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Potent activity against K562 cells by polyamide–seco-CBI conjugates targeting histone H4 genes

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ABSTRACT

We designed and synthesized conjugates between pyrrole–imidazole polyamides and *seco*-CBI that alkylate within the coding regions of the histone H4 genes. DNA alkylating activity on the histone H4 fragment and cellular effects against K562 chronic myelogenous leukemia cells were investigated. One of the conjugates, **5-CBI**, showed strong DNA alkylation activity and good sequence specificity on a histone H4 gene fragment. K562 cells treated with **5-CBI** down-regulated the histone H4 gene and induced apoptosis efficiently. Global gene expression data revealed that a number of histone H4 genes were down-regulated by **5-CBI** treatment. These results suggest that sequence-specific DNA alkylating agents may have the potential of targeting specific genes for cancer chemotherapy.

1. Introduction

DNA alkylating agents have shown high anticancer activity against a variety of solid tumors and leukemias and been used as drugs for chemotherapy.[1,2] However, classical DNA alkylating agents exhibit little sequence specificity, which causes significant DNA damage not only in cancer cells but also in normal cells. The undesired side effects in normal tissues and hematopoietic progenitor cells limit the clinical dose for treatment of cancer.[3–6] DNA alkylating agents that recognize DNA sequences at a single base-pair level can target specific genes and address the problems generated by non-specific DNA alkylation. Pyrrole (Py)–imidazole (Im) polyamides are a class of small molecules binding to the DNA minor groove at predetermined sequences.[7,8] The recognition rules are that an antiparallel pairing of Im opposite Py (Im–Py) recognizes a G–C base pair; Py–Im recognizes a C–G base pair; and, Py–Py recognizes A–T or T–A base pairs. Py–Im polyamides are cell permeable and inhibit a variety of transcription factors binding to DNA. Conjugates between DNA alkylating agents and polyamides selectively alkylate DNA in the proximity of the sequences recognized by polyamides.[9–11] Dervan and co-workers demonstrated that a polyamide–*seco*-CBI conjugate that alkylates SV40 viral DNA inhibited DNA replication under both cell-free and cellular conditions.[12,13] Starting from the cooperative alkylation by duocarmycin A and distamycin A,[14] we have demonstrated that conjugates between Py–Im polyamides and segment A of duocarmycins have efficient DNA alkylation activity at specific sequences.[15] We have also demonstrated that selective alkylation by an alkylating Py–Im polyamide on the template strand of a gene coding region resulted in the inhibition of transcription and the silencing of reporter luciferase gene.[16,17] The Dervan and Gottesfeld groups constructed a small library of conjugates between polyamides with different recognition sequences and chlorambucil (Chl). One of these conjugates, **1R-Chl**, arrested the growth of several cancer cell lines. They demonstrated that **1R-Chl** alkylated at the coding region of the histone H4c gene in vitro and in cultured cells, and inhibited its transcription.[18] H4c is highly expressed in SW620 cells, K562 cells, and in several cancer cell lines in comparison to its expression in normal cells.[18,19] Treatment with H4c siRNA affected the morphology and proliferation without effect on cell viability.[18] Further studies revealed that the depletion of histones modified chromatin structure to arrest cell proliferation at the G_2/M phase by extensive DNA alkylation.[20] Recently, **1R-Chl** also showed inhibition of proliferation against chronic myelogenous leukemia (CML) cell line K562 and high dose tolerance in the murine model.[21] These studies suggested that alkylating agents targeting histone genes can be effective for cancer chemotherapy.

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Recently, we have introduced the synthetic alkylating agent *seco*-CBI with an indole linker for conjugation with DNA alkylating Py–Im polyamides.[22] In this article, we report polyamide–*seco*-CBI conjugates with an indole linker that target the coding region of the histone H4 gene. Polyamide–*seco*-CBI conjugates showed efficient DNA alkylation activity and cytotoxicity against K562 cells. One of the conjugates, **5-CBI**, showed potent activity on H4 gene down-regulation and apoptosis induction compared with polyamide–Chl conjugates. It was found that **5-CBI** down-regulated a number of histone H4 genes. These results indicated that high alkylating activity and cellular effects are the advantage of a polyamide–*seco*-CBI conjugate **5-CBI**. Moreover, these results suggested that the efficient inhibition of the specific cancer-related gene is important toward the development of the novel anticancer agents.

2. Design and synthesis of the conjugates

We designed Py–Im polyamide–*seco*-CBI conjugates (Fig. 1a) targeting the template strand of the coding region of the histone H4 gene (GenBank accession number NM_003542). Polyamide–Chl conjugates, **1R-Chl** and **6R-Chl**, alkylate at the 5′-side of adenines of 5′-ACACCT-3′ and 5′-AGCACA-3′ in the coding region of the histone H4c gene. We designed polyamide–*seco*-CBI conjugates to overlap these sites (Supplementary data Fig. 1). Based on the property that *seco*-CBI conjugates alkylate adenines at the 3′-side of the sequence, we anticipated that conjugate **1-CBI** and **2-CBI** would alkylate at the sequence 5′-AGCACA-3′ (Supplementary data Fig. 1). Conjugate **2-CBI** had β-alanine substituted for Py. Incorporating β-alanine between two imidazoles improved the binding affinity and the sequence specificity.[23,24] Conjugates **3-CBI** and **4-CBI** had Y-shape motifs to extend the recognition sequence up to nine base pairs.[25] We anticipated that conjugates **3-CBI** and **4-CBI** would alkylate at 5′-TTAAGCACA-3′ sequences. Conjugate **5-CBI** targeted another site, 5′-ACGCCA-3′, also within the coding region of the histone H4 gene. These conjugates were synthesized according to reported procedures.[26]

3. DNA alkylating activity

We investigated DNA alkylation activity of polyamide–*seco*-CBI conjugates on the template strand within the coding region of the histone H4c gene. DNA alkylation of polyamide–*seco*-CBI conjugates was performed using a 5′-Texas Red-labeled 223 bp DNA fragment that includes part of the histone H4c gene. DNA fragments cleaved at the alkylation sites with heat treatment were analyzed using high-resolution denaturing polyacrylamide gel electrophoresis. The results are shown in Figure 2. Each of the polyamide–*seco*-CBI conjugates alkylated the histone H4c fragment; however, their alkylation sites were different. **5-CBI** mainly alkylated at the expected sequence, 5′-ACGCCA-3′ (site 1) with slight alkylation at the unexpected sequence, 5′-AGAAAA-3′ (site 4) On the other hand, **1-CBI**, **2-CBI**, **3-CBI**, and **4-CBI** mainly alkylated at an unexpected site, 5′-AGAAAA-3′ (site 4) with slight alkylation at the expected sequence (weak bands over the site 2) regardless of the different motifs of the polyamides. Unexpected alkylation at site 4 indicates that conjugates would prefer to bind in an extended formation to alkylate AT-rich sequences. Substitution on

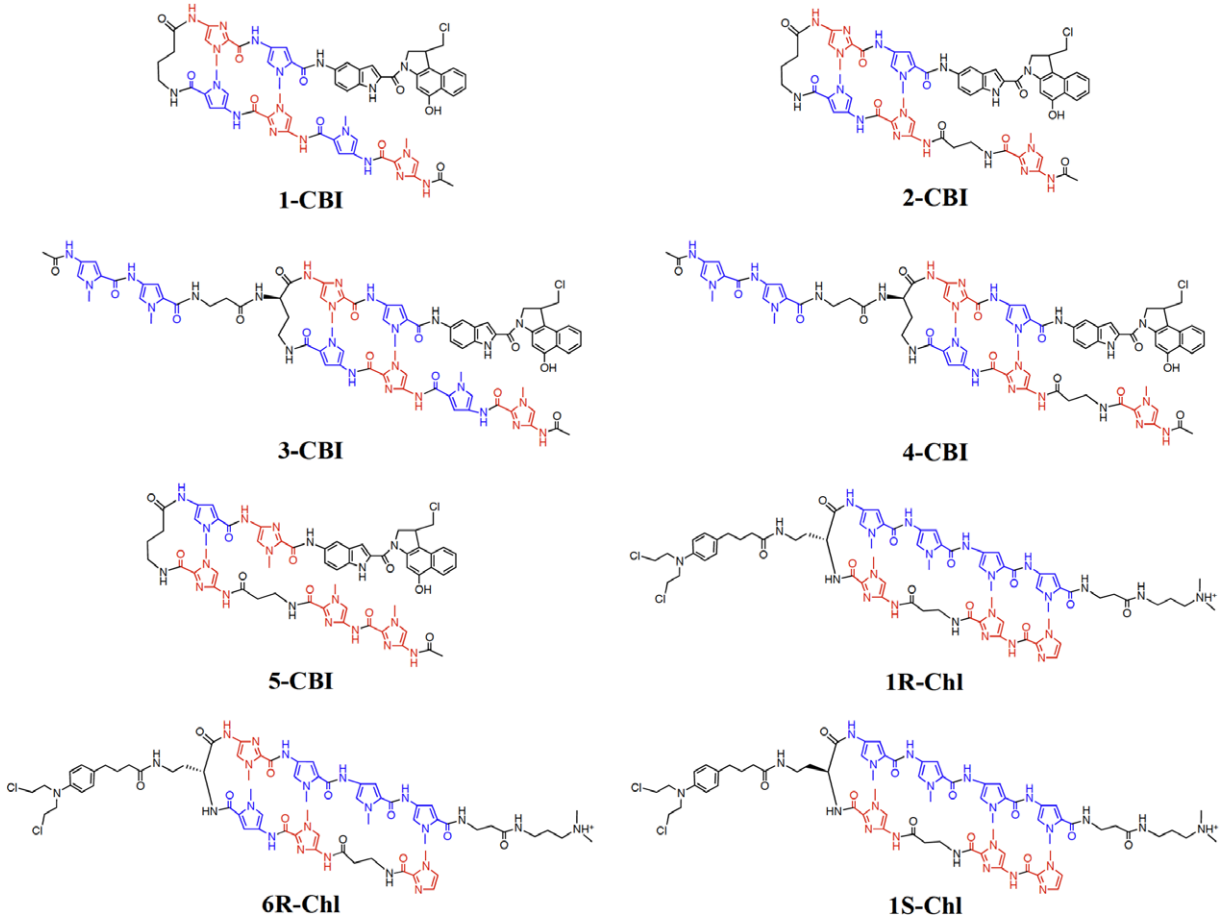

Figure 1. Chemical structures of polyamide–*seco*-CBI conjugates and polyamide–chlorambucil conjugates.

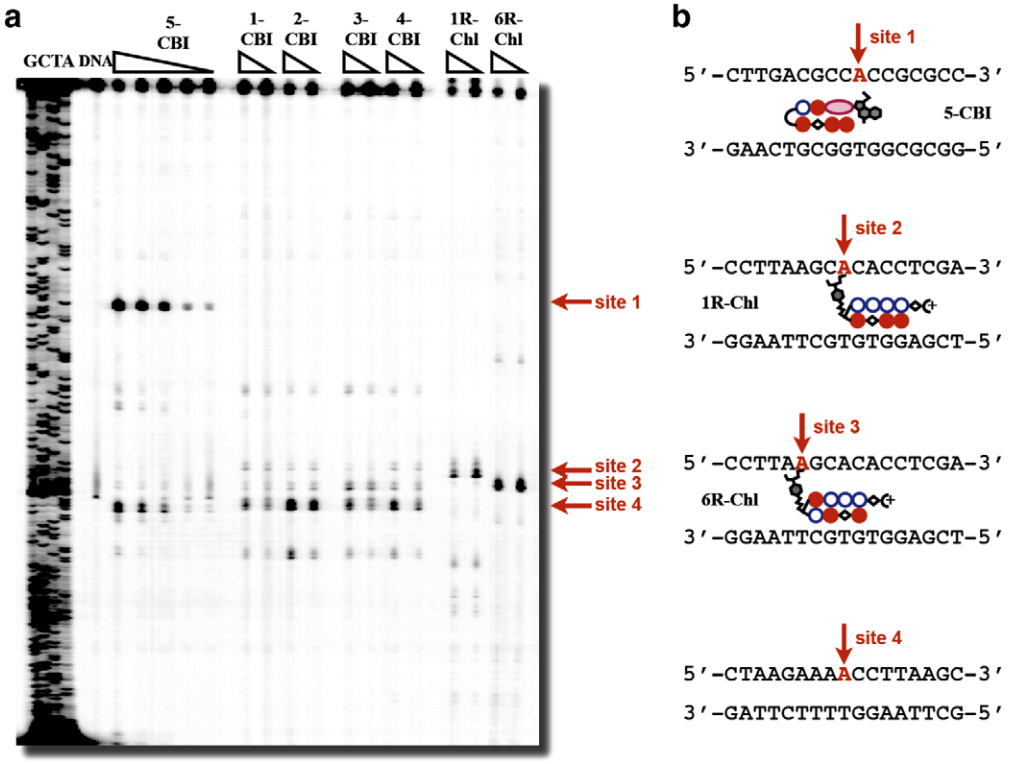

Figure 2. (a) Thermally induced strand cleavage of 5′-Texas Red-labeled 223 bp DNA fragments including the histone H4c coding region by alkylating polyamides. The concentrations of conjugates were 100, 50, 25, 10, and 5 nM for **5-CBI**, 100 and 50 nM for **1-CBI**, **2-CBI**, **3-CBI**, and **4-CBI**, 2000 and 1000 nM for **1R-Chl** and **6R-Chl**, respectively. (b) Schematic representations of the alkylation sites 1–4. Blue open circles represent Py, red closed circles Im, a pink ellipse indole linker, and diamonds β-alanine. Arrows indicate the alkylation sites.

Table 1
EC_{50} values of alkylating polyamides against K562 cells

Polyamide–*seco*-CBi conjugates	**1-CBI**	**2-CBI**	**3-CBI**	**4-CBI**	**5-CBI**
EC_{50} (M)	$3.3(\pm0.7)\times10^{-8}$	$1.1(\pm0.2)\times10^{-8}$	$1.7(\pm0.5)\times10^{-8}$	$3.8(\pm1.3)\times10^{-8}$	$9.7(\pm2.7)\times10^{-8}$
Polyamide–chlorambucil conjugates		1R-Chl			6R-Chl
EC_{50} (M)		$3.0(\pm1.5)\times10^{-7}$			$8.1(\pm2.4)\times10^{-8}$

the γ-turn unit locks the polyamide into a hairpin formation[27] and may improve the specificity for the expected sites. **1R-Chl** and **6R-Chl** alkylated at the expected sequences, 5′-ACACCT-3′ (site 2), and 5′-AGCACA-3′ (site 3), respectively. The alkylation activity of polyamide–*seco*-CBI conjugates at 25 nM was similar to that of polyamide–Chl conjugates at 1000 nM, suggesting that polyamide–*seco*-CBI conjugates have about 40-fold higher activity on DNA alkylation than polyamide–Chl conjugates. Unexpectedly, we found that only **5-CBI** alkylated at its expected the match sequence with precise recognition. Other polyamide–*seco*-CBI conjugates did not alkylate mainly at the match sequence in the histone H4c fragment, although **1-CBI** and **2-CBI** alkylated at the predetermined sequence on another DNA fragment (see Supplementary data Fig. 2).

4. Cytotoxicity against K562 cells

We evaluated the cytotoxicity of polyamide–*seco*-CBI conjugates against K562 cells using the MTS assay. Polyamide–*seco*-CBI conjugates showed inhibition of proliferation at nanomolar concentrations, with EC_{50} values of 33 (±7) nM for **1-CBI**, 11 (±3) nM for **2-CBI**, 17 (±5) nM for **3-CBI**, 38 (±13) nM for **4-CBI**, and 97 (±27) nM for **5-CBI** (Table 1). Previous results for polyamide–Chl conjugates **1R-Chl** and **6R-Chl** were 300 (±15) and 81 (±24) nM, respectively.[21] The antiproliferative activities of **1-CBI**, **2-CBI**, **3-CBI**, and **4-CBI** were stronger than those of the polyamide–Chl conjugates. **5-CBI** showed slightly reduced activity compared to the other polyamide–*seco*-CBI conjugates. The EC_{50} of **5-CBI** was

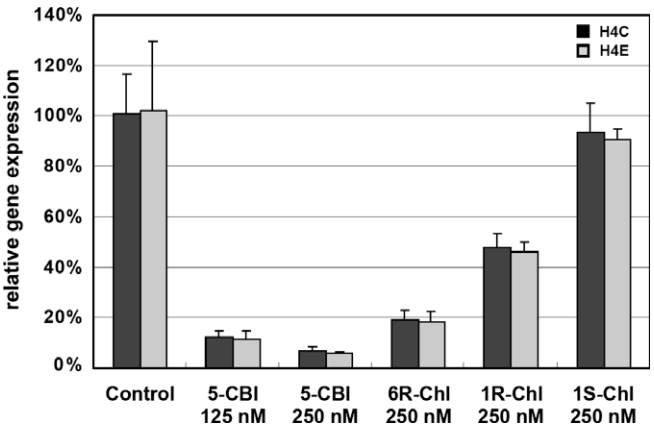

Figure 3. Quantification of histone H4c (shown in dark gray bars) and H4e (shown in light gray bars) mRNA expressed in K562 cells treated with **5-CBI**, **6R-Chl**, **1R-Chl**, and **1S-Chl** for 48 h using real-time PCR measurements. Error bars represent the standard deviation.

similar to that of **6R-Chl**. The cytotoxicity assay revealed that *seco*-CBI conjugates, except for **5-CBI**, had more potent toxicity against K562 cells than polyamide–Chl conjugates.

The reduced cytotoxicity of **5-CBI** might be the result of the different sequence specificity of this conjugate for its predicted DNA alkylation site. We previously demonstrated that alkylating polyamides with different alkylation site specificities showed distinct patterns of cytotoxicity against various cancer cell lines.[28,29] Since **1-CBI**, **2-CBI**, **3-CBI**, and **4-CBI** showed unexpected alkylation results, these conjugates would have high cytotoxicity derived

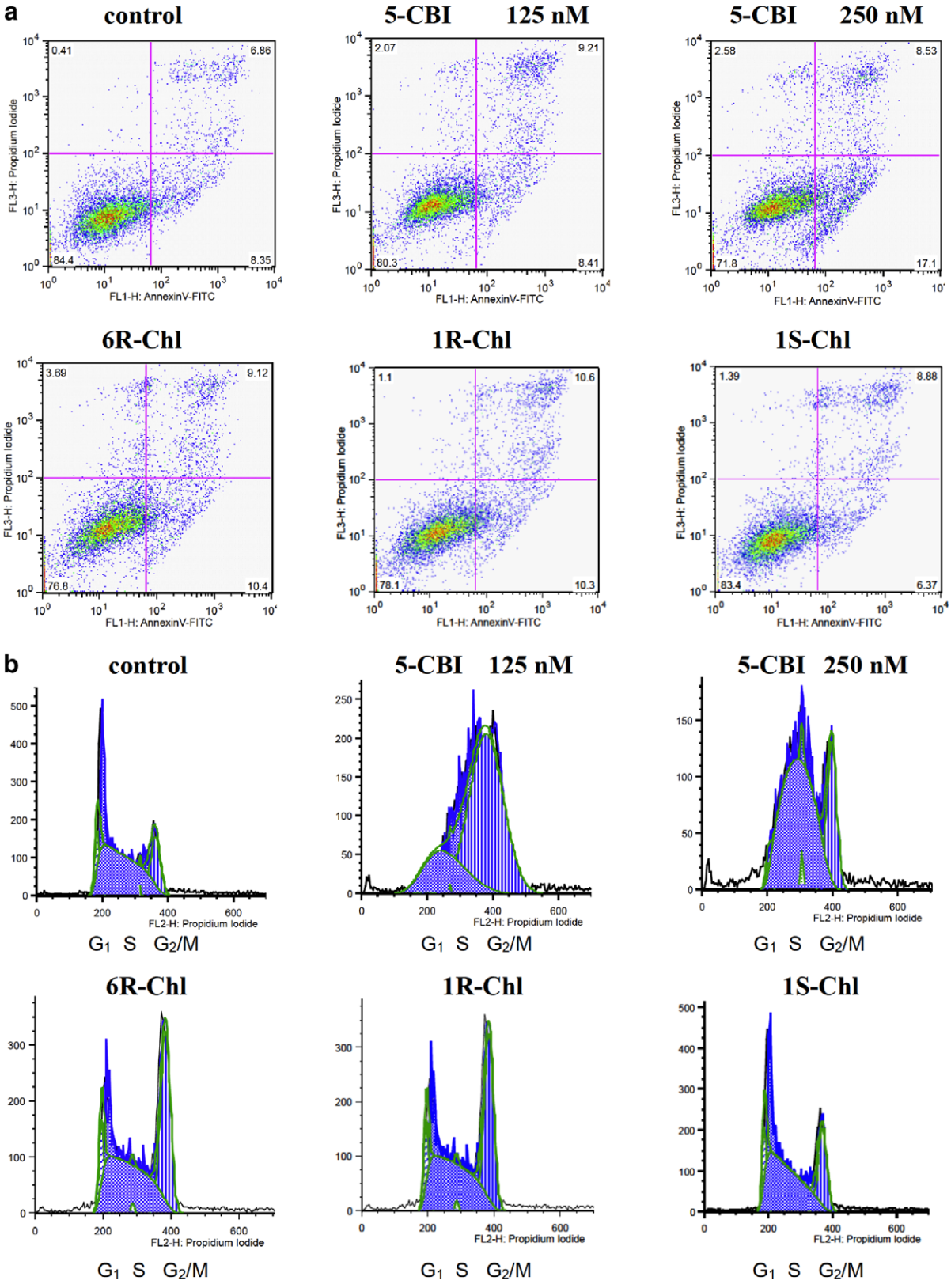

Figure 4. (a) Two-dimensional FACS analysis of K562 cells treated with **5-CBI**, **6R-Chl**, **1R-Chl**, and **1S-Chl** for 48 h. Cells were fixed and stained by Annexin V/propidium iodide. (b) FACS analysis of K562 cells treated with **5-CBI**, **6R-Chl**, **1R-Chl**, and **1S-Chl** for 48 h. Cells were fixed and stained with propidium iodide.

from non-specific alkylation. We focused on a more specific conjugate, **5-CBI** in further investigation.

5. Inhibition of histone H4 mRNA

A quantitative real-time PCR experiment was performed to confirm the effect of the polyamide conjugates on histone H4 gene expression (Fig. 3). Expression of histone H4c and H4e mRNAs was decreased by treatment with **5-CBI**, **1R-Chl**, and **6R-Chl**, whereas it was unchanged by **1S-Chl**, a conjugate that does not alkylate the histone H4c gene. These results suggest that DNA alkylation activity of histone H4 would correlate with the cellular effects. **5-CBI**, **1R-Chl**, and **6R-Chl** (at 250 nM concentration) down-regulated 93%, 52% and 81% of the histone H4c gene, respectively. These results indicate that **5-CBI** inhibited the H4 mRNA expression more efficiently than the polyamide–Chl conjugates.

6. The effect on cell cycle and apoptosis

Next, we investigated the influence of polyamide–*seco*-CBI and polyamide–Chl conjugates on cell cycle progression using fluorescence-activated cell sorting analysis (Fig. 4a). Treatment with polyamide–*seco*-CBI conjugate **5-CBI** for two days decreased the number of cells in G_1 phase and increased the number of cells in both the S and G_2/M phases. Polyamide–Chl conjugates increased cell numbers in the G_2/M phase. The influence on cell cycle was slightly different between polyamide–*seco*-CBI and polyamide–Chl conjugates. **5-CBI**-treated cells passed slowly through the S phase leading to G_2/M arrest because of the strong inhibition of DNA synthesis. Annexin V-FITC and propidium iodide staining analysis showed that treatment with polyamide–*seco*-CBI and polyamide–Chl conjugates increased apoptotic dead cells (Fig. 4b). **5-CBI** resulted in increased Annexin V-positive and propidium iodide-negative populations and Annexin V-positive and propidium iodide-positive populations compared with polyamide–Chl conjugates. Treatments with **1S-Chl** did not result in such an effect, corresponding with the previous result.[21] **5-CBI** showed a similar cytotoxicity against K562 cells but more efficient down-regulation of histone H4 mRNA and apoptosis induction than polyamide–Chl conjugates. These results may contribute to the superior properties of polyamide–*seco*-CBI conjugates. CBI is a chemically stable alkylating agent in the cell culture medium. Low nucleophilicity of CBI prevents the conjugates from inactivation by the reaction with water or other nucleophiles, which results in improved efficacy in cells.[30,31] In addition, polyamide–*seco*-CBI conjugates have high alkylation activity against DNA. We designed the polyamide–*seco*-CBI conjugates based on the potent antibiotic compounds, CC-1065 and duocarmycins that strongly alkylate DNA.[31–33] Introduction of an indole linker between polyamides and *seco*-CBI improved DNA alkylating activity.[22]

7. DNA microarray analysis

We analyzed the global effects of **5-CBI** on gene expression using an Affymetrix GeneChip Human Gene 1.0 ST array. Treatment with 10 nM **5-CBI** for 48 h affected the expression of 127 transcripts by at least twofold compared with the control cells (Fig. 5, transcripts were shown in Supplementary data). Time-course analysis was also performed in the above 127 transcripts and histone transcripts. Among the 127 transcripts, 28 transcripts relating to histone and seven transcripts relating to histone H4 were changed at least twofold. Most down-regulated transcripts relating to histone H4 are listed in Table 2. Microarray analysis showed that most of the histone H4 genes were down-regulated

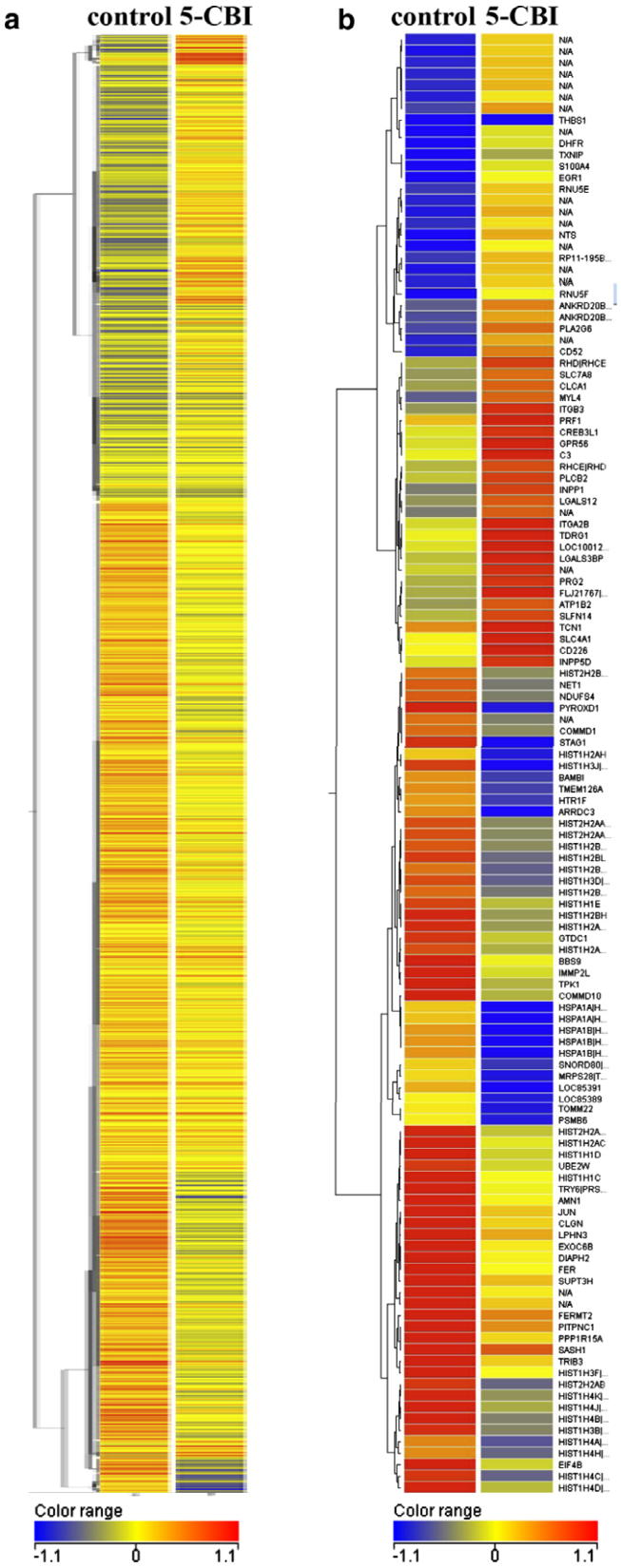

Figure 5. Global effects on transcripts in K562 cells interrogated by using Affymetrix GeneChip Human Gene 1.0 ST Arrays. (a) Agglomerative clustering of expression changes for the all transcripts up or down-regulated by twofold or more by 10 nM of **5-CBI** treatment. (b) Clustering of expression changes for 127 transcripts up or down-regulated by twofold or more by 10 nM of **5-CBI**.

Table 2
Representative list of histone H4 genes down-regulated over twofold by 10 nM of **5-CBI**

Symbol	GenBank/RefSeq	Fold change	Alkylation site (in the coding region)
HISTIH4B	NM_003544	−3.91	2
HISTIH4C	NM_003542	−2.86	2
HISTIH4K	NM_003541	−2.71	1
HISTIH4J	NM_021968	−2.61	1
HISTIH4D	NM_003539	−2.43	1
HISTIH4A	NM_003538	−2.25	1
HISTIH4H	NM_0035433	−2.11	1

by treatment with **5-CBI**. **1R-Chl** also down-regulated histone H4c mRNAs in cancer cells. The two most down-regulated histone H4 transcripts were histone H4b and H4c. Interestingly, both transcripts have the DNA alkylation sites for **5-CBI** (5'-ACGCCA-3') on the template strand of the coding region. Other transcripts have the alkylation sites on the non-template strand of the coding region. These results matched with the importance of alkylation at template strand of coding region for gene silencing.[16,17,34] However, the number of DNA alkylation sites in the coding region did not correlate completely with the extent of down-regulation, suggesting that the accessibility of chromatin by DNA alkylating agents could be a factor in inhibiting gene expression. Histone H4 is encoded by a large gene family in human cells and qRT-PCR experiments confirmed that the majority of the H4 mRNAs are indeed down-regulated by polyamide–alkylator conjugates (data not shown).

8. Conclusions

We investigated the activity of polyamide–*seco*-CBI conjugates that alkylated in the coding region of the histone H4 gene in K562 cells. One of the polyamide–*seco*-CBI conjugates, **5-CBI**, specifically alkylated the template strand of the coding region of histone H4c. **5-CBI** showed potent activity for histone H4 gene down-regulation and apoptosis induction more efficiently than polyamide–Chl conjugates. Microarray studies showed that a number of histone genes were down-regulated by **5-CBI**. We believe that specific histone-targeting polyamide–*seco*-CBI conjugates may be novel DNA alkylating agents for cancer chemotherapy.

In the chemotherapy of CML, the tyrosine kinase inhibitor imatinib mesylate (Gleevec) has been approved for first-line treatment.[35,36] Imatinib targets the bcr-abl fusion protein responsible for CML.[37] In spite of the great success, many CML patients show resistance against this drug.[38] Recently, **1R-Chl** showed an additive effect in combination with imatinib and toxicity against imatinib-resistant cells.[39] More potent polyamide–*seco*-CBI conjugates could have the potential for CML treatment. Moreover, targeting the bcr-abl fusion region in genomic DNA with alkylating polyamides may be an attractive approach to overcome the imatinib-resistant CML.

9. Experiments

9.1. Synthesis and characterization of pyrrole–imidazole polyamide–*seco*-CBI conjugates

Pyrrole–imidazole polyamides were synthesized by standard solid-phase methods.[40–42] Polyamide–*seco*-CBI conjugates were synthesized as previously described.[26] Conjugates were purified by high-performance liquid chromatography using a PU-2080 HPLC pump (Jasco), a UV-2075 HPLC UV/VIS detector (Jasco), and a Chemcobond 5-ODS-H column (Chemco Scientific). The eluent for the purification was H_2O with 0.1% AcOH containing 15–35% CH_3CN over a linear gradient for 30 min at a flow rate of 6 mL/min. Electrospray-ionization time-of-flight mass spectrometry was performed to confirm the polyamide–*seco*-CBI conjugates using a BioTOF II (Bruker Daltonics).

1-CBI: ESI-TOF-MS *m/z* calcd for $C_{61}H_{61}ClN_{19}O_{10}$ $[M+H]^+$ 1254.45; found 1254.64.

2-CBI: ESI-TOF-MS *m/z* calcd for $C_{57}H_{59}ClN_{19}O_{10}$ $[M+H]^+$ 1204.44; found 1204.00.

3-CBI: ESI-TOF-MS *m/z* calcd for $C_{78}H_{82}ClN_{25}O_{14}$ $[M+2H]^{2+}$ 813.81; found 814.18.

4-CBI: ESI-TOF-MS *m/z* calcd for $C_{75}H_{81}ClN_{24}O_{14}$ $[M+2H]^{2+}$ 788.30; found 788.64.

5-CBI: ESI-TOF-MS *m/z* calcd for $C_{57}H_{59}ClN_{19}O_{10}$ $[M+H]^+$ 1204.44; found 1204.50.

9.2. Preparation of 5′-labeled DNA

The 5′-Texas Red-modified 223 bp DNA fragment was prepared by PCR. GoTaq Green Master Mix (Promega), 5′-Texas Red-modified primer (5′-Texas Red-CCCTGACGTTTTAGGGCATA-3′), primer (5′-GGGATAACATCCAGGGCATT-3′), and 1 ng/μL of pMFST6 inserted histone H4c coding region were mixed. The reaction mix was incubated at 95 °C for 5 min then followed by 30 incubation cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s with a final extension step of 72 °C for 7 min using a Dice minithermal cycler (Takara). Products were purified by GenElute™ PCR Clean-up Kit (Sigma Aldrich), and their concentrations were determined by UV absorption.

9.3. DNA alkylating experiment

The 5′-Texas Red-labeled DNA fragments (10 nM) were alkylated by various concentration of conjugates in 10 μL of 5 mM sodium phosphate buffer (pH 7.0) containing 10% DMF at 23 °C. After incubation for 15 h, the reaction was quenched by the addition of 10 μg of calf thymus DNA and heating for 10 min at 95 °C. The solution was concentrated by vacuum centrifugation. The pellet was dissolved in 7 μL loading dye (formamide with fuchsin red), heated at 95 °C for 25 min, and then immediately cooled to 0 °C. A 2 μL aliquot was subjected to electrophoresis on a 6% denaturing polyacrylamide gel using a Hitachi SQ5500-E DNA sequencer.

9.4. Cell culture

The human CML lymphoblast cell line K562, which contains the b3a2 Bcr-Abl translocation (purchased from the American Type Culture Collection) was used and maintained in the standard mammalian cell culture conditions as recommended by the American Type Culture Collection. Direct phase-contrast microscopic visualization was used to monitor the effects of polyamide–Chl and polyamide–*seco*-CBI conjugates on cell growth rates and cell morphology.

9.5. Cytotoxicity assay

Promega CellTiter 96 Aqueous One Solution Cell Proliferation assay [using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) conversion to formazan to examine mitochondrial activity], was used to determine cell proliferation (EC_{50}).

9.6. Quantitative real-time PCR experiment

RNA from polyamide-treated cells was extracted using the Absolutely RNA Miniprep kit (Stratagene). Reverse transcription-PCR (RT-PCR) was performed using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad Laboratories) in accordance with the manufacturer's instructions. Sequences for primers were adopt

from PrimerBank (http://pga.mgh.harvard.edu/primerbank/) and primer specificity was confirmed by amplicon sequencing. The primer sequences used in qRT-PCR are listed below.

GAPDH	
Forward primer	GAGTCAACGGATTTGGTCGT
Reverse primer	GAGGTCAATGAGGGGTCAT
HIST1H4C	
Forward primer	GGGATAACATCCAGGGCATT
Reverse primer	CCCTGCCGTTTTAGGGCATA
HIST1H4E	
Forward primer	GCGGAAAGGGACTGGGTAAAG
Reverse primer	AGTCACAGCATCACGAATCAC

Levels of H4c and H4e transcripts were quantified by amplifying a segment of their respective mRNAs with appropriate primer sets. (The reverse transcription reaction was carried out at 50 °C for 10 min followed by iTaq hot-start DNA polymerase activation by heating at 95 °C for 15 min. Three-step cycling was performed: denaturation for 15 s at 95 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C for 45 cycles. All gene expression levels were normalized by parallel amplification and quantification of mRNA levels for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an endogenous reference.

9.7. Annexin V-propidium iodide staining and fluorescence-based cell sorting analysis

Annexin V-FITC/propidium iodide apoptosis staining (BD PharMingen) was used to determine the initiation of apoptosis. The effects of conjugates on cell cycle progression were monitored by flow cytometry analysis in the Scripps fluorescence-activated cell sorting core facility. Polyamide-treated cells (250 nmol/L of polyamide in culture medium for 24 h) were collected by centrifugation (200*g* for 5 min). Cell pellets were resuspended in 500 μL PBS and fixed with addition of 4.5 mL of prechilled 70% ethanol, stained with propidium iodide (50 μg/mL), and analyzed for DNA content, reflecting the fraction of cells at each point in the cell cycle (G_0–G_1, S, and G_2–M). Cells with less than a 2C DNA content are indicative of DNA fragmentation and apoptosis.

9.8. Gene expression profiling with oligonucleotide microarrays

K562 cells were treated with 10 nM **5-CBI**. Cells were collected 6, 12, 24, and 48 h after treatment. Control cells without treatment were collected after 48 h. RNA isolation and further experiment was carried out at Moritex Corporation. Labeled mRNA was hybridized on Affymetrix Genechip Human Gene 1.0 ST Array. Gene expression was analyzed using GeneViewer software (Moritex Corporation).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.005.

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