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Sequence-Specific Gene Silencing in Mammalian Cells by Alkylating Pyrrole−Imidazole Polyamides

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Abstract: Gene silencing was examined by sequence-specific alkylation of DNA by *N*-methylpyrrole (Py)-*N*-methylimidazole (Im) hairpin polyamides. Polyamides ImImPyPyγImImPyLDu86 (A) and ImImPyPyγImPyPyLDu86 (B) selectively alkylated the coding regions of the renilla and firefly luciferases, respectively, according to the base pair recognition rule of Py−Im polyamides. Two different plasmids, encoding renilla luciferase and firefly luciferase, were used as vectors to examine the effect of alkylation on gene silencing. Transfection of the alkylated luciferase vectors-by polyamide A or B-into HeLa, 293, and NIH3T3 cells demonstrated that these sequence-specific DNA alkylations lead to selective silencing of gene expression. Next, the vectors were cotransfected into HeLa cells and the cells were treated with polyamide A or B. Selective reduction of luciferase activities was caused by both polyamides. On the basis of this sequence-specific alkylation and gene silencing activity, these alkylating Py−Im polyamides thus have potential as antitumor drugs to target specific gene expression in human cells.

Introduction

Numerous diseases, including malignant lymphoma and cancer, are better understood at a sequence level in this era of rapidly growing genomic information,[1] and thus many areas of medical science, such as diagnosis, treatment, and prevention, are changing dramatically.[2] Because mutations often cause these diseases, regulation of specific gene expression by epigenetic mechanisms including synthetic transcription factors and activators,[3] triple helix forming oligonucleotides,[4] and small interfering RNA (siRNA) molecules[5] have emerged as promising approaches for designing drugs.

Minor groove-binding *N*-methyl pyrrole (Py) and *N*-methyl imidazole (Im) polyamide uniquely recognize each of the four Watson−Crick base pairs. Antiparallel pairing of imidazole with pyrrole (Im/Py) recognizes a G−C base pair, whereas a Py/Py pair recognizes either an A−T or T−A base pair.[6] The binding constant and sequence-specificity of the Py-Im hairpin polyamide is comparable to that of a transcription factor.[7] Thus, the genes of the human immunodeficiency virus (HIV) are silenced by competitive binding of Py−Im hairpin polyamides to their regulatory sequences.[8] It is believed that gene silencing occurs via inhibition of gene expression by the Py−Im polyamides binding to regulatory sequences. Therefore, the inhibition of

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transcription by simple Py–Im hairpin polyamides at coding region is difficult, because the polyamides are removed from duplex DNA during transcription.[9]

DNA alkylating agents, which include well-known anticancer agents such as cisplatin and mitomycin C, are routinely used in antitumor treatments. However, these drugs are also toxic for normal cells because they do not have the ability to recognize sequences in mutated genes of transformed cells. One important question to be addressed is whether the introduction of sequence selectivity to an alkylating agent can improve its efficacy as an anticancer agent. To address this question, we and others have designed and synthesized a series of sequence-specific alkylating agents.[10] We have demonstrated that hybrids between segment A of DU-86 and Py–Im hairpin polyamides selectively alkylate at target sequences according to the recognition rule of Py–Im polyamides.[10a,b] Recently, we showed that the alkylating Py–Im polyamide, which recognizes specific sites on template strand of coding region in the green fluorescent protein, effectively inhibits transcription in an in vitro transcription system.[11] In contrast, we demonstrated that alkylation of the nontemplate strand of the coding region does not inhibit the transcription. These results suggest that the alkylating polyamides targeting the template strand of coding region have the ability to silence specific gene expression in vitro.

In this study, we designed two different alkylating Py–Im polyamides, ImImPyPyγImImPyLDu86 (A) and ImImPyPyγ-ImPyPyLDu86 (B) that predominantly target coding regions of renilla luciferase and firefly luciferase, respectively, to investigate whether these polyamides can suppress the expression of luciferase activity in living cells. To examine whether these polyamides can suppress the expression of luciferase specifically, the firefly and renilla luciferase coding vectors were alkylated with polyamide A or B, respectively, in vitro, and mammalian cells were transfected with the modified vectors. Next, both firefly and renilla luciferase coding vectors were cotransfected into HeLa cells and the cells were treated with polyamides. Measurements of luciferase activities revealed that the alkylation of luciferase genes by these polyamides induced sequence-specific gene silencing.

Results and Discussion

Polyamides A and B (Figure 1a) were designed to alkylate selectively at the template strands of the coding regions of renilla and firefly luciferases, respectively. Polyamide A has four match sites (1194, 1384, 1499, and 1688) at the template strands of coding region of renilla luciferase and B has two match sites (425 and 885) for firefly luciferase. The putative alkylation sites by the polyamides in the coding regions of the pRL-TK and pGL3-control vectors are presented in Figure 1b. In addition to these sites, polyamide B has three match sites in the promoter region and one match site in the linker region of renilla luciferases, and polyamde A has three match sites in the promoter region of firefly luciferase and three match sites in the promoter region of renilla luciferase. These properties will provide valuable information on the effects of the site of alkylation on the gene silencing. The polyamides were synthesized as described previously.[10a,b]

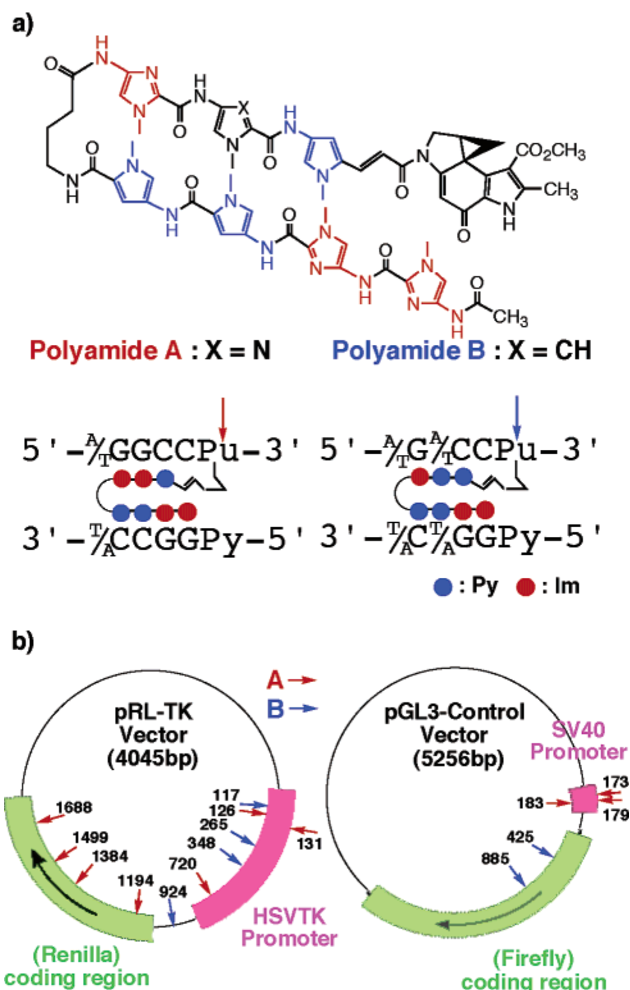

Figure 1. (a) Structures of polyamide A and B and their schematic representation of DNA alkylation. (b) Map of renilla and firefly luciferase gene and promoter region in pRL-TK and pGL3-control vector. The putative alkylation sites in promoter region on both strand, intron and coding region on template strand by polyamide A and B are indicated by the red and blue arrows, respectively. Detailed alkylation sites are described in Figures 1S and 2S.

To confirm the sequence-specific alkylation by polyamides A and B, we used 5′-Texas Red-labeled DNA fragments that encode renilla or firefly luciferase genes. The sites of alkylation were determined using an automated DNA sequencer as described previously (Figure 2).[12] The polyamide A alkylated at three matched sites (IX, X, and XII) with one mismatch sites (XI) on the renilla luciferase template strand of coding region, whereas the polyamide B alkylated at one mismatch site (VIII) on the renilla luciferase template strand (Figure 2a). In the sequence of firefly luciferase (Figure 2b), the polyamide B alkylated at the two match sites (IV and V) with one mismatch site (III) and polyamide A alkylated at one mismatch site (VI)

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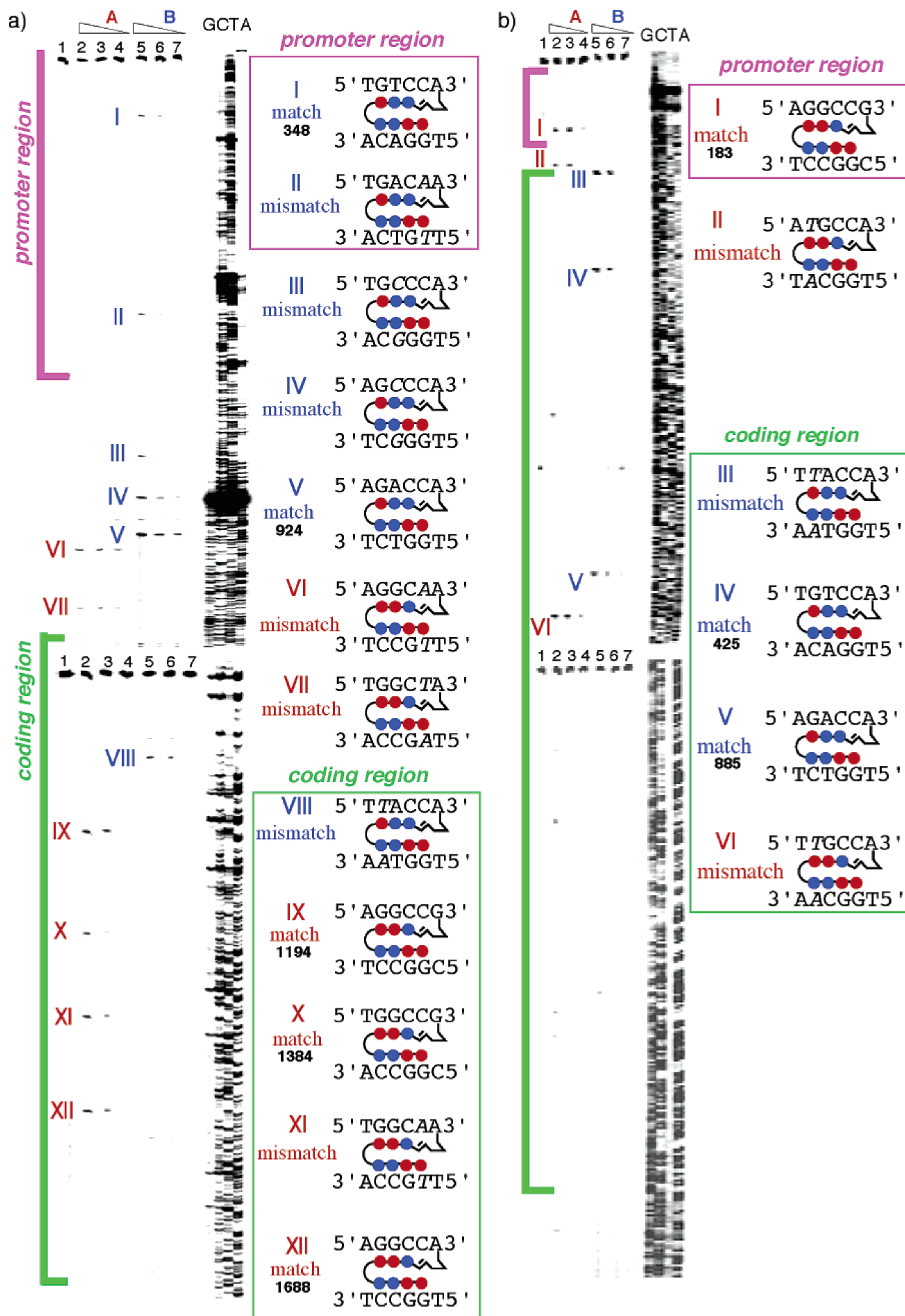

Figure 2. Thermally induced strand cleavage of 5′-Texas Red-labeled DNA fragments of renilla and firefly luciferase-template strands alkylated by polyamides A or B. (a) DNA fragments derived from the 5′-end-labeled renilla-luciferase template strand and (b) the 5′-end-labeled firefly luciferase template strand are shown. Lane 1: DNA control; lane 2−4: 50, 25, 12.5 nM of A; lane 5−7: 50, 25, 12.5 nM of B. Lanes G, C, T, and A contain Sanger-sequencing products.

on the template strand of coding region. The observed sequence-specificity on linear DNA may not be the same as the results on supercoiled DNA; however, our previous results of the alkylation of supercoiled DNA by alkylating Py−Im polyamides

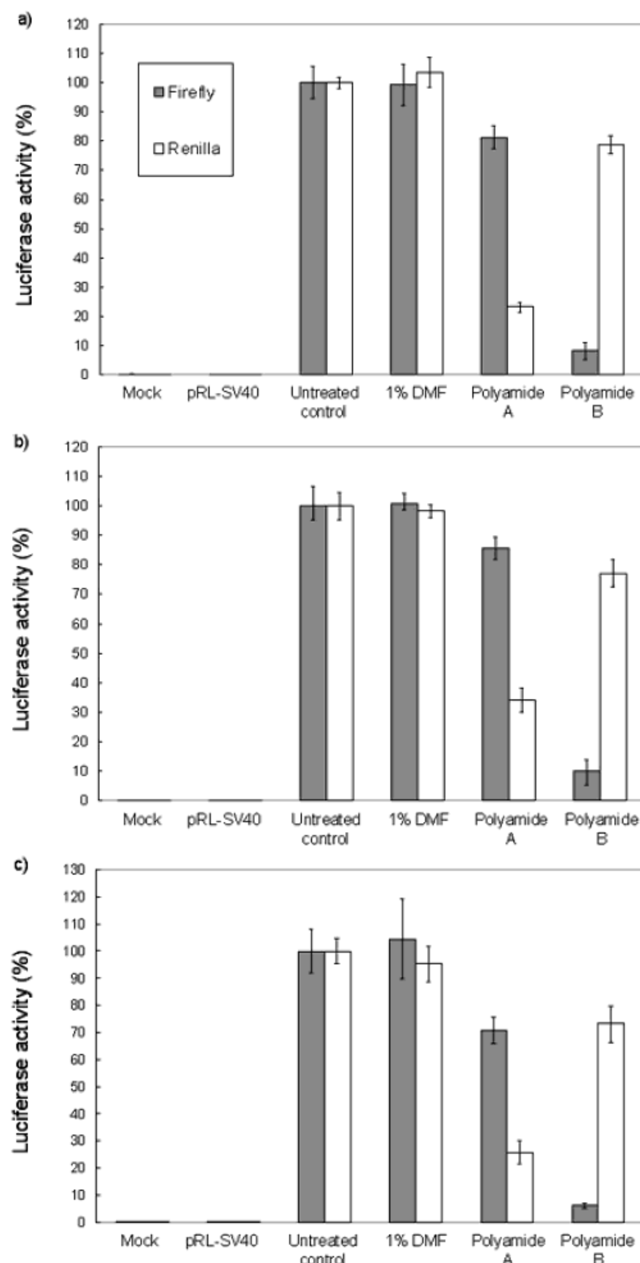

Figure 3. DNA alkylation of luciferase-template region by Py−Im polyamides induced specific inhibition of luciferase activity. The vectors were pre-alkylated in 1 μM polyamide solution containing 1% DMF in vitro. The pre-alkylated renilla or firefly luciferase vectors were transfected into HeLa (a), 293 (b), or NIH3T3 cells (c). The pRL-SV40 vector was used as a nonluciferase vector. The gray and white bars represent firefly and renilla luciferase activity, respectively. Data shown represent the means of three independent experiments; error bars indicate SDs.

suggest that the present sequence specificities observed on linear DNA fragments roughly correspond with those on supercoiled DNA.[13]

To examine the effects of these alkylation on intracellular transcription, each luciferase vector was alkylated by either polyamide A or B in vitro, and then each alkylated vector was transfected into HeLa, 293, and NIH3T3 cells by lipofection. After 24 h incubation, the individual luciferase activities were measured (Figure 3). The renilla luciferase vector alkylated with

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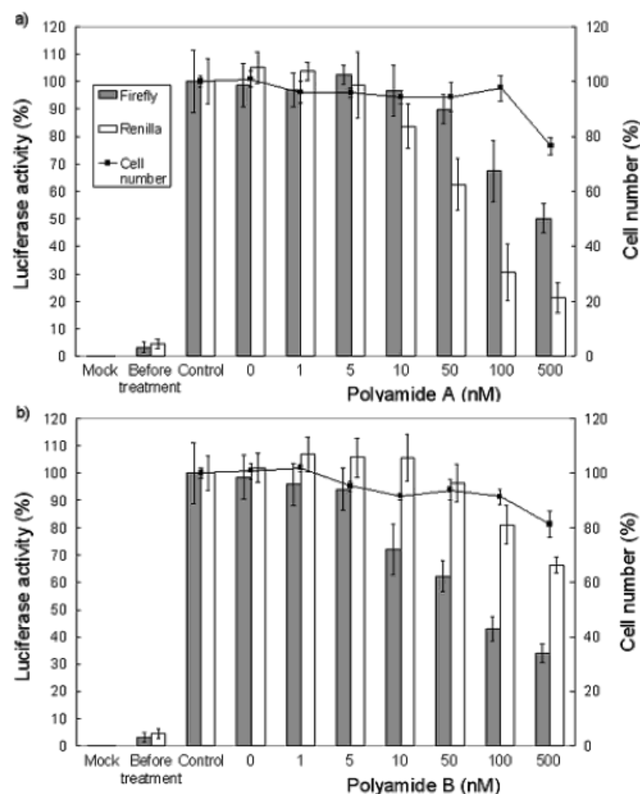

Figure 4. Selective silencing of renilla and firefly luciferase genes in polyamide-treated HeLa cells. Transfection of both luciferase vectors into HeLa cells was performed for 2 h (before treatment), and then the cells were cultured in a fresh growth medium containing 0−500 nM polyamides A (a) or B (b). Results are shown before and after treatment for 24 h. The control contained no DMF (1%) as a solvent for polyamides. The gray and white bars represent firefly and renilla luciferase activity, respectively, and the filled squares indicate cell numbers. Data shown represent the means of three independent experiments; error bars indicate SDs.

polyamide A led to reduction of activities to 26%, 34%, and 23%, of controls in HeLa, 293, and NIH3T3 cells, respectively. In marked contrast, the renilla luciferase vector alkylated by polyamide B caused weak reduction. Similarly, the activity of firefly luciferase alkylated by polyamide B was reduced to 8%, 10%, and 6% compared with controls in the HeLa, 293, and NIH3T3 cells, respectively. These results confirmed that the sequence-specific DNA alkylation of the luciferase-coding region shown in Figure 2 consistently led to the selective inhibition of gene expression. Although the polyamide B alkylated at least two sites on promoter region (I and II) and three sites on linker region (III−V) of renilla luciferase, polyamide A exhibited stronger gene silencing. This suggests that alkylation of the template strand of the coding region may cause stronger gene silencing, and that the promoter and linker regions might weakly influence luciferase gene expression.

To examine the selective gene silencing by polyamides A or B in vivo, HeLa cells were cotransfected with firefly and renilla luciferase vectors, and then the cells were treated with various concentrations of polyamides A or B for 24 h (Figure 4). The activities of the firefly and renilla luciferase in the transfected cells were then analyzed. The activity of renilla luciferase was reduced after treatment with polyamide A (Figure 4a) to 61%, 32%, and 21% of untreated controls after treatment with 50, 100, and 500 nM of polyamide A, respectively. In contrast, treatment with polyamide B showed opposite results to poly-

amide A (Figure 4b). There was significant reduction of the firefly luciferase activity to 72%, 62%, 42%, and 34% of controls at concentrations of 10, 50, 100, and 500 nM of polyamide B, respectively. Because the luciferase activity was not affected by the presence of alkylating polyamides (data not shown), this selective reduction of luciferase activity directly reflects selective gene silencing. Cell numbers were also assessed in treatment with polyamides A or B (Figure 4, filled squares).

The cell numbers were only slightly reduced (<25%) at concentrations of 500 nM for both polyamides A and B. These results suggest that treatment with the polyamides at a concentration of less than 500 nM is likely to have no effect on the proliferation of HeLa cells.

We observed here that nanomolar concentrations of Py−Im polyamides effectively cause specific gene silencing in mammalian cells by alkylation on coding regions of DNA. This specificity is remarkable, as two different hairpin polyamides require concentrations in the order of micromoles to inhibit HIV-1 replication.[8] However, our present results are limited to exogenous DNA sequence-specific DNA alkylation. In endogenous genes in chromatin, many kinds of chromosomal proteins are likely to interfere with DNA alkylation by the polyamides.

The endogenous repair of DNA alkylation sites should also be taken into account. To improve gene silencing, the design of a new type of alkylating Py−Im polyamides with expanding sequence specificity is currently under investigation.

Experimental Section

General Methods. The plasmids pGL3-Control (firefly luciferase), pRL-TK (renilla luciferase), pRL-SV40, and luciferase assay kits (Dual-Luciferase Reporter Assay System and Dual-Glo Luciferase Assay System) were purchased from Promega. Luciferase activity was assessed using an automatic luminometer model LUMINOUS CT-9000D (DIA-IATRON). The *Ex Taq* DNA polymerase was from Takara. The Thermo Sequenase Primer Kit for DNA sequencing was from Amersham Biosciences. MinElute PCR Purification Kit was from QIAGEN. The transfection reagents LipofectAMINE and E-Gel 1.2% agarose gel were from Invitrogen. The cell number assay reagent WST-8 was from Dojindo. Colorimetric assays were performed using a microplate reader model MPR-A4I (Tosoh). Inorganic pyrophosphatase and other biochemicals were from Sigma. All DNA oligomers were from JBioS. Electrospray ionization time-of-flight (ESI-TOF) mass spectra were recorded on a BioTOF II (Bruker Daltonics) mass spectrometer. High-resolution PAGE of Texas Red-labeled DNA was performed using a Hitachi 5500-S DNA sequencer. Polyamides were purified by high-performance liquid chromatography (HPLC) with a JASCO PU-980 HPLC pump, a UV-975 HPLC UV/VIS detector, and a Chemcobond 5-ODS−H column (4.6 × 150 mm). Polymerase chain reactions (PCR) were performed using a Perkin-Elmer GeneAmp PCR System 2400. The following conjugates were prepared by the previously reported procedures.[10a,b] **ImImPyPyγImImPyLDu86 (A):** HPLC purification using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 0−50% linear gradient, 33.7 min/40 min, 254 nm); ^1H NMR (500 MHz, [D_6]-DMSO) δ 12.33 (brs, 1H; NH), 10.31 (s, 2H; NH), 10.28 (s, 1H; NH), 9.91 (s, 2H; NH), 9.32 (s, 1H; NH), 9.31 (s, 1H; NH), 8.02 (brt, 1H; NH), 7.56 (d, J = 15.0 Hz, 1H; C*H*=CHCO), 7.53 (s, 1H; CH), 7.49 (s, 2H; CH), 7.39 (s, 1H; CH), 7.36 (s, 1H; CH), 7.26 (s, 1H; CH), 7.17 (s, 1H; CH), 7.15 (s, 1H; CH), 7.00 (s, 1H; CH), 6.89 (s, 1H; CH), 6.54 (d, J = 15.0 Hz, 1H; CH=C*H*CO), 6.51 (s, 1H; CH), 4.26 (m, 1H; NCH*H*), 4.17 (m, 1H; NC*H*H), 4.00 (s, 6H; NCH_3), 3.97 (s, 9H; NCH_3), 3.84 (s, 3H; NCH_3), 3.80 (s, 3H; NCH_3), 3.71 (s, 3H; OCH_3), 3.49 (m, 1H; CH), 3.18 (m, 2H; CH_2), 2.46 (s, 3H; CH_3), 2.37 (m, 2H; CH_2), 2.07 (m, 1H; CH*H*), 2.02 (s, 3H; $COCH_3$), 1.80 (m, 2H; CH_2), 1.13 (m, 1H; C*H*H); ESI-TOF−MS (monoisotopic) [M+H] 1270.502 (calcd 1270.504 for $C_{60}H_{64}N_{21}O_{12}$). **ImImPyPyγImPyPyLDu86 (B):** HPLC purification using a Chemcobond 5-ODS−H column (0.1% AcOH/CH_3CN 0−50% linear gradient, 34.1 min/40 min, 254 nm); ^1H NMR (500 MHz, [D_6]DMSO) δ 11.80 (brs, 1H; NH), 10.30 (s, 1H; NH), 10.27 (s, 1H; NH), 10.24 (s, 1H; NH), 9.94 (s, 1H; NH), 9.90 (s, 2H; NH), 9.32 (s, 1H; NH), 8.00 (brt, 1H; NH), 7.57 (d, J = 14.5 Hz, 1H; C*H*=CHCO), 7.56 (s, 1H; CH), 7.50 (s, 1H; CH), 7.45 (s, 1H; CH), 7.37 (s, 1H; CH), 7.27 (s, 2H; CH), 7.17 (s, 1H; CH), 7.15 (s, 2H; CH), 6.89 (s, 2H; CH), 6.55 (d, J = 14.5 Hz, 1H; CH=C*H*CO), 6.48 (s, 1H; CH), 4.27 (m, 1H; NCH*H*), 4.19 (m, 1H; NC*H*H), 4.03 (s, 3H; NCH_3), 4.00 (s, 3H; NCH_3), 3.97 (s, 3H; NCH_3), 3.85 (s, 6H; NCH_3), 3.80 (s, 3H; NCH_3), 3.72 (s, 3H; OCH_3), 3.70 (s, 3H; NCH_3), 3.50 (m, 1H; CH), 3.16 (m, 2H; CH_2), 2.47 (s, 3H; CH_3), 2.35 (m, 2H; CH_2), 2.14 (m, 1H; CH*H*), 2.03 (s, 3H; $COCH_3$), 1.75 (m, 2H; CH_2), 1.29 (m, 1H; C*H*H); ESI-TOF-MS (monoisotopic) [M+H] 1269.513 (calcd 1269.509 for $C_{61}H_{65}N_{20}O_{12}$).

Preparation of 5′-Texas Red-Modified DNA Fragments. The 5′-Texas Red-modified four DNA fragments renilla former sequence, renilla latter sequence, firefly former sequence and firefly latter sequence were prepared by PCR using following 5′-Texas Red-modified 20-mer primers: 5′- CCAGAGTGTGACCTTTTCGG-3′ and 5′-*GCGCTACT-GGCTCAATATGTG-3′ (for 1000 bp renilla former sequence), 5′-CATGGTAACGCGGCCTCTTC-3′ and 5′- *CTTGTTTATTGCAGC-TTATAATGG-3′ (for 936 bp renilla latter sequence), 5′-GCGATC-TGCATCTCAATTAGTC-3′ and 5′-*CTCCTCAGAAACAGCTCT-TCTTC-3′ (for 1065 bp firefly former sequence), 5′-GATATGTGG-ATTTCGAGTCGTC-3′ and 5′-*CATGTCTGCTCGAAGCGG-3′ (for 925 bp firefly latter sequence). Fragments were purified by filtration using QIAGEN and their concentrations were determined by UV absorption. The asterisk indicates Texas Red-modification and the nucleotide numbering starts with the replication site.

High-Resolution Gel Electrophoresis. The 5′-Texas Red-labeled DNA fragments (10 nM) were alkylated by various concentrations of polyamide **A** and **B** in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at room temperature for 5 h. The reaction was quenched by the addition of calf thymus DNA (1 mM, 1 μL) and heating for 5 min at 90 °C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 7 μL loading dye (formamide with fuchsin red), heated at 95 °C for 20 min and then immediately cooled to 0 °C. A 2-μL aliquot was subjected to electrophoresis on a 5% denaturing polyacrylamide gel using a Hitachi SQ5500-E DNA Sequencer.

Cell Lines. HeLa, 293, and NIH3T3 are human cervical epithelial carcinoma, human kidney epithelial, and mouse fibroblast cell lines, respectively. These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in 95% air.

Preparation of Alkylated Plasmids. The pGL3-Control and pRL-TK vectors (20 nM each) were alkylated with 1 μM polyamide in a 50 mM HEPES buffer (pH 7.0) containing 1% dimethylformamide (DMF) at 37 °C for 2 h. After incubation, the modified vectors were precipitated by ethanol, and then dried by vacuum centrifugation (the yield of vectors was more than 90%). The pellet was dissolved in TE buffer and stored at −20 °C until use. Alkylation of the vectors was confirmed by thermally induced cleavage production.

Transfection and Luciferase Assay. HeLa, 293, and NIH3T3 cells were cultured on 24-well plates at 1.0×10^5, 1.2×10^5, and 5.0×10^4 cells/well, respectively. Either 0.4 μg each of alkylated pGL3-Control and nonalkylated pRL-TK, or alkylated pRL-TK and nonalkylated pGL3-Control vectors were cotransfected into the cells with 1 μL LipofectAMINE reagent per well. The nonalkylated vectors were transfected as internal controls in the dual luciferase assay system. The pRL-SV40 plasmid was employed as a negative control vector. The

medium containing transfection reagent was removed 2 h later, and cells were washed once with phosphate-buffered saline (PBS), then covered with fresh growth medium. After 24 h culture, the cells were treated with 100 μL of lysis buffer, and luciferase activity was determined using the manufacturer's instructions for the Dual-Luciferase Reporter Assay System and an automatic luminometer CT-9000D.

Treatment of HeLa Cells with Polyamide. Both 0.1 μg pGL3-Control and 0.1 μg pRL-TK vectors were transfected into HeLa cells at a density of 5×10^3 cells/well (using 96-well plates) with 0.2 μL LipofectAMINE reagent. After 2 h incubation, the medium was removed; cells were washed once with PBS, and then cultured in a fresh growth medium containing polyamides A or B (0, 1, 5, 10, 50, 100, and 500 nM with 1% DMF). After 24 h, luciferase activity was determined using the manufacturer's instruction by the Dual-Glo Luciferase Assay System and an automatic luminometer model CT-9000D. Cell number was also assessed by colorimetric assay with WST-8. Optical density was measured using a microplate reader MPR-A4I at wavelengths of 450 and 600 nm.

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Supporting Information Available: The putative alkylating sites of renilla and firefly luciferase gene and promoter region in pRL-TK (Figure 1S) and pGL3-control (Figure 2S) vector. (PDF). This material is available free of charge via Internet at http://pubs.acs.org.

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