameter, data processing was performed with an appropriate fitting model using BLAevaluation 4.1 program. The predefined models (1:1 binding model with mass transfer or with drafting baseline) were used for fitting the sensorgrams of polyamide 12, 13, and 15 to give better fitting. For polyamide 11, in order to represent dynamics in forming the complexes with two polyamides bound side by side in the same region, the sensorgram was fitted with the following model (eq 1):

\[ A + B \rightleftharpoons A + B \]

A and Conc A correspond to the polyamide close to the surface which is capable of binding to the DNA ligand and the bulk concentration of the polyamide. \( k_0 \) and \( k_2 \) represent the rates of association of the first and second polyamide with the ligand, respectively; \( k_{-2} \) and \( k_{-1} \) represent the rates of dissociation of the polyamide from a 1:1 complex and 2:1 complex, respectively. \( k_i \) is the empirical constant for mass transfer. The closeness of the fit is described by the statistical value \( \chi^2 \) (eq 2):

\[ \chi^2 = \frac{\sum (r_i - R_i)^2}{\eta - p} \]

where \( r_i \) is the experimental value at a given point, \( R_i \) is the theoretical value at the same point, \( \eta \) is the number of data points, and \( p \) is the number of fitted parameters

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at http

References and notes

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