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DNA Interstrand Cross-Links by H-pin Polyamide (*S*)-*seco*-CBI Conjugates

Chuanxin Guo^[a], Sefan Asamitsu^[a], Gengo Kashiwazaki^[a], Shinsuke Sato^[b], Toshikazu Bando^{*, [a]}, Hiroshi Sugiyama^{*, [a], [b]}

Abstract: Although DNA interstrand cross-links (ICLs) agents are widely used as antitumor drugs, DNA sequence-specific ICLs agents are quite rare. In this study, H-pin imidazole-pyrrole polyamide 1-(chloromethyl)-2,3-dihydro-1H-benzo[e]indol-5-ol (*seco*-CBI) conjugates that produce sequence-specific DNA ICLs have been designed and synthesized. Conjugates with H-pin polyamide and *seco*-CBI moieties were constructed for the recognition of a 7 bp DNA sequence and their reactivity and selectivity in DNA alkylation were evaluated by using high-resolution denaturing gel electrophoresis and sequence-specific plasmid cleavage. Conjugate **6**, which contained a chiral (*S*)-*seco*-CBI, exhibited higher sequence-specific ICLs activity toward the target DNA sequence and was appropriately cytotoxic to a cancer cell line. Molecular modeling studies indicated that the higher activity of **6** results from the relative orientation of the cyclopropane group in the (*S*)-CBI unit.

Use of DNA interstrand cross-linking (ICL) agents as a tool for antitumor therapy is based on their ability to inhibit both transcription and replication by preventing the separation of DNA strands.^[1] However, it cannot be overlooked that there is a limitation in ICL antitumor drugs arising from side effect to untargeted normal cells. Tumor cells always share many common features with normal cells. Consequently, traditional ICL drugs cannot achieve selective toxicity to cancer cells.^[2] Targeting oncogenes have contributed to the development of modern antitumor therapy.^[3] The common theme in development of ICL drugs is to address the limitations in current chemotherapy and to design new drugs with mechanisms that are based on traditional agents.^[4] For the goals of reducing side effect and increasing selectivity, many new types of cross-linking agents have been investigated extensively.^[5] As a member of cyclopropapyrroloindole family, bizelesin (NSC-615291), a bifunctional alkylating agent that contains two alkylating CPIs linked by a flexible methylene chain.^[6] Based on its preference for AT-rich sequence and antitumor activity,^[7] the National Cancer Institute has selected bizelesin for potential chemotherapeutic agent.^[8] Compared with the CPI, the *seco*-CBI moiety has greater reactivity and selectivity towards DNA and it is also more stable in aqueous solution.^[9] The *seco*-CBI dimer was able to form DNA ICLs in cell nucleus and showed extreme cytotoxicity to cancer cell lines.^[10] We conjugated the alkylation moieties with minor-groove-binding polyamides containing N-methylimidazole (Im) and N-methylpyrrole (Py), which uniquely recognize each of the four Watson-Crick base pairs^[11], and realized programmable sequence-specific DNA alkylation^[12] and ICLs.^[13] In

this research, conjugates of H-pin type^[14] Im-Py polyamide and enantiomeric or racemic *seco*-CBI were designed and synthesized. Both compounds target 5'-TGATTCA-3' DNA sequence, and the CBI subunit efficiently alkylated two adenine sites to form an ICL at the predetermined DNA sequence. We also compared the efficiency of the racemic *seco*-CBI and (*S*)-*seco*-CBI alkylating subunits to generate ICL.

Scheme 1. The synthetic route to H-pin type Im-Py polyamide (*S*)-*seco*-CBI conjugate **6** (Bottom left). The bottom right was racemic type *seco*-CBI moiety.

The synthetic steps to the H-pin type Im-Py polyamide (*S*)-*seco*-CBI conjugate were shown in Scheme 1. The H-pin type polyamide carboxylic acid **5**, which was obtained through liquid-phase synthesis, was directly coupled with indole-(*S*)-*seco*-CBI alkylating subunits by using PyBOP as coupling agent. Product conjugate **6** was purified by HPLC for use in DNA ICLs studies. By the same method, racemic type indole-*seco*-CBI was coupled with **5** to obtain the diastereomeric mixture **7**. The (*S*)-*seco*-CBI^[15] and racemic *seco*-CBI^[16] were prepared by previously reported method.

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To investigate whether the H-pin type polyamide indole-*seco*-CBI conjugates were able to form ICLs structure, a 40 bp dsDNA fragment containing the target sequence of 5'-TGATTCA-3' was incubated with either **6** or **7** and then subjected to 15% denaturing polyacrylamide gel electrophoresis.^[17] SYBR Green II staining was used to visualize the DNA fragments in the gel.

As shown in Figure 1, incubation of the DNA with conjugate **6** at 10 nM (1 equiv), 50 nM (5 equiv) and 100 nM (10 equiv) provided prominent slower migrating band. According to previous reports, these bands were considered as DNA ICLs structure.^[18] However for the conjugate **7**, ICLs formation bands could only be observed at the highest concentration (100 nM, 10 equiv). Significantly low ICLs reactivity of **7** might be explained by the low content of **6**. These results suggested that the conjugate **6** was able to form DNA ICLs structure effectively by alkylating the two adenines in opposite strands of DNA.

Figure 1. a) The DNA sequences of the forward and reverse fragments. b) Alkylation induced ICLs of 40 bp dsDNA fragment (10 nM) by conjugates **6** and **7** after incubation for 18 h at 23 °C. DNA and concentrations of the conjugates: Lane 1 = dsDNA; lane 2 = ssDNA of forward fragment; lane 3 = ssDNA of reverse fragment; lanes 4-6 = dsDNA mixed with conjugate **6** (10, 50, 100 nM). Lanes 7-9 = dsDNA mixed with conjugate **7** (10, 50, 100 nM). Lane 10 = dsDNA mixed with DMF. SYBR Green II staining was used to visualize the DNA fragments and ICLs DNA. The red arrows on the right of the gel represent ICLs DNA and ssDNA bands. c) Schematic representation of the ICLs DNA structure formed by conjugate **6**.

To confirm the sequence specificity of ICLs against the target DNA sequence, DNA alkylating reaction was evaluated by using 5'-Texas Red labeled 197-bp forward/reverse DNA fragments and a DNA sequencer. The forward T7 strand contained the 5'-TGATTCA-3' sequence, and the reverse SP6 strand contained the 5'-TGAATCA-3' sequence. After the forward and reverse fragments respective incubated with **6** and **7**, DNA alkylation sites were visualized by thermal cleavage and analyzed by denaturing polyacrylamide gel electrophoresis. Sanger sequencing was used to identify the alkylation sites.^[12]

Figure 2 showed the DNA alkylation induced by conjugates **6** and **7** on both strands. Attributed to high DNA sequence specificity of conjugate **6**, the alkylation occurred predominantly at adenine sites in both the forward 5'-TGATTCA-3' sequence and reverse 5'-TGAATCA-3' sequence. However, conjugate **7** provided very faint adenine site thermal cleavage bands on both strands. Higher ICLs reactivity of **6** compared with **7** was consistent with the result shown in Figure 1.

Figure 2. Thermally induced strand-cleavage of 197 bp forward T7 and reverse SP6 strand DNA fragments labeled with 5'-Texas Red (10 nM) by conjugates **6** and **7** that were incubated for 18 h at 23 °C at the following concentrations: a) T7 strand DNA, lane 1 = DNA control; lanes 2-4 = 62.5, 125, and 250 nM of conjugate **6**; lanes 5-7 = 62.5, 125, and 250 nM of conjugate **7**. b) SP6 strand DNA, lane 1 = DNA control; lanes 2-4 = 62.5, 125, and 250 nM of conjugate **6**; lanes 5-7 = 62.5, 125, and 250 nM of conjugate **7**. The central schematic shows a representation of the recognition and alkylation models of conjugates **6** and **7**. The red arrows indicated the alkylation sites that were inferred from sequencing gel analysis.

To further explore the sequence specificity of the H-pin type polyamide indole-*seco*-CBI conjugates, recombinant plasmid pGEM-T Easy/ICLs containing the ICLs target sequence was incubated with **6**, **7**, and hairpin Im/Py polyamide (S)-*seco*-CBI conjugate **8** (Figure S1), respectively. After incubation, the conjugates **6**, **7** and **8** will bind at target sequences and alkylate adenine sites. Thermal treatment induced the recombinant plasmids cleavage at alkylation sites. DNA double-strand or single-strand cleavage led to plasmid form changing. Different plasmid formations were separated by 1% agarose gel electrophoresis. As shown in Figure 3, conjugate **6**'s bifunctional alkylation and double-strands thermal cleavage led to the visualization of linear formation plasmid (Form III) in a concentration-dependent manner. However, conjugate **7** showed weaker double-strands thermal cleavage activity than conjugate **6**. The conjugate **8** which induced mono-alkylation and single-strand cleavage at match sequence cannot produce Form III, the linear formation plasmid.^[19] Importantly, the plasmid pGEM-T Easy/Control, without the target sequence, did not produce any Form III plasmid by conjugates **6**.^[20] These results demonstrated that conjugate **6** was superior to induce DNA sequence-specific ICLs and undergo further double-strands cleavage at brief heating conditions.

To gain insight into the mechanism of alkylating reactivity of the H-pin type Im/Py polyamide *seco*-CBI conjugates, molecular modeling studies of the target DNA fragment-conjugate complexes was carried out.^[21] Alkylation of the adenine by *seco*-CBI occurred through the formation of a cyclopropane unit on the 1,2,9,9a-tetrahydrocyclopropa [1,2-c] benz [1,2-e] indol-4-one (CBI) moiety (Figure 4a). Conjugates **9S** and **9R** were used for the molecular modeling studies. The two alkylation sites were indicated with red arrows in Figure 4b. As shown in Figure 4c, for complex **9S**, the distance between the N3 atom of each adenine moiety and the C9 atom of the CBI cyclopropane unit was only 3.56 and 3.58 Å, respectively. The N-terminal Im-Py-Py

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structure in both legs of the H-pin polyamide retained a planar conformation, thereby allowing efficient binding to occur between the

Figure 3. a) Alkylation and thermal cleavage induced form change of recombinant plasmid pGEM-T Easy/ICLs (20 nM) by conjugates **6**, **7** and **8**. DNA and conjugates concentration: Lane 1 = Marker; lane 2 = pGEM-T Easy/ICLs; lane 3 = pGEM-T Easy/ICLs mixed with DMF; lane 4-6 = pGEM-T Easy/ICLs mixed with conjugate **6** (20, 100, 200 nM); lane 7-9 = pGEM-T Easy/ICLs mixed with conjugate **7** (20, 100, 200 nM); lane 10-12 = pGEM-T Easy/ICLs mixed with conjugate **8** (20, 100, 200 nM); lane 13 = pGEM-T Easy/ICLs digested by *Pst* I; lane 14 = pGEM-T Easy/Control mixed with DMF; lane 15 = pGEM-T Easy/Control mixed with DMF conjugate **6** (200 nM). Lane 3-12, 14, 15 were treated by heat. b) Images of pGEM-T Easy Vector/ICLs containing match site for **6** and **8**, and pGEM-T Easy Vector/Control containing match site for **8**. c) Schematic representation of the conjugate **6** recombined match site and formed ICLs. d) Schematic representation of the conjugate **8** recombined the match site and formed mono-alkylation.

polyamide and DNA. This planar conformation would also contribute to stabilizing the *S*-form CBI unit in the leading H-pin for efficient alkylation. Furthermore the angles of the N3-C9-C8b for the **9S** were 118° and 116°. Thus the cyclopropane rings were positioned in an efficient orientation for alkylation. However, for the *R*-form CBI unit, the cyclopropane ring was positioned on the opposite side, away from the DNA minor groove. In this case, the distances between the N3 atom of the adenine moiety and the C9 atom of the CBI cyclopropane were 4.42 and 4.57 Å, respectively. These distances were considered too long to allow effective DNA alkylation to occur. Additionally the angles of the N3-C9-C8b for **9R** were turn to inefficient orientation (62° and 77°)^[10] Thus, the ICLs activity of conjugate **7**, bearing the racemic form of *seco*-CBI moiety, was weaker than conjugates **6**.

For the demonstration of the biological property of **6** as an ICL agent, the cytotoxicity assay was performed against breast adenocarcinoma, MDA-MB-231. The results revealed that conjugate **6** (IC₅₀ = 110 nM) showed higher cytotoxicity than **7** (IC₅₀ = 1.5 μM), which is parallel with ICLs ability observed in Figures 1, 2 and 3. However, the cytotoxicity of hairpin **8** (IC₅₀ = 21.2 nM) exhibited the highest cytotoxicity of the three kinds of conjugates (Figure S2). The reason why cytotoxicity of conjugate **6** weaker than **8** was unknown, however it might be attributed to the difference of cell permeability between H-pin and hairpin. For comparison, ICL agents that are used as chemotherapeutic drugs, such as cisplatin, have a similar IC₅₀ range (0.54 μM to 1.6 mM for cisplatin).^[22]

In summary, H-pin imidazole-pyrrole polyamide CBI conjugates have been designed and synthesized as sequence-specific ICLs agents.

We have shown that the conjugate **6** can target a 7 bp DNA sequence, 5'-TGATTCA-3', with high reactivity and selectivity to form an ICL formation. Conjugate **6** had higher activity than **7** because of the chiral form of the *seco*-CBI moiety. Our findings suggested that alkylating H-pin imidazole-pyrrole polyamide derivatives could be used to target specific gene sequences and form ICLs structure. These findings also provide the opportunity to develop a new generation of oncogene-targeting ICLs chemotherapeutic drugs.^[23]

Figure 4. Molecular models of the target DNA-conjugate complex. a) H-pin polyamides with the *S*- and *R*-cyclopropane CBI derivatives, **9S** and **9R**, respectively. b) Energy-minimized structure of the 5'-dGGTGATTACACC-3'/5'-dGGTGAATACACC-3'-**9S** complex. The two alkylation sites were indicated with red arrows. The distance between the N3 atom of the adenine moiety and the C9 atom of the CBI cyclopropane unit, the angle between the N3, C9 and C8b in the *S*-form (c) and *R*-form (d) of CBI were measured. Bottom table showed the distances and angles.

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Keywords: Im-Py Polyamide • Interstrand-crosslink • Sequence-specific DNA Alkylation • (*S*)-*seco*-CBI

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DNA Interstrand Cross-Links by H-pin
Polyamide (*S*)-*seco*-CBI Conjugates

H-pin imidazole-pyrrole polyamide *seco*-CBI conjugates that can be used to form sequence-specific DNA interstrand cross-links (ICLs) have been designed and synthesized. In particular, conjugate **6**, which contained a chiral (*S*)-*seco*-CBI moiety, exhibited higher sequence-specific ICL activity toward the target DNA sequence and was appropriately cytotoxic to a cancer cell line.

Supporting information

Sequence-Specific DNA Interstrand Cross-Links by an Imidazole-Pyrrole Polyamide (*S*)-seco-CBI Conjugate

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Experimental Section

General methods. Reagents and solvents were purchased from commercial suppliers and used without further purification. Solid-phase peptide synthesis was performed by using a PSSM-8 peptide synthesizer (Shimadzu, Kyoto). Water was purified with a Millipore Direct-Q (MQ) system. HPLC was performed with a Jasco PU-2089 HPLC pump, a UV-2075 HPLC UV/Vis detector, and either a Chemco-bond 5-ODS-H column (Chemco Scientific, 4.6 mm×150 mm, 0.1% TFA in water/MeCN, flow rate: 1.0 ml/min, linear gradient elution of 0–100% MeCN over 20 min, detection at 254nm) or a Chemcobond 5-ODS-H column (Chemco Scientific, 10 mm×150 mm, 0.1% TFA in water/MeCN, flow rate: 3.0 ml/min, linear gradient elution of 0–100% MeCN over 20 min, detection at 254 nm). TOF-MS (ESI) was performed on a BioTOF II (Bruker Daltonics) mass spectrometer in positive-ionization mode. UV spectra were recorded on a Nanodrop ND-1000 spectrophotometer. The DNA oligonucleotides and the 5'-Texas-Red-labeled primers were purchased from Sigma-Aldrich. A GenElute plasmid mini prep kit and a Genelute PCR clean-up kit were purchased from Sigma-Aldrich. A Thermo Sequence core-sequencing kit was purchased from GE Healthcare. Polymerase chain reaction (PCR) was performed on an iCycler (Bio-Rad). Using a HITACHI SQ5500E DNA sequencer performed polyacrylamide gel electrophoresis and the data were analyzed by using FLAGLYS version 2 software (HITACHI). Denatured polyacrylamide electrophoresis gel was scanned by FLA-3000 (Fujifilm). NMR spectra were recorded on a JEOL JNM ECA-600 NMR spectrometer with tetramethylsilane as an internal standard. ¹H NMR spectra (600 MHz) were recorded in parts per million (ppm) downfield of tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet), and br (broad). NH₂-indole-seco-CBI and NH₂-indole-(*S*)-seco-CBI were synthesized according to a literature procedure.

Synthesis of compound 2. K_2CO_3 (0.350 g, 2.50 mmol), 1,6-dibromohexane (64 μ l, 0.416 mmol) were added to the DMF solution (2 mL) of compound **1** (200 mg, 0.832 mmol) and it was stirred vigorously at 40 °C for 66 h. The reaction mixture was filtered and the remaining solution was evaporated to remove DMF solvent. After adding cold water (100 mL) to the reaction mixture, the solid was filtered and dried under vacuum to obtain the crude product **2** (198 mg, 0.352 mmol, yield 84%) as brown powder. Compound **2** was used in the next step without any further purification. 1H NMR (600 MHz, $DMSO-d_6$): δ 9.11 (s, 2H; $NH \times 2$), 7.13 (s, 2H; $CH \times 2$), 6.62 (s, 2H; $CH \times 2$), 4.18 (t, 4H, $J = 7.2$ Hz; $CH_2 \times 2$), 3.70 (s, 6H; $CH_3 \times 2$), 1.59 (m, 4H; $CH_2 \times 2$), 1.43 (s, 18H; $CH_3 \times 6$), 1.24 (m, 4H; $CH_2 \times 2$). ESI-TOFMS: m/z calcd for $C_{28}H_{43}N_4O_8$: 563.3075 $[M+H]^+$, found: 563.2790; HPLC : $t_R = 20.1$ min (0.1% TFA/MeCN, linear gradient 0-100 %, 0-20 min).

Synthesis of compound 3. Compound **2** (198 mg, 0.352 mmol) was dissolved in 4 N hydrochloric acid/ethyl acetate (2 mL) and the solution was stirred at rt for 30 min. After evaporation and drying under vacuum compound **3** was obtained as brown powder (124 mg, 0.342 mmol, yield 98%). Compound **3** was used in the next step without any purification. 1H NMR (600 MHz, $DMSO-d_6$): δ 9.98 (br, 4H; $NH_2 \times 2$), 7.30 (d, 2H, $J = 1.4$ Hz; $CH \times 2$), 6.81 (d, 2H, $J = 1.4$ Hz; $CH \times 2$), 4.27 (t, 4H, $J = 7.2$ Hz; $CH_2 \times 2$), 3.75 (s, 6H; $CH_3 \times 2$), 1.63 (qu, 4H, $J = 6.5$ Hz; $CH_2 \times 2$), 1.22 (m, 4H; $CH_2 \times 2$). ESI-TOFMS: m/z calcd for $C_{18}H_{27}N_4O_4$: 363.2072 $[M+H]^+$, found: 263.2014; HPLC: $t_R = 8.4$ min (0.1% TFA/MeCN, linear gradient 0-100 %, 0-20 min).

Synthesis of compound 4. ImPyCO₂H was synthesized following the previous paper.² 1H NMR (600 MHz, $DMSO-d_6$): δ 12.18 (br, 1H; OH), 10.47 (s, 1H; NH), 7.47 (d, 1H, $J = 1.4$ Hz; CH), 7.38 (s, 1H; CH), 7.03 (s, 1H; CH), 6.98 (d, 1H, $J = 2.0$ Hz; CH), 3.98 (s, 3H; CH_3), 3.82 (s, 3H; CH_3). A DMF solution (5 mL) of ImPyCO₂H (169 mg, 0.684 mmol), DIEA (*N,N*-diisopropylethylamine) (474 μ l, 2.74 mmol), PyBOP ((benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate) (353 mg, 0.684 mmol) and compound **3** (124 mg 0.342 mmol) was stirred at 23 °C for 12 h, then the mixture was evaporated. After adding 0.1 N NaOH (120 mL) at 0 °C, the solvent was removed by filter to obtain compound **4** (259 mg, 0.315 mmol, yield 92%) as a white powder. 1H NMR (600 MHz, $DMSO-d_6$): δ 10.46 (s, 2H; $NH \times 2$), 9.95 (s, 2H; $NH \times 2$), 7.50 (d, 2H, $J = 1.4$ Hz; $CH \times 2$), 7.39 (s, 2H; $CH \times 2$), 7.28 (d, 2H, $J = 2.0$ Hz; $CH \times 2$), 7.18 (d, 2H, $J = 2.0$ Hz; $CH \times 2$), 7.04 (s, 2H; $CH \times 2$), 6.92 (d, 2H, $J = 2.0$ Hz; $CH \times 2$), 4.24 (t, 4H, $J = 7.6$ Hz; $CH_2 \times 2$), 3.99 (s, 6H; $CH_3 \times 2$), 3.84 (s, 6H; $CH_3 \times 2$), 3.73 (s, 6H; $CH_3 \times 2$), 2.61 (qu, 4H, $J = 7.2$ Hz; $CH_2 \times 2$), 1.26 (m, 4H; $CH_2 \times 2$). ESI-TOFMS: m/z calcd for $C_{40}H_{47}N_{12}O_8$: 823.3634 $[M+H]^+$, found: 823.3418; HPLC: $t_R = 12.8$ min (0.1% TFA/MeCN,

linear gradient 0-100%, 0-20 min).

Synthesis of compound 5. Compound **4** (54 mg, 68 μ mol) in 3 ml of THF : 2 M aq. NaOH (1 : 1), which was stirred at 40 °C for 48 h. After the reaction mixture was neutralized by acetic acid until pH = 6, the solvent was evaporated. The residue was suspended by 0.1 N HCl at 0 °C, then the solvent was removed by filter to obtain the crude and completely dried. Subsequently the solid was washed with a solution of MeOH : CH₂Cl₂ (1 : 1) (3 ml) then with THF (3 ml). Compound **5** (25.9 mg, 32.6 μ mol, yield 48%) was collected as a gray solid, which was used in the next step without any purification. ¹H NMR (600 MHz, DMSO-*d*₆): δ 10.45 (s, 2H; NH $\times$ 2), 9.92 (s, 2H; NH $\times$ 2), 7.45 (d, 2H, *J* = 2.3 Hz; CH $\times$ 2), 7.39 (s, 2H; CH $\times$ 2), 7.27 (d, 2H, *J* = 1.6 Hz; CH $\times$ 2), 7.17 (d, 2H, *J* = 2.1 Hz; CH $\times$ 2), 7.04 (s, 2H; CH $\times$ 2), 6.86 (d, 2H, *J* = 2.1 Hz; CH $\times$ 2), 4.24 (t, 4H, *J* = 7.6 Hz; CH $\times$ 2), 3.99 (s, 6H; CH $\times$ 2), 3.84 (s, 6H; CH $\times$ 2), 2.61 (qu, 4H, *J* = 1.8 Hz; CH $\times$ 2), 2.38 (qu, 4H, *J* = 1.8 Hz; CH $\times$ 2). ESI-TOFMS: *m/z* calcd for C₃₈H₄₃N₁₂O₈: 795.3321 [M+H]⁺, found: 795.4049; HPLC: *t*_R = 12.8 min (0.1% TFA/MeCN, linear gradient 0-100%, 0-20 min).

Synthesis of conjugates 6 and 7. (*S*)-seco-CBI¹ and NH₂-indole-(*S*)-seco-CBI were synthesized by following previously reported procedure. A DMF solution (0.2 mL) of compound **5** (2.0 mg, 2.5 μ mol), DIEA (2.5 μ l, 15.1 μ mol), PyBOP (5.2 mg, 10.0 μ mol) was stirred at 23 °C for 30 min. After the formation of the activated 1-hydroxybenzotriazole ester was checked by analytical HPLC, NH₂-indole-(*S*)-seco-CBI (3.0 mg, 7.6 μ mol) was added to reaction vessel, and the reaction mixture was stirred at 23 °C for 10 h. Reaction solution was diluted by Et₂O (1 mL), and the precipitate was collected by filtration and washed with Et₂O (3 mL) and CH₂Cl₂ (1 mL). The crude was purified by reverse-phase HPLC (0.1% TFA/ MeCN, linear gradient 55-80%, 0-20 min) to obtain conjugate **6** as a yellow solid (0.8 mg, 0.5 μ mol, yield 21%). ESI-TOFMS: *m/z* calcd for C₈₂H₇₆Cl₂N₁₈O₁₀²⁺: 771.2679 [M+2H]²⁺, found: 771.2650; HPLC: *t*_R = 28.5 min (0.1% TFA/MeCN, linear gradient 0-100%, 0-40 min).

Similar synthetic procedure was used for the synthesis of conjugate **7** (0.5 mg, 0.3 μ mol, yield 10%). ESI-TOFMS: *m/z* calcd for C₈₂H₇₆Cl₂N₁₈O₁₀²⁺: 771.2679 [M+2H]²⁺, found: 771.2708; HPLC: *t*_R = 28.5 min (0.1% TFA/MeCN, linear gradient 0-100%, 0-40 min).

Synthesis of hairpin Im-Py polyamide conjugate 8.

Figure S1. The synthetic route to hairpin Im-Py polyamide (*S*)-seco-CBI conjugate **8**. SPPS*: Solid-phase peptide synthesis.

Hairpin Im-Py polyamide carboxylic acid was prepared in a stepwise method by using a previously reported Fmoc solid-phase procedure by using CLEAR Acid resin, Fmoc-Py-COOH, Fmoc-Im-COOH, Fmoc-PyIm-COOH, Fmoc- β -COOH, O-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), DIEA and DMF. Cleavage from the CLEAR-Acid resin was performed with acidic conditions (950 μl TFA, 25 μl triisopropylsilane, 25 μl H_2O , rt, 30 min) (Wako). The corresponding crude samples were used as coupling partners with $\text{NH}_2\text{-Indole-(S)-seco-CBI}$ without further purification. (10.7mg, 12.1 μmol , total 54% yield, from 22.2 μmol of Im-CLEAR-Acid resin). ^1H NMR (600 MHz, $\text{DMSO-}d_6$): δ 10.40 (d, 1H, $J = 10.8$ Hz; NH), 10.32 (d, 1H, $J = 10.8$ Hz; NH), 10.31 (d, 1H, $J = 10.2$ Hz; NH), 9.91 (d, 1H, $J = 10.8$ Hz; NH), 9.61 (d, 1H, $J = 10.2$ Hz; NH), 9.33 (d, 1H, $J = 10.2$ Hz; NH), 10.40 (d, 1H, $J = 10.8$ Hz; NH), 8.02 (br, 1H; NH), 7.62 (d, 1H; $J = 11.4$ Hz; CH), 7.58 (d, 1H; $J = 12.0$ Hz; CH), 7.51 (m, 2H; $\text{CH}\times 2$), 7.49 (d, 1H; $J = 10.8$ Hz; CH), 7.16 (m, 2H; $\text{CH}\times 2$), 6.89 (d, 1H; $J = 9.6$ Hz; CH), 4.01 (d, 3H; $J = 11.4$ Hz; CH_3), 3.99 (d, 3H; $J = 12.6$ Hz; CH_3), 3.94 (d, 3H; $J = 12.6$ Hz; CH_3), 3.93 (d, 3H; $J = 11.4$ Hz; CH_3), 3.85 (d, 3H; $J = 10.8$ Hz; CH_3), 3.80 (d, 3H; $J = 11.4$ Hz; CH_3), 3.18 (m, 2H; CH_2), 2.36 (m, 2H; CH_2), 2.03 (d, 3H; $J = 11.4$ Hz; CH_3), 1.78 (m, 2H; CH_2), ESI-TOFMS: m/z calcd for $\text{C}_{36}\text{H}_{44}\text{N}_{17}\text{O}_9^+$: 882.3502 $[\text{M}+\text{H}]^+$, found: 882.2945; HPLC: $t_R = 12.0$ min (0.1% TFA/MeCN, linear gradient 0–100%, 0–20 min).

Hairpin Im-Py polyamide carboxylic acid (2.0 mg, 2.3 μmol), DMF (200 μl), DIEA (2.3 μl , 13.6 μmol), and PyBOP (3.5 mg, 6.8 μmol) were stirred for 30 min at rt. The formation of the activated 1-hydroxybenzotriazole ester was checked by analytical HPLC ($t_R = 14.3$ min, 0.1% TFA/MeCN, linear gradient 0–100%, 0–20 min); then, $\text{NH}_2\text{-indole-(S)-seco-CBI}$ (2.7 mg, 6.8 μmol) was added and the mixture was stirred overnight at rt. Et_2O was added to the mixture and the precipitate was collected by centrifugation and washed with Et_2O and CH_2Cl_2 , followed by HPLC purification (0.1% TFA/MeCN, linear gradient 50–80 %, 0–20 min). After collecting the main peak fraction and lyophilization, conjugate **8** was obtained as a yellow solid (0.9 mg, 0.7 μmol , 32% yield) ESI-TOFMS: m/z calcd for $\text{C}_{60}\text{H}_{61}\text{ClN}_{20}\text{O}_{10}^{2+}$: 628.2279 $[\text{M}+2\text{H}]^{2+}$, found: 628.3353; HPLC: $t_R = 14.3$ min (0.1% TFA/MeCN, linear gradient 0–100%, 0–20 min).

Denatured polyacrylamide gel electrophoresis. 10 nM 40 bp dsDNA fragment (5'-GAATTCGATTATCCGGTGATTCACCGGATAATCACTAGTG-3' / 3'-CTTAAGCTAATAGGCCACTAAGTGGCCTATTAGTGATCAC-5') was incubated with various concentrations of H-pin type polyamide conjugates 10, 50, 100 nM in DMF with 5 mM sodium phosphate buffer (pH 7.0) at 23 °C for 18 h. After incubation, the reaction mixture was concentrated by using vacuum centrifugation. The pellet was dissolved in 5 µl formamide loading dye. A 2.0 µl aliquot was subjected to electrophoresis on a 15 % denaturing polyacrylamide gel with 6 M urea. The electrophoresis carried out at 4 °C for about 180 min. The gel was stained by SYBR Green II for 10 min and then scanned by FLA-3000 (Fujifilm). In order to analyze the data quantitatively, the percentage of ICLs DNA bands were evaluated by Multi Gauge V3.1 (Fujifilm). For the lane 4, 5 and 6, the percentage of ICLs DNA bands were 14%, 36% and 57% that formed by **6**. For the lane 7, 8 and 9, the percentages of ICLs bands were 0, 9% and 16% that formed by **7**. For the lanes 5 and 8, the ICLs ratio was 25% (9%/36%). For the lanes 6 and 9 the ratio was 28% (16%/57%). The values roughly correspond to expected yield.

Preparation of pGEM-T Easy/ICLs.

All of the oligonucleotides (5'-ATCCGGTGATTCACCGGATA-3', 5'-ATCCGGTGAATCACCGGAT-3') were purchased from Sigma-Aldrich, annealed at a concentration of 10 µM, and ligated into the pGEM-T Easy vector (Promega). *Escherichia coli* JM109 competent cells (Promega) were transformed by the recombined plasmid and cultured on an LB plate with 100 µg/ml ampicillin, X-gal (32 µg, 20 mg/ml), and IPTG (25 µl, 100 mM) overnight at 37 °C. White colonies were identified by colony PCR in 20 µl of reaction mixtures that contained 250 nM of each primer (T7 primer, 5'-TAATACGACTCACTATAGGG-3'; SP6 primer, 5'-TATTTAGGTGACACTATAG-3'), 200 µM dNTPs (Sigma Aldrich), 2 units of Taq polymerase, and ThermoPol reaction buffer (New England Bio Labs). Amplification of the DNA fragments was performed by incubating at 95 °C for 5 min, followed by 35 cycles at 95 °C for 35 s, 50 °C for 35 s, 72 °C for 30 s, and a final extension step of 72 °C for 7 min. The appropriate colony was selected for transfer into LB medium (5 ml) with 100 mg/ml ampicillin and cultured overnight at 37 °C. The recombined plasmid pGEM-T Easy/ICLs was extracted by using a GenElute Plasmid Miniprep Kit (Sigma-Aldrich) and identified by using PCR (program and reaction mixtures were the same as above).

Cloning of a 197 bp DNA fragment. The forward T7 5'-Texas-Red-modified 197 bp DNA fragments was prepared by PCR with 5'-Texas Red-modified T7 primer (5'-Texas Red-TAATACGACTCACTATAGGG-3') and SP6 primer

(5'-TATTTAGGTGACACTATAGA-3') from 1 µg/ml of the recombined plasmid pGEM-T Easy/*ICLs*. The reverse SP6 5'-Texas-Red-modified 197 bp DNA fragments was prepared by PCR with 5'-Texas Red-modified SP6 primer (5'-Texas Red-TATTTAGGTGACACTATAGA-3') and T7 primer (5'-TAATACGACTCACTATAGGG-3') from 1 µg/ml of the recombined plasmid pGEM-T Easy/*ICLs*. The 5'-Texas-Red-modified forward and reverse 197 bp DNA fragments purified by GenElute PCR Clean-up Kit (Sigma Aldrich) and their concentrations were determined by UV absorption. The sequence of forward T7 5'-Texas-Red-modified 197 bp DNA fragments containing 5'-TGATTCA-3' region: 5'-TexasRed-TAATACGACTCACTATAGGGCGAATTGGGCCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTATCCGGTTGATTCACCGGATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATA-3'. The sequence of reverse SP6 5'-Texas-Red-modified 197 bp DNA fragments containing 5'-TGAATCA-3' region: 5'-Texas Red-TATTTAGGTGACACTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCAGTGTGATTATCCGGTGATCACCCGGATAATCGAATTCCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTA-3'.

High-resolution gel electrophoresis. The forward and reverse 197 bp DNA fragments (6 nM) were alkylated by the polyamide conjugates at various concentrations from 62.5 nM to 250 nM in DMF with 5 mM sodium phosphate buffer (pH 7.0) at 23 °C for 18 h. After incubation, the reaction mixture was quenched by calf thymus DNA and heated for 5 min at 95 °C. The solution was concentrated by using vacuum centrifugation. The pellet was dissolved in 7 µl of loading dye, heated at 95 °C for 20 min, and then immediately cooled to 0 °C. A 0.8 µl aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel by using a Hitachi SQ5500E DNA sequencer.

Preparation of pGEM-T Easy/*Control*.

The oligonucleotides (5'-GAATTCTTCTTCTGCTGTTGAATTCTTCTTCAGCAGTTA-3', 5'-AACTGCTGAAGAAGAATTCAACAGCAGAAGAAGAATTCA-3') were purchased from Sigma-Aldrich, annealed at a concentration of 10 µM, and ligated into the pGEM-T Easy vector (Promega). *Escherichia coli* JM109 competent cells (Promega) were transformed by the plasmid and cultured on an LB plate with 100 µg/ml ampicillin, X-gal (32 µg, 20 mg/ml), and IPTG (25 µl, 100 mM) overnight at 37 °C. White colonies were identified by colony PCR in 20 µl of reaction mixtures that contained 250 nM of each primer (T7 primer, 5'-TAATACGACTCACTATAGGG-3'; SP6 primer, 5'-TATTTAGGTGACACTATAG-3'),

200 μ M dNTPs (Sigma Aldrich), 2 units of Taq polymerase, and ThermoPol reaction buffer (New England Bio Labs). Amplification of the DNA fragments was performed by incubating at 95 °C for 5 min, followed by 35 cycles at 95 °C for 35 s, 50 °C for 35 s, 72 °C for 30 s, and a final extension step of 72 °C for 7 min. The appropriate colony was selected for transfer into LB medium (5 ml) with 100 mg/ml ampicillin and cultured overnight at 37 °C. The recombinant plasmid pGEM-T Easy/*Control* was extracted by using a GenElute Plasmid Miniprep Kit (Sigma-Aldrich) and identified by using PCR (program and reaction mixtures were the same as above).

Plasmid alkylation and thermal cleavage.

20 nM pGEM-T Easy/*ICLs* was incubated with various concentrations of Im-Py polyamide *seco*-CBI conjugates (**6**, **7** and **8**) 0, 20, 100, 200 nM in DMF with 5 mM sodium phosphate buffer (pH 7.0) at 23 °C for 24 h. 20 nM pGEM-T Easy/*Control* was incubated with 0 and 200 nM conjugate **6** in DMF with 5 mM sodium phosphate buffer (pH 7.0) at 25 °C for 24 h. After incubation, the reaction mixture was heated at 90 °C for 15 min. Lane 1 was 20 nM pGEM-T Easy/*ICLs* plasmid without any treatment. Lane 13 was *Pst* I (TAKARA) single digested 20 nM pGEM-T Easy/*ICLs* plasmid, which used as linear form plasmid control. A 5.0 μ l aliquot was subjected to electrophoresis on a 1% agarose gel. The electrophoresis carried out with 100 V at rt for about 45 min. The gel was stained by SYBR Green I for 20 min and then scanned by FLA-3000 (Fujifilm).

Cell culture and cytotoxicity assay. The human Caucasian breast adenocarcinoma MDA-MB-231 cell line was purchased from European collection of cell cultures. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/ml streptomycin at 37°C in 5% CO₂. Cytotoxicity assay using Cell Count Reagent SF were performed on 96-well plates. A 50 μ l amount of each cell suspension was added at a density of 1×10^4 cells/well. Im-Py polyamide conjugates **6**, **7** and **8** were dissolved in DMSO, and 50 μ l of each solution in the medium (final DMSO concentration was 1%) was added. After treatment for 48 h, 10 μ l of WST-8 reagent was added to each well and incubated at 37 °C. Absorbance was then measured at 450 and 600 nm using an MPR-A4i microplate reader (Tosoh). The results were shown at Figure S2.

Figure S2. Cell viability assays conducted with MDA-MB-231 cells and conjugates a) **6**, b) **7** and c) **8**. Cells were treated with the conjugates for 48 h. The data are means $\pm$ SD calculated for six replicates of each experimental point.

Molecular Modeling Studies. Molecular modeling studies on the target DNA–conjugate complexes were conducted using previously reported method with small modifications. In brief, the starting B-form Watson-Crick DNA structure 5'-dGGTGATTACACC-3'/5'-dGGTGAATCACC-3' was constructed by the builder module with standard bond lengths and angles using Discovery Studio 4.5 program (BIOVIA). Conjugate **9S** and **9R** were manually inserted into the DNA minor groove of the target sequence 5'-dTGATTCA-3'/5'-dTGAATCA-3'. Based on the polyamide-DNA recognition rules, the conjugate-DNA complexes were pre-minimized to maintain the interaction distance of hydrogen bonds between the polyamide part and DNA base pairs. For final minimization, the resulting complexes were solvated by explicit boundary model, and 5 mM of sodium cations and phosphate anions were placed in the system. The whole structures were then minimized to the stage where the RMS was less than 0.001 kcal/mol·Å by smart minimizer algorithm with harmonic restraints of the DNA.

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