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# Alkylation of template strand of coding region causes effective gene silencing

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## ABSTRACT

**We recently developed a new type of pyrrole (Py)–imidazole (Im) polyamide–tetrahydrocyclopropabenzindolone (CBI) conjugate with an indole linker as a stable sequence-specific alkylating agent. In this study, we investigated the gene silencing activities of polyamides A, B and C, which selectively alkylate specific sequences in the promoter region, non-coding strand and coding strand, respectively, of the green fluorescent protein (GFP) gene. GFP vectors were transfected into human colon carcinoma cells (HCT116), and the cells were treated with 100 nM of the polyamides for 24 h. Fluorescence microscopy indicated that a significant reduction of GFP fluorescence was only observed in the cells that were treated with polyamide C. In clear contrast, polyamides A and B did not show such activity. Moreover, real-time PCR demonstrated selective reduction of the expression of GFP mRNA following treatment with polyamide C. These results suggest that alkylating Py–Im polyamides that target the coding strand represent a novel approach for sequence-specific gene silencing.**

## INTRODUCTION

Minor groove-binding N-methyl pyrrole (Py) and N-methyl imidazole (Im) polyamides uniquely recognize each of the four Watson–Crick base pairs (1–6). 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. The binding constant and sequence-specificity of the Py–Im hairpin polyamides are similar to that of a transcription factor (7–10). Therefore, many genes, including vascular endothelial growth factor (11) and human estrogen-related receptor 2 (12), are silenced by competitive binding of Py–Im hairpin polyamides to their regulatory sequences (13,14). Gene expression is usually controlled by a combination of multiple common trascription factors. Inhibition of gene expression through the binding of Py–Im polyamides to regulatory sequences must be unique to a specific gene, it should contain part of the recognition sequence of the transcription factor together with the unique flanking sequences. In contrast, targeting Py–Im polyamide to the coding region is more straightforward when selecting a unique sequence. However, the inhibition of transcription by the binding of simple Py–Im hairpin polyamides in the coding region is difficult, because the polyamides are removed from duplex DNA during transcription (15). To overcome this problem, we and others have designed and synthesized various alkylating Py–Im polyamides that show sequence-specific DNA alkylation (16–20). 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 rules of the Py–Im polyamides (16,19). Recently, we demonstrated that the alkylation of the template strand in the coding region by Py–Im polyamide–cyclopropylpyrroloindole (CPI) conjugates with a vinyl linker resulted in the production of truncated mRNA, effectively inhibiting transcription *in vitro* (21). Furthermore, we demonstrated that alkylating Py–Im polyamides selectively induced gene silencing of renilla and firefly luciferases in mammalian cells by specific alkylation (22). These results suggest that alkylating polyamides have the ability to suppress specific gene expression. Recently, we developed new types of Py–Im tetrahydrocyclopropabenzindolone (CBI) conjugates with indole linkers. Because indole–CBI has increased chemical stability under acidic and basic conditions, the conjugates were readily synthesized by coupling of Py–Im polyamides prepared by automated solid phase synthesis with indole and CBI (23). In this study, we used fluorescent microscopy and real-time PCR to examine whether the new types of alkylating polyamides selectively induce green fluorescent protein (GFP) gene silencing.

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## MATERIALS AND METHODS

### Cell culture and transfection

HCT116 cells were maintained in RPMI1640 (Nacalai Tesque) supplemented with 10% fetal bovine serum (JRH Biosciences), and penicillin (100 U/ml)–streptomycin (100 μg/ml) at 37°C in a 5% $CO_2$ atmosphere. Cells were transfected with GFP vectors (pAce-Green N1, Novagen) using the LipofectAMINE2000 reagent (Invitrogen), following the manufacturer's instructions.

### Cytotoxicity assay

The compounds PyImPyPy-γ-ImPy–indoleCBI (A), ImImPyPy-γ-ImPy–indoleCBI (B) and ImPyPyPy-γ-ImPy–indoleCBI (C) were synthesized by previously reported procedures (18,19). Colorimetric assays using WST-8 (Dojindo) were performed on 96-well plates. HCT116 cells were plated on the well at ~40% confluence in 50 μl of culture medium. One day later, the medium was changed to 100 μl of fresh medium containing 10, 50, 100, 500 and 1000 nM of polyamides and 0.1% *N*,*N*-dimethyl formamide (DMF). After treatment with the polyamides for 24 h, 10 μl of WST-8 reagent was added into each well and incubated for 2 h at 37°C. Absorbance was then measured at 450 and 600 nm using an MPR-A4I microplate reader (Tosoh).

### Treatment of HCT116 cells with polyamides and fluorescence microscopic analysis

The GFP vectors were transfected into HCT116 cells for 2 h. The medium was then removed, cells were washed once with phosphate-buffered saline (PBS) and then cultured in culture medium containing 100 nM of polyamides A–C with 0.1% DMF. After 24 h, GFP fluorescence was visualized under ultraviolet (UV) illumination using a CKX41 microscope equipped with an FITC filter set, a DP30BW digital camera and DP Manager Software (Olympus).

### DNA amplification

All primers for DNA amplification were purchased from Proligo. To obtain the DNA fragment encoding the GFP gene, PCR was performed in a final volume of 50 μl containing 1 ng of GFP vector DNA, 200 nM deoxynucleotide primer set, 200 μM deoxynucleotide triphosphates, 2 U *Taq* DNA polymerase and 1× ThermoPol Reaction Buffer (New England Biolabs) according to the following conditions: 94°C for 5 min followed by 35 cycles of denaturing at 94°C for 30 s, annealing at the set temperature for each primer set for 30 s, and extension at 72°C for 60 s, followed by a final extension step at 72°C for 7 min. Unless otherwise noted, the PCR primers and annealing temperatures were as follows: for the promoter region (pGFP-F: 5′-TAGTTATTAATAGTAATCAAT-3′, pGFP-R: 5′-CAGATGAACTTCAGGGTCAG-3′ and 55°C) and for the coding region (cGFP-F: 5′-AAGCTTCCACCATGAGCAAGG-3′, cGFP-R: 3′-AAGCTTTCAGCTCATCTTGTA-5′ and 60°C). Amplification cycles were carried out with an iCycler (Bio-Rad). Products were identified by separation on 1.0% TBE agarose gels with 0.5 μg/ml ethidium bromide and using a 100 bp ladder marker (New England Biolabs) and visualization under UV illumination.

### Cloning of amplified DNA fragments

The PCR products were ligated into pGEM-T Easy vectors (Promega) according to the manufacturer's instructions. *Escherichia coli* DH5α competent cells were transformed and cultured on Luria–Bertani (LB) plates with 100 μg/ml ampicillin and 32 μg X-gal/400 μg Isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 37°C. White colonies were identified by colony-direct PCR using the following primer sets: pGFP-F and T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′), pGFP-R and T7, cGFP-F and T7, cGFP-R and T7, in the same reaction mixtures as above. The reaction mixtures were incubated at 94°C for 5 min, followed by 30 incubation cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, with a final extension step of 72°C for 7 min. The appropriate colonies were selected for transfer to 5 ml of LB medium with 100 μg/ml ampicillin and cultured overnight at 37°C. The plasmids with inserts were extracted using GenElute™ Plasmid Miniprep Kit (Sigma Aldrich) and identified by PCR (program and reaction mixtures as above).

### Preparation of 5′-Texas Red-modified DNA fragments and high-resolution gel electrophoresis

5′-Texas Red-modified DNA fragments of GFP were prepared by PCR using primer sets of 5′-Texas Red-labeled T7 promoter primer and either pGFP-F (template strand of promoter region), pGFP-R (complementary strand of promoter region), cGFP-F (template strand of coding region) or cGFP-R (complementary strand of coding region), and containing 1 ng of each plasmid. PCR steps and other reagents were as above. The resultant fragments were purified using the GenElute™ PCR Clean-up Kit (Sigma Aldrich) and concentrations were determined by 260/280 nm absorbances. The 5′-Texas Red-labeled DNA fragments (10 nM) were alkylated by various concentrations of polyamides A–C in 10 μl of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at room temperature for 10 h. The reaction was quenched by the addition of 1 μg of calf thymus DNA followed by heating for 5 min at 90°C. The DNA was recovered by vacuum centrifugation. The pellet was dissolved in 5 μl loading dye (formamide with fuchsin red), heated at 95°C for 30 min and then immediately placed on ice. A 2 μl aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a SQ5500-E DNA Sequencer (Hitachi).

### RNA extraction and first strand cDNA synthesis

The GFP vectors were transfected into HCT116 cells and treated with polyamides as above. The SV Total RNA Isolation System (Promega) was used for the isolation of total RNA from the HCT116 cells (about $2 \times 10^6$ cells). The purity of the RNA preparations was checked using the 260/280 nm ratio (range 1.8 ± 0.2). First strand cDNA synthesis was carried out with Omniscript RT kit (Qiagen) and random primers (Promega) in a total volume of 80 μl. Reverse transcription was performed at 37°C for 60 min followed by 72°C for 15 min. We confirmed that there was no contaminant genomic or vector DNA in the cDNA solution (Supplementary Figure 1).

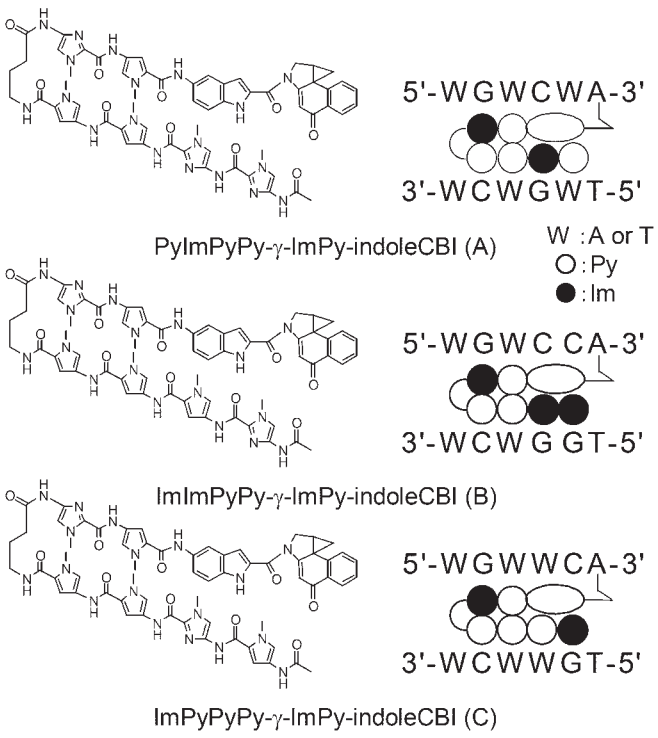


**Figure 1.** Structures of indole–CBI conjugated Py–Im polyamides **A**–**C**, and schematic representations of their DNA alkylation. Open circles, filled circles and ellipses indicate pyrroles, imidazoles and indoles, respectively. W indicates A or T. CBI, which is an alkylating moiety, adducts to the adenine N3 position.

### Real-time PCR

Real-time PCR and subsequent calculations were performed with the 7300 Real-Time PCR System (Applied Biosystems), which detects the signal emitted from fluorogenic probes during PCR. Primers and probes were designed according to guidelines from Applied Biosystems with the help of the Primer Express 3.0 software (Applied Biosystems). The specificity of the nucleotide sequences chosen was confirmed by BLAST searches. Real-time PCR was performed with TaqMan Universal PCR Master Mix reagents (Applied Biosystems). The PCR mixture contained 1× TaqMan Universal PCR Master Mix, 200 nM forward and reverse primers, 100 nM probe and 0.25 μM AmpErase uracyl *N*-glycosylase (UNG) in a total volume of 25 μl. PCR was performed using 2.5 μl of the first strand cDNA mix. After 2 min at 50°C, to permit UNG cleavage, AmpliTaq Gold was activated by a 10 min incubation at 95°C. Each of the 40 PCR cycles consisted of a 15 s denaturation step at 95°C and a hybridization step, with probes and primers and for DNA synthesis, for 1 min at 60°C. The primer sets were as follows: for GFP (rtGFP-F: AACTACAACGCCCACAATGTGT, rtGFP-R: CGGATCTTGAAGTTCACCTTGAT and probeGFP: 5′FAM-CATCATGACCGACAAGGCCAAGAATG-3′TAMRA), and for β-actin (rtACTB-F: 5′-CCCAGCCATGTACGTTGCTA-3′ rtACTB-R: 5′-TCACCGGAGTCCATCACGAT-3′ and probeACTB: 5′FAM-ACGCCTCTGGCCGTACCACTGG-3′TAMRA). The standard curve was obtained using 2.5 μl volumes of serial dilutions of pooled first strand cDNA from 0.1% DMF-treated control cells.

## RESULTS AND DISCUSSION

### Sequence-specific alkylation by Py–Im polyamide CBI conjugates with indole linker and their cytotoxicities

PyImPyPy-γ-ImPy–indoleCBI (A), ImImPyPy-γ-ImPy–indoleCBI (B) and ImPyPyPy-γ-ImPy–indoleCBI (C) were respectively designed to selectively alkylate the promoter, complementary strand and template strand regions of the GFP gene based on the recognition rules of Py–Im polyamides. Polyamides A, B and C recognize the sequence of 5′-WGWCWA-3′, 5′-WGWCWA-3′ and 5′-WGWCWA-3′ (W = A or T), respectively, and adduct to the adenine N3 position, as described in Figure 1. We first examined the sequence-specificity of polyamides A–C using denaturing polyacrylamide gel electrophoresis. The sequences of the alkylated regions were determined by thermal cleavage of the DNA strand at the alkylated sites. All N3-alkyl adducts are cleaved quantitatively to produce cleavage bands under the heating conditions used. We confirmed the alkylation sites of polyamides A–C by using 5′-Texas Red-labeled DNA fragments of GFP alkylated by polyamides A–C, cleaved by heating and followed by sequence gel electrophoresis (Figures 2 and 3). Importantly, it was revealed that the template strand of the GFP sites in the coding region was specifically alkylated only by polyamide C. Polyamide C displayed four (698, 1166, 1331, 1385) alkylation sites, whereas polyamides A and B did not alkylate any site in this region. Conversely, it was observed that polyamides A, B and C showed one (555), two (297, 362) and one (39) sites in the promoter region of the complementary strand of GFP, respectively, and polyamides A, B and C displayed one (1017), five (703, 760, 817, 1285, 1303) and one (817) sites in the coding region, respectively. Moreover, polyamides A, B and C alkylated four (345, 408, 459, 573), three (51, 345, 365) and one (200) sites in the promoter region, respectively. Thus, it was clearly demonstrated that polyamides A, B and C predominantly alkylate the promoter region, complementary strand and template strand of GFP, respectively. In addition, we investigated the cytotoxic effects of these polyamides (Figure 4). HCT116 cells were treated with 10, 50, 100, 500 and 1000 nM polyamides for 24 h. It was shown that all three polyamides were highly toxic at 500 nM, whereas the cell viability was slightly reduced at 100 nM. Moreover, the viability of HCT116 cells was substantially reduced (∼50%) by treatment with 10 nM polyamides for 48 h (data not shown). Therefore, we considered that using the polyamides at 100 nM and treating for 24 h was optimum for the analysis of gene silencing effects in this study.

### Effective GFP gene silencing was induced by specific alkylation of template strand of coding region

Using these alkylating Py–Im polyamides, we next investigated whether polyamide C can induce specific gene silencing of GFP. In this experiment, GFP vectors were transfected into HCT116 cells followed by treatment with polyamides A–C. After 24 h incubation, the cells were monitored by fluorescent microscopy (Figure 5). Transfection of HCT116 cells with pAce-Green N1 followed by 24 h incubation resulted in expression of GFP protein (Figure 5d). When cells were treated with the polyamides (100 nM) after transfection, significant polyamide-mediated GFP gene silencing was observed in

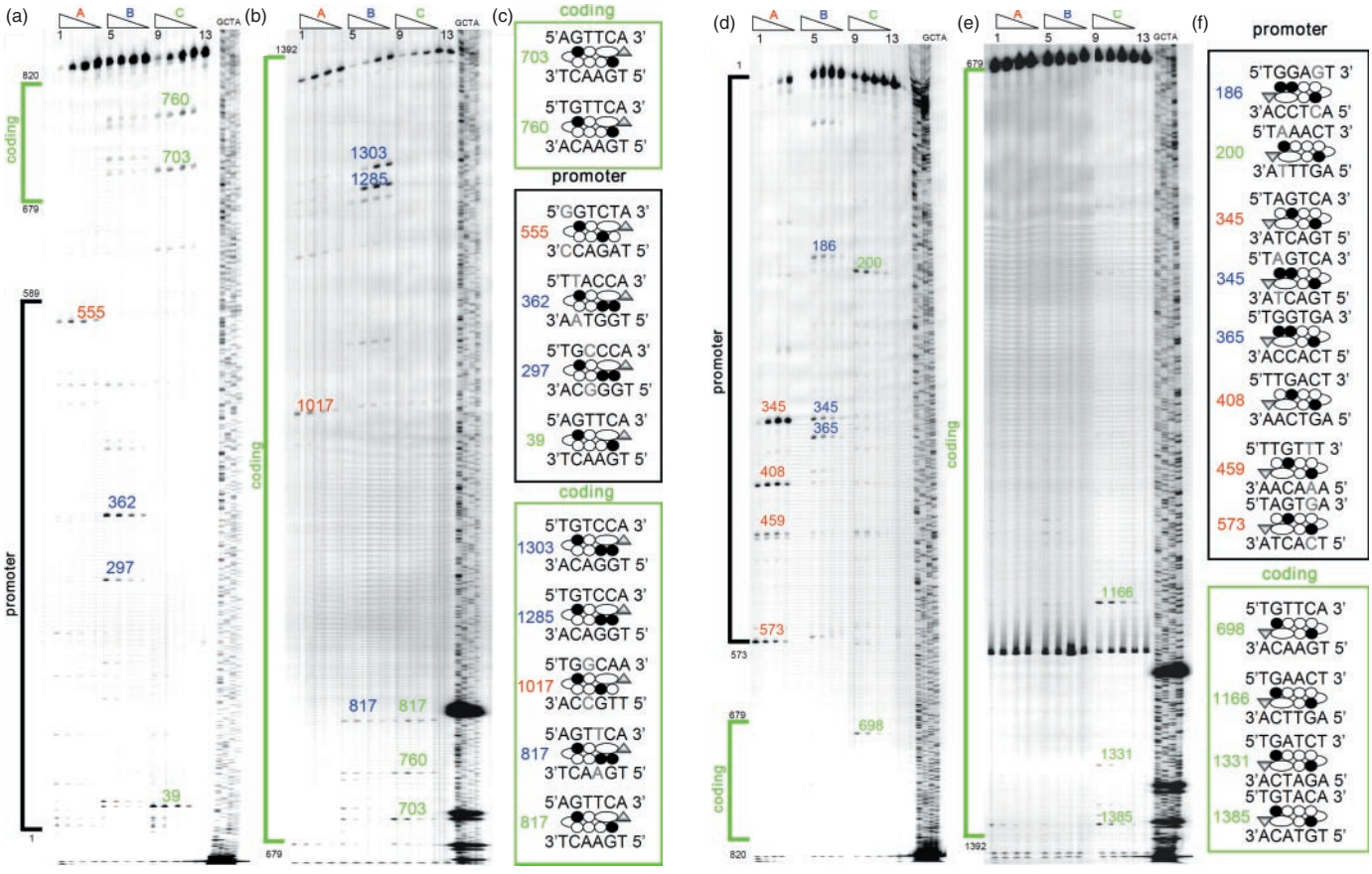


**Figure 2.** Thermally induced strand cleavage of 5′-Texas Red-labeled DNA fragments of GFP former (**a** and **d**) and latter (**b** and **e**) sequences of GFP alkylated by polyamides A, B and C. Results in the sequence of complementary strand (a–c) and coding strand (d-f) are displayed. Lanes 1–4: 100, 50, 25, 12.5 nM of A; lanes 5–8: 100, 50, 25, 12.5 nM of B, lanes 9–12: 100, 50, 25, 12.5 nM of C and lane 13: DNA control. Lanes G, C, T and A contain Sanger-sequencing products (**c** and **f**). Sequences containing alkylation sites are represented. Mismatched sequences are indicated by gray letters.

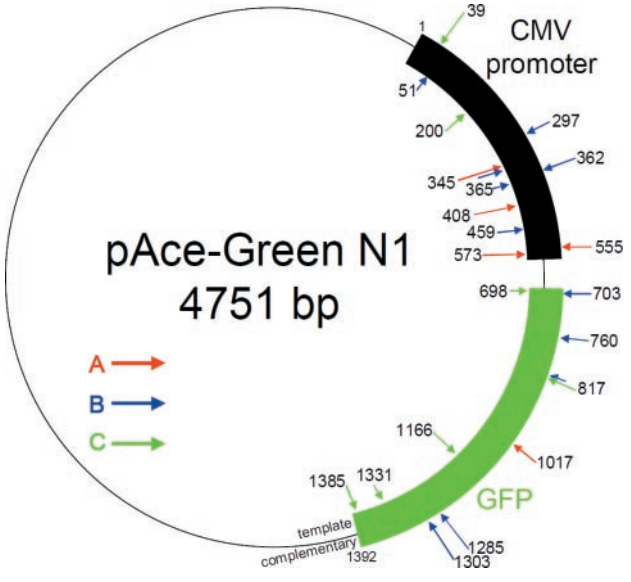


**Figure 3.** Map of the GFP gene and promoter region in the pAce-Green N1 vector. The alkylation sites of polyamides A, B and C in Figure 2 are indicated by the red, blue and green arrows, respectively. The arrows outside and inside indicate the complementary and template strands, respectively. The sizes of the arrows indicate the efficiency of alkylation.

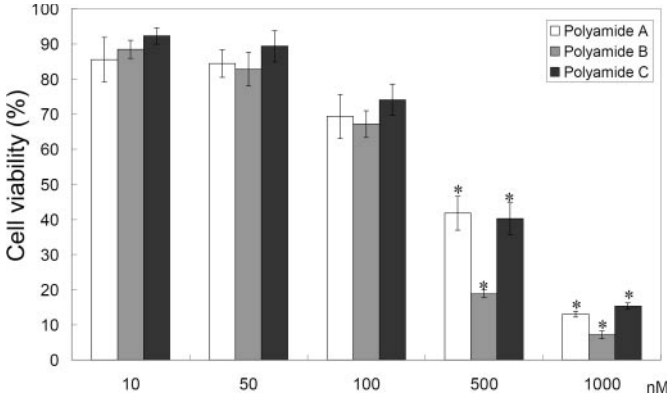


**Figure 4.** Cytotoxic effects of indole–CBI conjugated Py–Im polyamides. HCT116 cells were treated with Indole–CBI conjugated Py–Im polyamides containing 0.1% DMF for 24 h. After treatment, cell viability was determined by colorimetric assay using WST-8 and estimated by comparison with untreated control as 100%. White, gray and black blocks indicate the treatment of polyamide A, B and C, respectively. Error bars, standard deviation of the means of triplicate samples. $*P < 0.05$ by unpaired Student's *t*-test compared with each 100 nM.

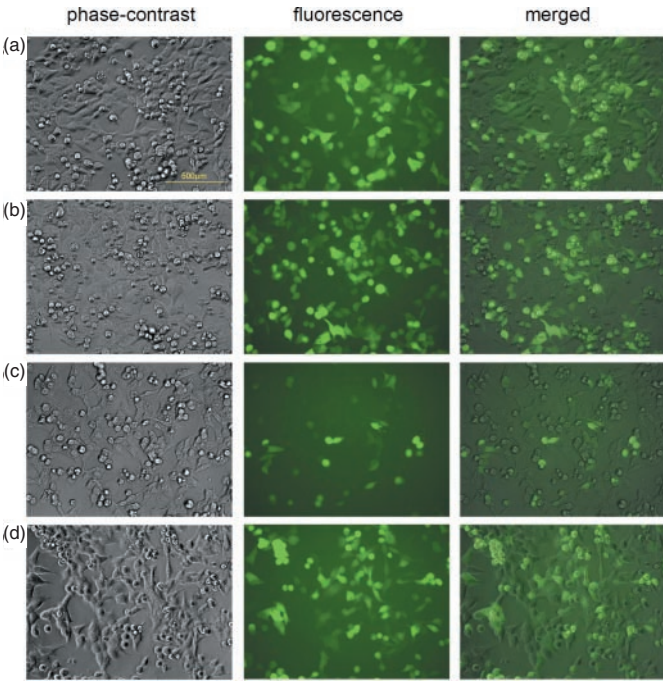


**Figure 5.** Selective silencing of GFP genes in polyamide-treated HCT116 cells was observed by fluorescent microscopy. Transfection of GFP vectors into HCT116 cells was performed for 2 h, and the cells were then cultured in fresh growth medium containing 0.1% DMF with 100 nM polyamides A (**a**), B (**b**), C (**c**) or no polyamide (**d**). Results are shown after treatment for 24 h. The left, middle and right panels show views from phase-contrast, fluorescent microscopy, and their merged image, respectively. The scale bar indicates 500 μm.

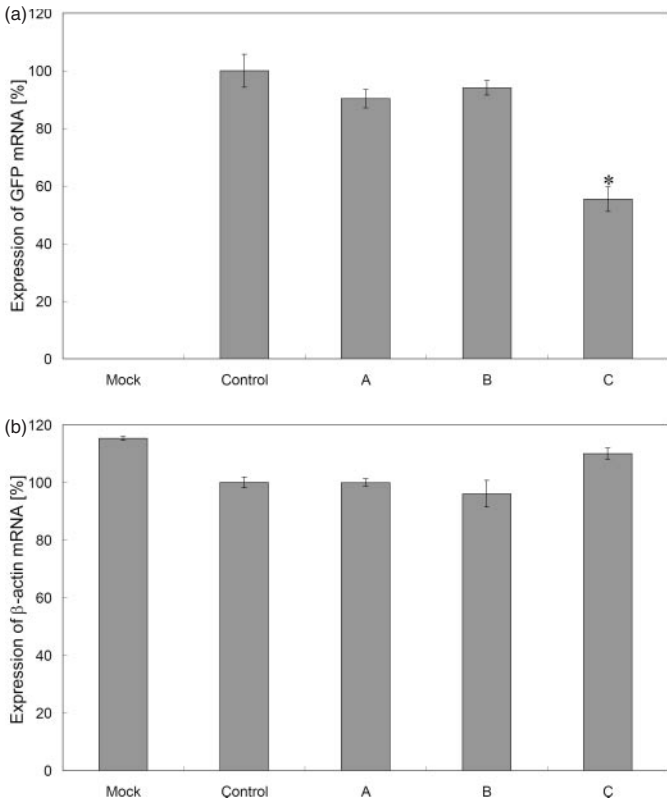


**Figure 6.** Results of real-time quantification of PCR GFP (**a**) and β-actin (**b**) mRNAs in HCT116 cells treated with polyamides A–C. To evaluate the amount of transcribed GFP mRNA, TaqMan real-time PCR was performed with the 7300 Real-Time PCR System. The amount of GFP and β-actin mRNAs in the HCT116 cells were measured using pairs of primers with the TaqMan probes for each gene. The amount of mRNAs in HCT116 cells treated with 100 nM polyamides for 24 h was calculated with reference to the cells treated with 0.1% DMF (Control), set as 100%. Compared with the control, HCT116 cells treated with polyamide C showed significantly decreased GFP mRNA expression. The cells which the GFP vectors were not transfected (Mock) did not express GFP mRNA. Error bars, standard deviation of the means of triplicate samples. $*P < 0.05$ by unpaired Student's *t*-test compared with control.

the case of polyamide C (Figure 5c), whereas polyamides A and B (Figure 5a and b, respectively) did not show such an effect. In lower concentration such as 50 and 10 nM, the effects of gene silencing by polyamide C were relatively reduced (data not shown).

## Quantification of GFP mRNA by real-time PCR

In addition, we evaluated the expression of GFP mRNA by real-time PCR. To examine the GFP mRNA levels, total RNA was collected from cells transfected with GFP vectors followed by treatment with polyamides A–C for 24 h, and cDNAs were synthesized by reverse transcription. To quantify the amount of GFP mRNA, we performed TaqMan-probe-mediated real-time PCR. GFP and β-actin mRNA levels were measured individually by means of FAM fluorescence (Figure 6). The amount of GFP mRNA was significantly decreased in the HCT116 cells treated with 100 nM polyamide C for 24 h (about 50%). In contrast, the amount of GFP mRNA was little reduced by polyamides A and B. Furthermore, the levels of β-actin mRNAs were not reduced significantly. These results were consistent with those shown in Figure 5. These results demonstrate that alkylation of the template strand of the coding region causes effective and specific gene silencing. In clear contrast, alkylation of the promoter region and the complementary strand of the coding region did not induce effective inhibition of transcription.

It is believed that artificial molecular switches that control the expression of any gene of interest will be powerful tools for studying the function of a gene. For example, the advent of RNA interference technology has led to extraordinary advances in via identifying the function of many genes (24). In addition, other techniques exist for sequestering the mRNA transcribed from DNA hybridization with a complementary base sequence, including using antisense oligonucleotide, ribozymes and peptide nucleic acids (PNA), which have been shown to prevent RNA translation *in vitro*. However, for therapeutic use these methods still need to clear several hurdles, such as their poor cell permeability, lack of safe delivery methods and unwanted side effects. Alkylating Py–Im polyamides are cell-permeable small molecules capable of recognizing specific, predetermined sequences of DNA (25). This study clearly demonstrates that they effectively cause specific gene silencing in mammalian cells by alkylation of coding regions of DNA as shown in Figure 5.

Recently, we demonstrated that alkylating Py–Im polyamides, which differ only in that the C–H bond is substituted by an N atom in the second ring, showed

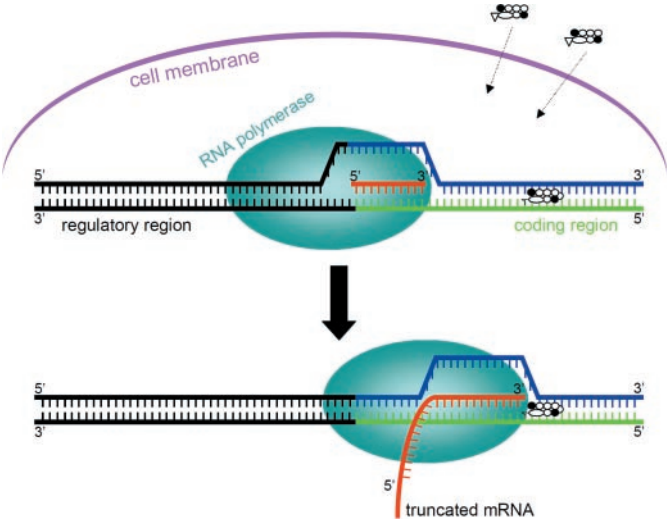


**Figure 7.** Schematic representation of the mechanism of gene silencing by alkylating polyamides. Alkylating Py–Im polyamides are small cell-permeable molecules that bind in the minor groove of double-stranded DNA. When the alkylating polyamides localize in the DNA, covalent binding between the alkylating moiety and the adenine N3 position are rapidly formed. When mRNA is synthesized by RNA polymerase on the DNA that is specifically alkylated on the template strand, RNA polymerization is efficiently inhibited at the alkylation site resulting in production of truncated mRNA. Thus, alkylating Py–Im polyamides effectively cause gene silencing. Black, blue, green and red lines indicate the DNA of the regulatory region, complementary strand, template strand and mRNA, respectively.

significantly different cytotoxicities in human cancer cell lines (26). Moreover, five analogous conjugates of indole–secoCBI and Py–Im polyamides, including A, B and C, showed different growth inhibition activity against 10 different cell lines (27). These results suggest that differences in sequence specificity of DNA alkylation leads to marked differences in biological activity and the alkylating Py–Im polyamides could lead to new types of cancer chemotherapy (Figure 7).

In conclusion, alkylating Py–Im polyamides are a new type of small-molecule-based gene silencer. Alkylation of the coding strand specifically induced the inhibition of transcription at the alkylation sites. Our results are currently limited to exogenous DNA sequence-specific DNA alkylation because the polyamides in this study have only 6 bp recognition sites. In this study we successfully confirmed by fluorescent microscopy and by the quantification of GFP mRNA by real-time PCR that the gene silencing was effectively caused by sequence-specific alkylation of the template strand of the coding region. Importantly, the evidence that alkylation of the template strand of the coding region causes effective gene silencing conclusively confirmed our previous observations, (22) as the template strand of the coding region of the GFP gene was specifically alkylated only by polyamide C. This is in contrast to our previous report, in which the luciferase activity of non-targeted sites was also reduced slightly, presumably due to the mismatched alkylation (22). Thus, this study reports an important advance in the targeting of endogenous cellular genes by Indole–CBI-type alkylating polyamides with long base sequence recognition. We are currently in the process of elongating the sequence-specificity of this new type of alkylating Py–Im polyamides, which recognizes more than 10 bp. Using these polyamides with longer sequence recognition, we are aiming to target specific genes and improve current chemotherapy.

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*Conflict of interest statement*. None declared.

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