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Synthesis and Biological Properties of Sequence-Specific DNA-Alkylating Pyrrole−Imidazole Polyamides

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ABSTRACT
In recent years, many diseases including cancer and hereditary and viral diseases have been understood at the DNA sequence level. Direct control of the expression level of a specific gene would provide a promising approach for knowledge-based therapy. *N*-Methylpyrrole (Py) and *N*-methylimidazole (Im) polyamides are a new type of small compound that precisely bind to the minor groove of the DNA duplex in a sequence-specific fashion and recruit alkylating agents to the target sequence. We designed and synthesized a series of sequence-specific alkylating Py−Im polyamide conjugates that selectively alkylate predetermined DNA sequences. We have demonstrated that sequence-specific alkylating agents possess gene-silencing activities and a promising potency against human cancer cell lines as well as against xenografts of human cancer cell lines. In this Account, we focus on recent progress in alkylating Py−Im polyamides with regard to sequence specificity and biological activities and the future direction of rational molecular design of genetic switches in the postgenome era is described.

Introduction

The chemical biology of the genome is having a major influence on medicinal chemistry and medical science. Because biological characteristics such as polymorphisms and gene-expression profiles are closely related to drug responses, tailor-made therapy might be realized by knowledge-based design.[1] Cancer is a serious threat to humans, because one-third of people die from this genomic disease. Many challenges such as genome-based drug development and tailored cancer chemotherapy based on individual genomic construction have attracted considerable attention.[2] Therefore, much effort has been directed at understanding biological profiles and the development of functional small molecules that selectively bind DNA and recognize base-pair sequences, allowing for the targeting of specific gene sequences.[3] For example, definitive success has been shown for *in vivo* gene-based therapies using specific gene vectors against severe combined immunodeficiency (SCID), adenosine deaminase (ADA) deficiency, and various terminal cancers. DNA-alkylating agents covalently bond with DNA and have been used as antitumor agents. The selectivity to cancer cells usually depends upon the rapid proliferation of cancer cells compared with normal cells, and severe side effects are caused by the nonspecific DNA alkylation of normal cells. One important question to consider is whether the introduction of sequence selectivity to DNA-targeting agents can improve their efficacy as anticancer agents. To address this question, we have designed and synthesized various types of sequence-specific DNA-alkylating agents by the conjugation of *N*-methylpyrrole (Py)−*N*-methylimidazole (Im) polyamides with DNA-alkylating agents. Py−Im polyamides are synthetic compounds that bind with sequence specificity to the minor groove of double-stranded DNA, with antiparallel-paired Im/Py uniquely recognizing G·C base pairs and Py/Py pairs recognizing either an A·T or T·A base pair.[3,4]

In this Account, we review recent progress in alkylating Py−Im polyamides with regard to their sequence specificity and biological activities. We expect that such progress in molecular design and the functional analysis of sequence-specific DNA-alkylating agents steadily approaches the goal of developing tailor-made anticancer agents.

Sequence-Specific DNA Alkylation by Py−Im Polyamides Conjugates

Sequence Readout by Small Molecules. DNA minor-groove binding by small molecules has been extensively examined for many years for biological activity as antitumor and antibacterial activities.[5] For example, distamycin A (Dist) and netropsin, containing a common three or two Py skeleton, respectively, are representative of classical minor-groove binders that recognize consecutive A·T base pairs in DNA (Figure 1).[6] Dickerson and colleagues solved the crystal structure of a 1:1 complex of netropsin and d(CGCGAATTCGCG), in which netropsin binds to the minor groove of the middle of the 5′-AATT-3′ sequence.[7] Three hydrogen bonds were observed to form between the amide of netropsin and the N-3 group of adenine and O-2 group of thymine.

On the basis of these contributions to the concept of sequence-specific recognition in the minor groove, Dervan and colleagues designed and demonstrated the concept of Py−Im polyamides as minor-groove-binding molecules and demonstrated by ^{1}H nuclear magnetic resonance (NMR) that the synthetic ligand ImPyImPy can bind to 5′-(T/A)GCGC(A/T)-3′ sequences through side-by-side ho-

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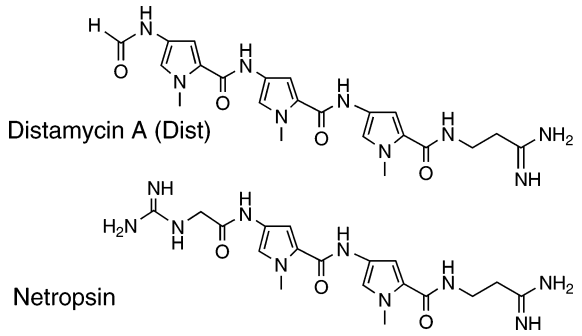


FIGURE 1. Chemical structures of Dist and netropsin.

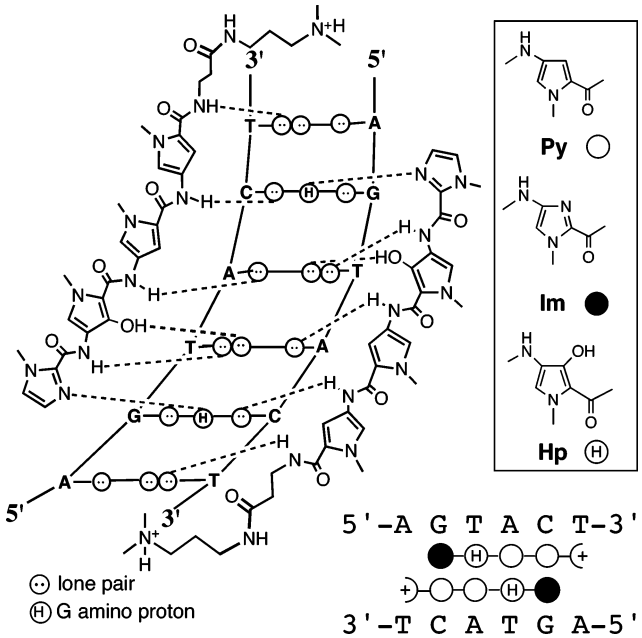


FIGURE 2. Binding model of Py−Im polyamides based on the recognition rule in the minor groove.

modimer formation.[8,9] The Dervan group discovered the general rule that Py−Im polyamides precisely recognize each of the four Watson−Crick base pairs.[10] Sequence-specific DNA recognition in the minor groove by Py−Im polyamides depends upon the sequence of side-by-side aromatic amino acid pairings oriented in the amino−carboxyl (N−C) direction with respect to the 5′−3′ direction of the DNA helix (Figure 2). An antiparallel pairing of Im opposite Py (Im/Py) recognizes a G·C base pair, whereas a Py/Py pair recognizes A·T or T·A base pairs. Even though a 3-hydroxypyrrole/Py pairing (Hp/Py) distinguishes T·A from A·T base pairs,[11] there are disadvantages in using Hp as a T-specific recognition element, such as the chemical instability of the Hp residue under acidic conditions or against free radicals and the lower affinity for T bases relative to Py. Including a β-alanine increases the affinity and specificity for sequences by relaxing the rigid curvature of the polyamides.[12]

The binding constants and sequence specificity of Py−Im hairpin polyamides are comparable to those of a transcription factor.[4,10] Thus, the expression of various genes has been silenced by competitive binding of Py−Im hairpin polyamides to their regulatory sequences.[13] Hairpin polyamides have advantages because of their

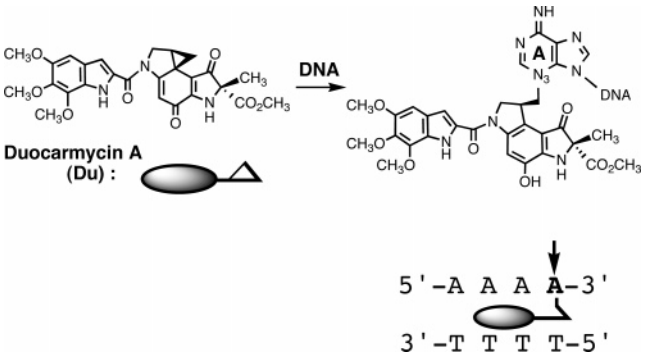


FIGURE 3. Chemical structure of dDu and a schematic representation of the adenine N3 alkylation in an AT-rich sequence by Du. The arrow indicates the site of alkylation.

characteristics, such as the ease and flexibility of their design, automation-driven solid-phase synthesis,[14,15] cell permeability because of their low molecular weight, the presence of flexible sites for covalent attachment to other molecules, and use in analytical applications. With standard liquid-phase peptide chemistry, Py−Im polyamides can be synthesized by coupling an amino group and a carboxylic acid of Py or Im residues, creating building blocks. The solid-phase synthesis of Py−Im polyamides has been achieved by t-Boc[14] and Fmoc[15] chemistry. Polyamides with a variety of functional tails can be synthesized by an alternative solid-phase method using oxime resin.[16]

Sequence-Specific DNA Alkylation. To develop a sequence-specific DNA-alkylating agent, we chose to use the sequence recognition ability of Py−Im polyamides and the alkylating moieties of antitumor antibiotics.[17] We were especially attracted by duocarmycin A (Du), a minor-groove-binding antitumor antibiotic produced by *Streptomyces* species that alkylates adenine N3 at the 3′ end of sequences of three or more consecutive A·T base pairs in DNA (Figure 3).[18] Importantly, Boger and colleagues recently demonstrated that duocarmycin derivatives efficiently alkylated A·T base pairs, even in the nucleosome core particle-bound DNA.[19] These results indicate that nucleosomal DNA is fully accessible to this class of minor-groove-alkylating agent and that the specificity and efficiency of DNA alkylation are relatively unaffected by nucleosome structure.

Several years ago, we discovered that the addition of Dist markedly modulates alkylation sites, primarily at the G residues in GC-rich sequences, by forming a cooperative heterodimer between Du and Dist.[20] The NMR-refined structure of a Du−Dist−d(CAGGTGGT)/d(ACCACCTG) complex demonstrated that heterodimers of Du and Dist tightly bind to the minor groove of DNA duplexes. Importantly, the replacement of Dist with various Py−Im triamides changes the sequence-specific alkylation by Du in a predictive manner,[21] with two Py units of Dist recognizing the complementary strand of the reacting octamer according to the base-pair recognition rule of Py−Im polyamides in the minor groove. These results suggest that Py−Im polyamides can be used as versatile sequence-

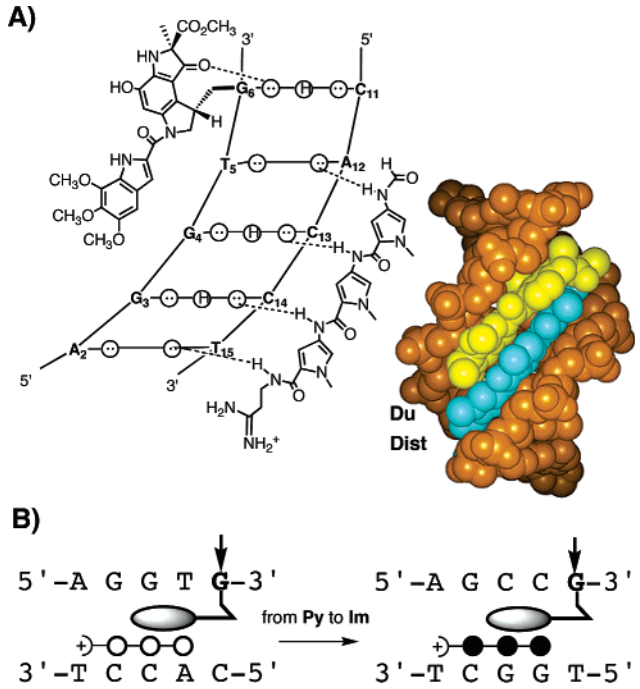


FIGURE 4. (A) ^{1}H NMR refined structure and (B) schematic presentation of alkylation by heterodimer formation between Du and Dist and an example of modulation of alkylation by substitution of Dist with ImImIm.

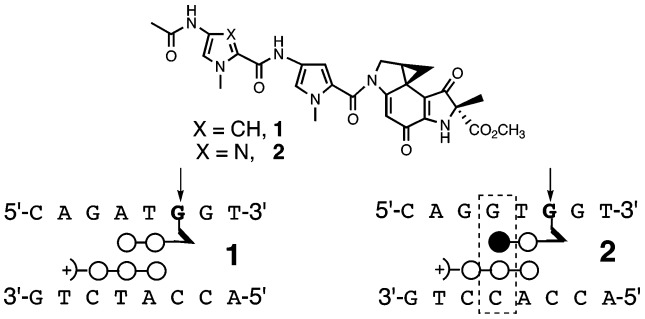


FIGURE 5. Chemical structures of Py−Im Du conjugates **1** and **2** and a schematic presentation of the specific alkylation by **1** and **2**.

recognition components of sequence-specific DNA-alkylating conjugates (Figure 4).

Thus, we synthesized sequence-specific alkylating Py−Im polyamide conjugates **1** and **2** by coupling Py−Im diamides and segment A of Du. The sequence specificity of DNA alkylation by compounds **1** and **2** was examined using 5′-Texas Red-labeled DNA fragments using an automated DNA sequencer. Labeled DNA was treated with compounds **1** and **2**, followed by quenching of the alkylation by the addition of an excess of calf thymus DNA. The samples were heated at 94 °C under neutral conditions for 20 min. The sequences of the alkylated regions were determined by thermal cleavage of the DNA strand at the alkylated sites. Under these heating conditions, all A and G residues containing purine N3−alkyl adducts are cleaved quantitatively to produce a band upon electrophoresis. We found that conjugates **1** and **2** selectively alkylated DNA in the presence of Dist at 5′-AGAT**G**-3′ and 5′-AGGT**G**-3′ sequences, respectively (Figure 5).[22] These results clearly demonstrate that the DNA-binding Py−Im

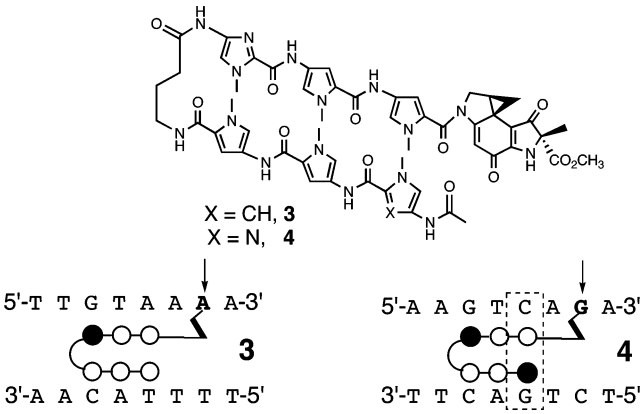


FIGURE 6. Chemical structures of Py−Im Du conjugates **3** and **4** and a schematic presentation of the specific alkylation by **3** and **4**.

polyamide moiety and the alkylating Du unit functioned perfectly. However, these conjugates were inadequate as specific alkylating agents because they required partner Py−Im triamides to achieve sequence-specific DNA alkylation and they alkylated at mismatch sequences in the absence of partner triamides. For example, the Im in **2** did not have the ability to recognize G·C base pairs. These results clearly indicate that the binding affinities of **1** and **2** were not sufficient for specific alkylation. Therefore, we decided to tether conjugates with partner Py−Im polyamides.

Py−Im hairpin polyamides with a γ-amiobutyric acid turn were demonstrated to be useful DNA-binding units, with both increased specificity and affinity, as confirmed by NMR spectroscopy.[23] In fact, the Py−Im hairpin polyamides played an important role in regulating gene expression by blocking transcription factors such as TFIIIA, TFIIB, and Ets-1.[24] Thus, we linked alkylating Py−Im polyamides and partner polyamides with a γ-aminobutyric acid linker to make **3** and **4**. Conjugate **3** mainly alkylated the A of the 5′-TGTAA**A**-3′ sequence within a 400 bp DNA fragment. Similarly, alkylation by conjugate **4** occurred at targeted sequences, such as the G of 5′-AGTCA**G**-3′, at nanomolar concentrations (Figure 6).[25]

Although highly specific alkylation was achieved by **3** and **4** in these systems, alkylation is rather slow, and after 7 days, the efficiency of DNA alkylation by **3** and **4**, the amount of DNA alkylation divided by the amount of the agent, was 1.6 and 7.4%, respectively. Molecular dynamic simulation of a **3**−octamer complex suggested that the position of cyclopropane is between two bases, which explains the low efficiency of the reactions by **3** and **4**. It was also suggested that the insertion of a vinyl linker (L) between the Py−Im polyamide and Du would adjust the location of the cyclopropane ring and improve the DNA-alkylating efficacy of the conjugates. Previously, Lown and colleagues pointed out that the insertion of L between Py and cyclopropylpyrroloindole (CPI) groups greatly enhanced alkylating activity and cytotoxicity.[26] However, their molecules were limited to PyPy dimers. Dervan and colleagues have also synthesized different types of Py−Im and *seco*-CBI- or dichloroethylamine conjugates, **5** and **6**, which have been examined by their sequence-specific

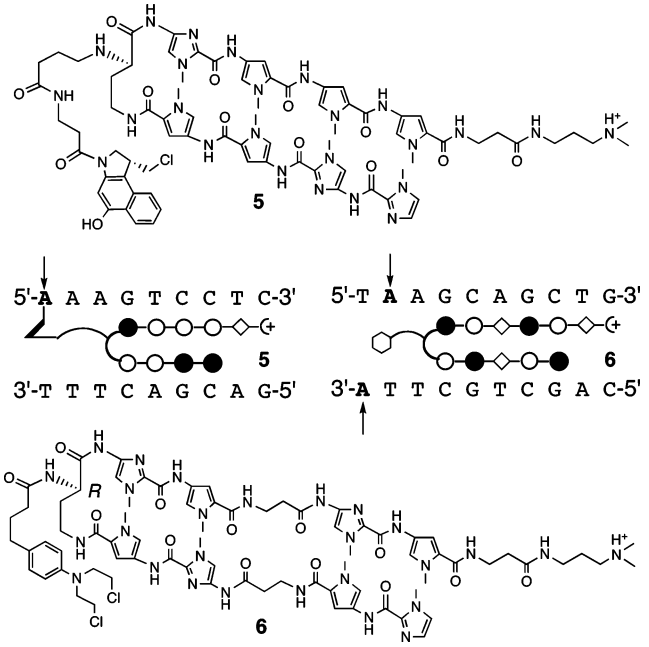


FIGURE 7. Chemical structures of Py−Im conjugates **5** and **6** and a schematic presentation of the specific alkylation by **5** and **6**.

DNA-alkylating activities (Figure 7).[27,28] Because the alkylating unit is set in the γ-turn moiety, conjugates **5** and **6** mainly alkylated adenines in the vicinity of each matching target site.

When the alkylation by **3** and **4** was taken into account, we first synthesized Py−Im diamide conjugates with a vinyl linker. Because of the low coupling yield of Du with the PyIm dimer with acrylic acid, we required more rigorous coupling conditions. We therefore introduced CPI, which is more stable than Du. The DNA-alkylating CPI moiety is prepared in five steps from the antibiotic duocarmycin B_2,[29] with an overall 25% yield. Thus, we synthesized ImPyLCPI (**7**) and found that **7** efficiently alkylated DNA at the 5′-PyG(A/T)C**Pu**-3′ sequence. Sequencing analysis using top and bottom strand labeled DNA fragments suggests that alkylation occurs through homodimer formation.[30] HPLC product analysis and ESI masses after alkylation of oligonucleotides confirm that the dialkylation occurs through highly cooperative homodimer formation (Figure 8).

Importantly, Py−Im triamide conjugates **8** and **9** sequence-specifically alkylate their target sequences in supercoiled plasmid DNA such as pQBI PGK (5387 bp). Hydrolysis by the restriction endonucleases *Bss*H II and *Eco*52 I is inhibited by the alkylation.[31] These results clearly demonstrate that **8** and **9** selectively alkylate DNA at matching sequences, even within several thousand base pairs of supercoiled DNA. These results also indicate that DNA alkylation in the minor groove by Py−Im polyamides strongly affects DNA−protein interactions.

The high efficiency of DNA alkylation by polyamides with vinyl linkers encouraged us to synthesize dimer molecules of **7** to develop DNA interstrand crosslinking agents with sequence specificity. Because DNA interstrand crosslinking agents directly inhibit both DNA replication and gene expression by preventing the melting of DNA

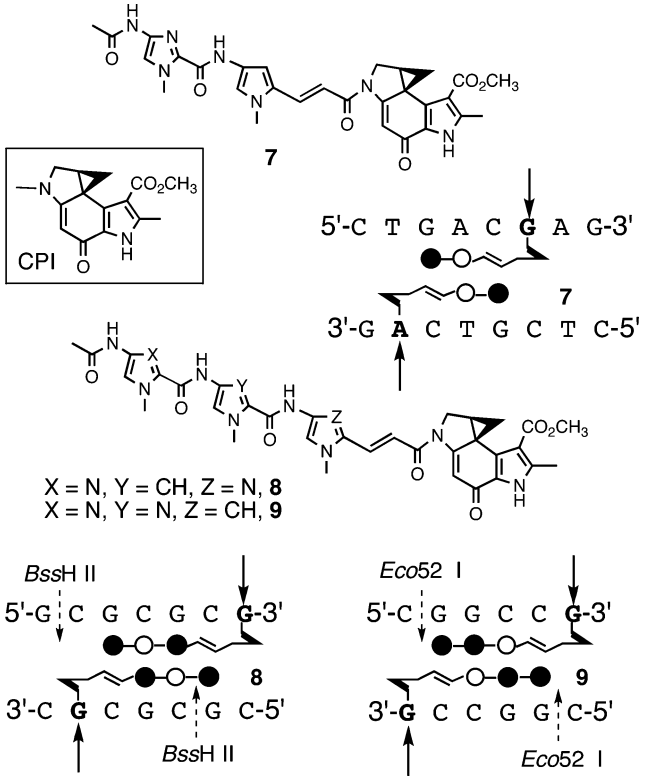


FIGURE 8. Chemical structures of CPI conjugates **7**−**9** and a schematic presentation of the specific alkylation by **7**−**9** through homodimer formation. Dotted arrows indicate the site of hydrolysis by restriction endonucleases.

strands, crosslinking agents are expected to have potent antitumor activity.[32] We synthesized several dimers of ImPyLCPI **7** and developed a novel DNA interstrand crosslinking agent (**10**) containing a tetramethylene linker. Compound **10** crosslinks double-stranded DNA at the nine-base-pair sequence, 5′-PyGGC(T/A)GCC**Pu**-3′, but only in the presence of the triamide ImImPy (Figure 9).[33,34] The present system consists of a 1:2 heterodimer complex of the alkylating agent and partner ImImPy in the DNA minor groove and causes interstrand crosslinking in a sequence-specific fashion according to the base-pair recognition rule of Py−Im polyamides. It is noteworthy that **10** efficiently induces interstrand cross-linking at the concentration at which **7** induces alkylation. Using biotin-labeled fragments, we demonstrated that **10** and ImImPy cause interstrand cross-linkages on longer DNA fragments.

Insertion of a vinyl linker (L) between the Py−Im polyamides and CPI adjusted the location of the reactive cyclopropane ring, dramatically enhancing DNA-alkylating reactivity. Consequently, we synthesized CPI and Py−Im polyamide hairpin conjugates (**11**−**14**) containing vinyl linkers. Sequencing gel analysis indicated that conjugates **11**−**14** effectively alkylated DNA at the N3 position of adenines and guanines in each matching sequence in the DNA fragment. Most of the DNA alkylation was completed within 5 min at nanomolar concentrations of the conjugate. In clear contrast, hairpin polyamides without vinyl linkers did not cause alkylation after 1 h of incubation.[35] The observation of efficient sequence-specific alkylation by conjugates **11**−**14** encouraged us to examine further

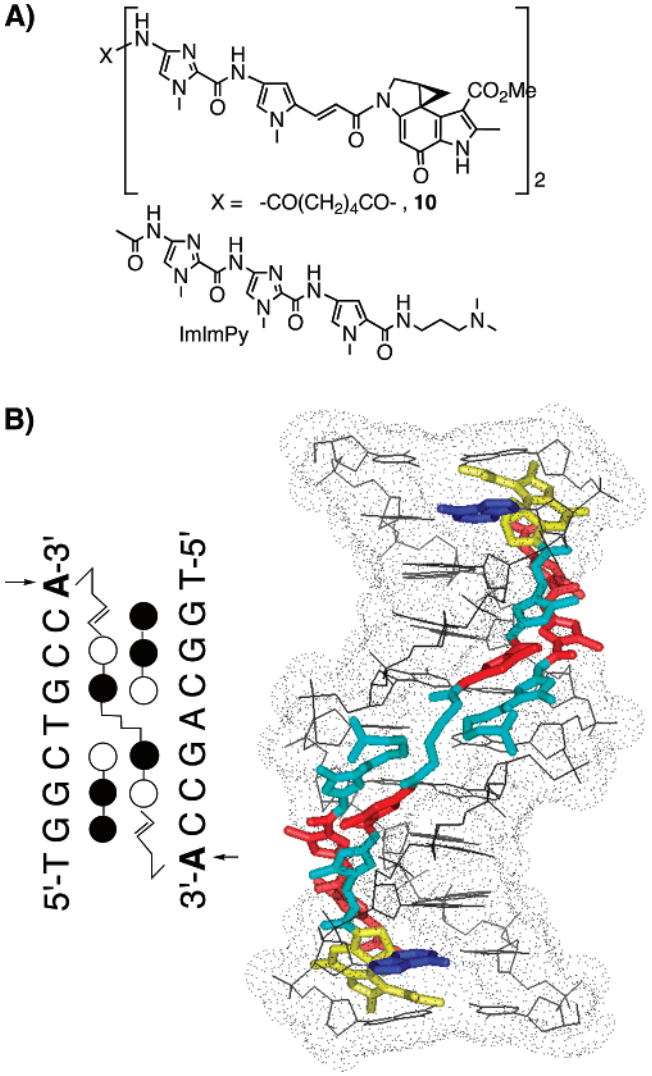


FIGURE 9. (A) Chemical structures of interstrand crosslinker **10** and ImImPy. (B) Schematic presentation of the sequence-specific interstrand crosslining by **10** in the presence of ImImPy (left). The energy-minimized model of the d(TGGCTGCCA)/d(TGGCAGCCA)−**10**−ImImPy complex (right).

the biological activity induced by specific DNA alkylation by these agents (Figure 10).

Improvement of the Alkylating Moiety. During the development of these efficient sequence-specific alkylating agents, we realized that improvements of the alkylating moiety and linker region were necessary for further studies. Although the insertion of a vinyl linker between CPI and the Py−Im polyamides dramatically enhanced the DNA-alkylating activity by more than 100-fold compared to with no vinyl linker,[35] it is impractical to synthesize sufficient quantities of Py−Im CPI conjugates for animal studies. To overcome this problem, we searched for an alternative alkylating moiety that is equivalent to CPI in terms of its DNA alkylation and that could be synthesized from commercially available starting materials using a general synthetic methodology.

Importantly, Boger and colleagues investigated the stability and cytotoxicity of various duocarmycin analogues and demonstrated that 1,2,9,9a-tetrahydrocyclo-

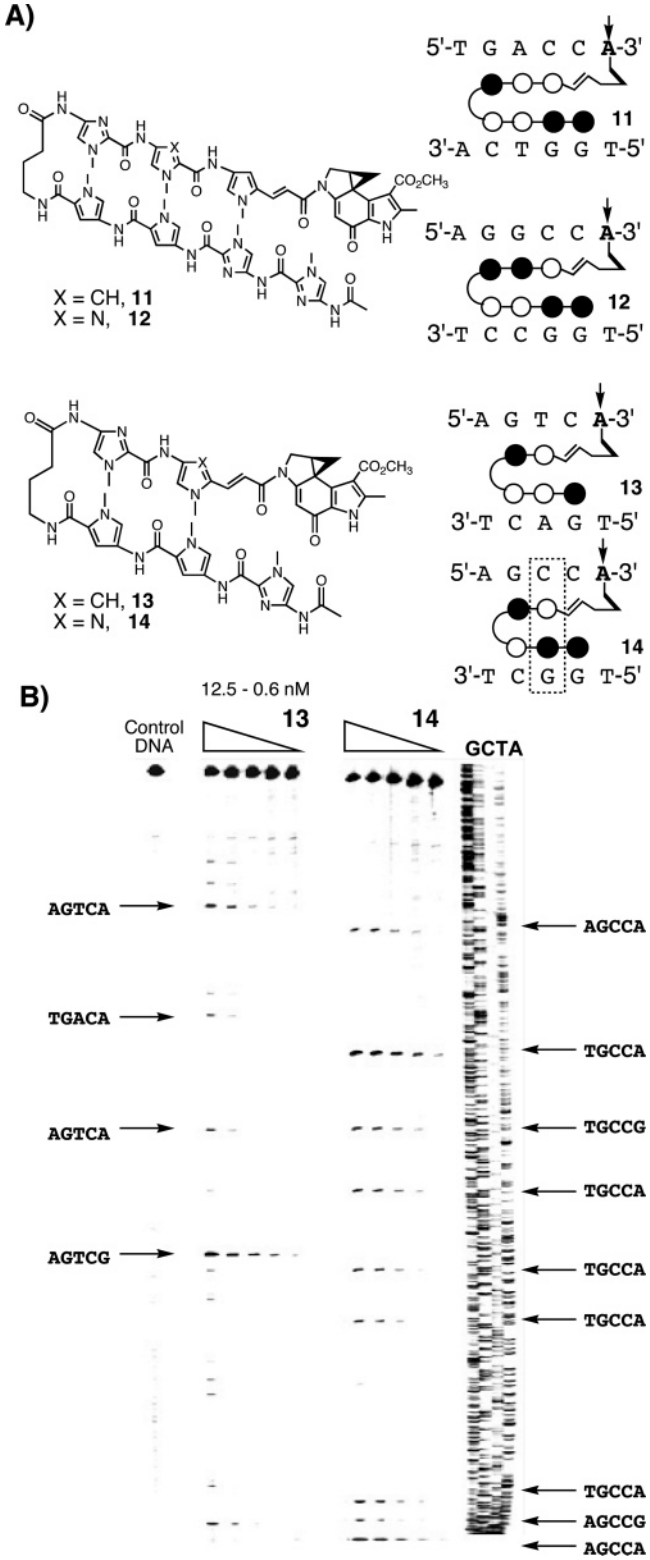


FIGURE 10. (A) Chemical structures and a schematic presentation of the specific alkylation of Py−Im hairpin conjugates **11**−**14** and (B) thermally induced sequence-specific strand cleavage by **13** and **14**.

propa[1,2-*c*]benz[1,2-*e*]indol-4-one (CBI) was more stable than CPI in aqueous solution and that DNA-alkylating conjugates with high stability at neutral pH have higher cytotoxicity. They had also already established an efficient synthetic route for CBI from commercially available 1,3-

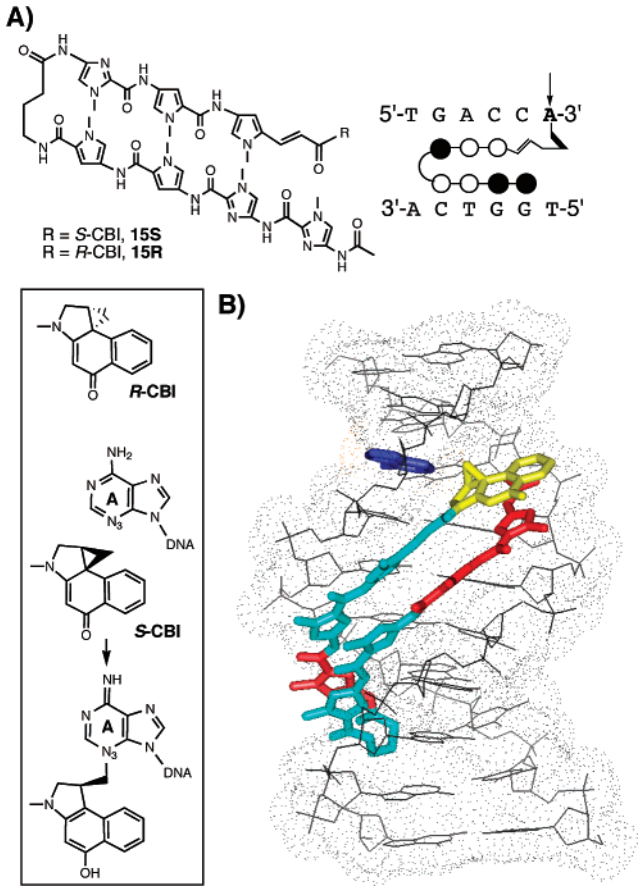


FIGURE 11. (A) Chemical structures of **15S** and **15R** and a schematic presentation of specific alkylation by Py−Im hairpin conjugate **15S**. (B) Energy-minimized model of the d(CTTGACCATG)/d(CATGGTCAAG)−**15S** complex.

naphthalenediol.[36,37] Therefore, we selected CBI as the DNA-alkylating unit and synthesized hairpin polyamides possessing both enantiomers of CBI, **15S** and **15R**. High-resolution denaturing gel electrophoresis indicated that **15S** efficiently alkylated the target sequence 5′-TGACCA-3′ in DNA fragments. The specificity and efficiency of DNA alkylation by **15S** were comparable to or even higher than those of the corresponding CPI conjugate **11**.[38] In contrast, the enantiomer **15R**, with an unnatural orientation of the cyclopropane ring showed very weak DNA-alkylating activity. Molecular modeling suggested that the different binding orientations of the *S*- and *R*-CBI units explained the significantly different reactivities of **15S** and **15R**.

Interestingly, despite having similar DNA-binding orientations, DNA alkylation CPI conjugate **11** occurred equally at N3 of both the A and G of the matching sequence, whereas CBI conjugate **15S** specifically alkylated only N3 of the A.[39] Importantly, it was also observed that CBI conjugates reduced the alkylation of mismatch sites. These results indicate that lowering the reactivity increases the base and sequence specificity, and the introduction of CBI further improved specificity to target sequences (Figure 11).

Improvement of the Linker Region. A vinyl linker between the Py−Im polyamides and CBI moiety is highly advantageous for DNA alkylation. However, the vinyl linker moiety has certain disadvantages in the synthesis of alkylating Py−Im polyamides. It is relatively unstable under acidic and basic conditions, and it suffers from low chemical yields. Because ethyl-3-(4-amino-*N*-methylpyrrol-2-yl)acrylate is extremely unstable, solid-phase synthesis of Py−Im polyamides with vinyl linkers was not successful. Therefore, we synthesized various alkylating Py−Im polyamides by a combination of time-consuming liquid-phase coupling reactions.[30,31,35,38,39] To overcome these problems, we searched for a new linker that is stable and could provide the same geometry as Py with a vinyl linker.

We chose a 5-amino-1*H*-indole-2-carbonyl linker (indole linker), because B3LYP/6-31G* level molecular orbital calculations indicate that the amide linkages of this unit are approximately superimposable with Py containing a vinyl linker. Thus, CBI conjugates **16** and **17** and *seco*-CBI conjugate **18** with an indole linker were synthesized by a combination of Fmoc solid-phase synthesis using an oxime resin[16] and a subsequent liquid-phase coupling procedure. Conjugates **16**, **17**, and **18** efficiently alkylate the specific sequences, 5′-WWWCC**A**-3′ and 5′-WGWCC**A**-3′ (W = A or T). Importantly, these results indicated that the indole linker acts as an appropriate substitute for the vinyl linker. In particular, *seco*-CBI conjugate **19** alkylates a specific nine-base-pair sequence, 5′-ACAAATCC**A**-3′ (Figure 12).[40] Introduction of an indole linker greatly facilitated the synthesis of sequence-specific alkylating Py−Im polyamides by enabling effective use of solid-phase Py−Im polyamide synthesis.

Gene Silencing by Sequence-Specific Alkylation by Py−Im Polyamide Conjugates

The regulation of specific gene expression by synthetic small molecules has emerged as a promising approach for gene-targeting drugs. Because the DNA-binding affinity and sequence specificity of the Py−Im polyamides are comparable to those of a transcription factor, silencing of gene expression, such as 5S RNA, the human immunodeficiency virus, hypoxia response element, and human transforming growth factor-β, was able to be achieved by competitive binding of Py−Im hairpin polyamides to regulatory sequences.[41−45] Because gene expression is generally controlled by the binding of common transcription factors to regulatory sequences, the design of polyamides for use in this approach has limitations. To obtain sufficient specificity for inhibition of the expression of certain genes, Py−Im polyamides need to include the unique flanking sequences of the binding sequences of transcription factors. In contrast, targeting Py−Im polyamides to the unique sequences in the coding region is relatively straightforward. However, the inhibition of transcription by binding Py−Im polyamides in the coding region is difficult, because the polyamides are removed from duplex DNA during pol II transcription.

Recently, we showed that an alkylating Py−Im polyamide **13**, which alkylates a specific site on the template strand of the coding region of green fluorescent protein

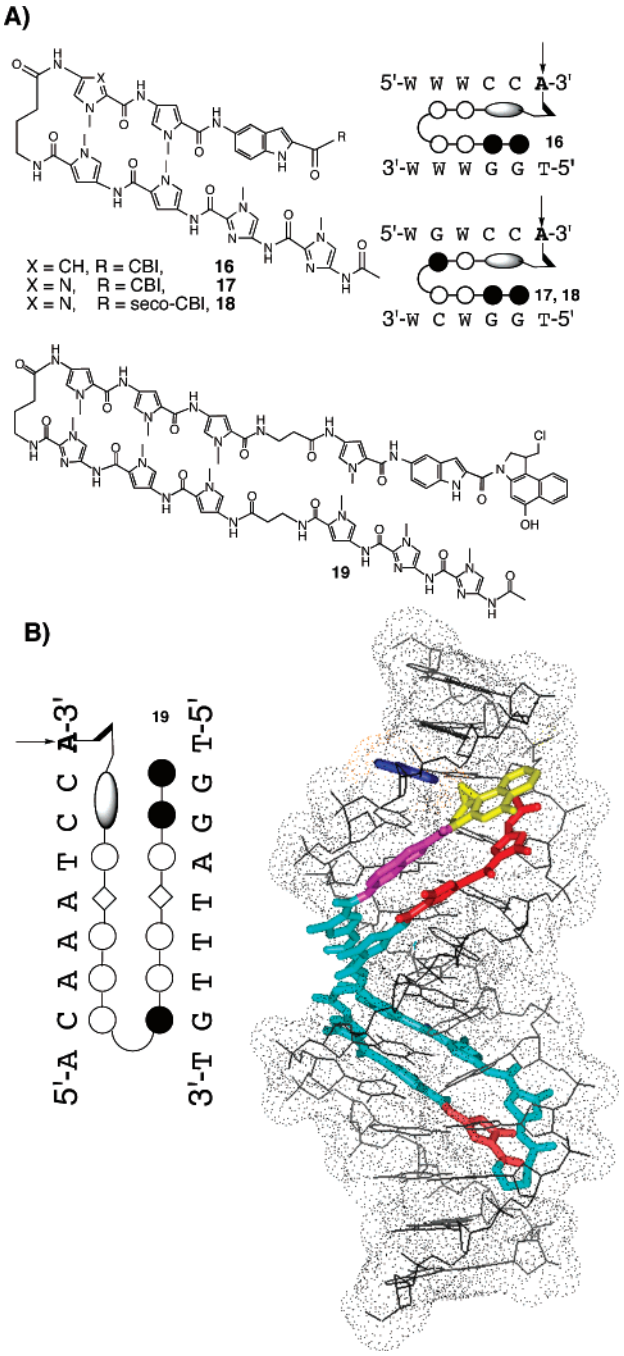


FIGURE 12. (A) Chemical structures and a schematic presentation of specific alkylation by Py—Im hairpin conjugates **16**—**19** and (B) energy-minimized model of the d(ATACAAATCCAAT)/ d(ATTGGATTTGTAT)—**19** complex.

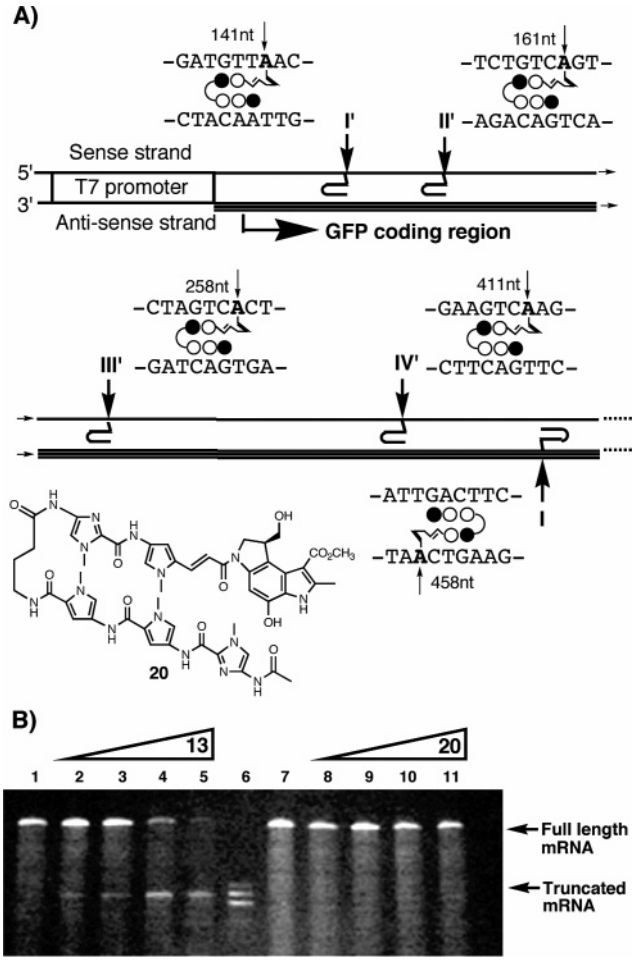


FIGURE 13. Inhibition of GFP mRNA expression by sequence-specific alkylation at 5′-AGTCA-3′ by conjugate **13**. (A) Schematic presentation of the sites of alkylation in the GFP gene under the control of the T7 promoter. (B) PAGE analysis of transcripts of **13**- or **20**-treated DNA. The three bands in lane 7 are RNA markers.

(GFP; 458 nt), effectively inhibited transcription by alkylation, producing truncated mRNAs in an *in vitro* transcription system.[46] In sharp contrast, alkylation in the nontemplate strand did not give such truncated products. The inhibition of transcription by deactivated conjugate **20** was not observed by polyacrylamide gel electrophoresis (PAGE) analysis, confirming the fact that noncovalent binding does not cause inhibition of transcription (Figure 13).

Sequence-specific gene silencing by the alkylating Py—Im conjugates **11** and **12**, which target the coding regions of renilla and firefly luciferases, respectively, was investigated. Two vector plasmids were transfected into HeLa cells, and the ability to silence luciferase expression was examined *in vitro*.[47] We expected that the alkylating Py—Im conjugates would not only target the promoter region but also the coding region, which dramatically increases its choice of target sequences as an antigene agent (Figure 14).

Selective Cytotoxicity of Sequence-Specific Alkylation by Py—Im Polyamide Conjugates

New types of anticancer agents that target mutated gene products are showing great promise, such as STI-571 (Glivec), which targets the Abelson leukemia viral oncogene kinase in patients with chronic myelogenous leukemia.[48] DNA-alkylating agents such as the nitrosoureas, mitomycin C, and cisplatin, which constitute a major class of antitumor drugs, have long been of interest for their biological properties and are routinely used for cancer therapy. These drugs are, in a manner, relatively toxic to normal 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. In addition, the question arises as to whether one

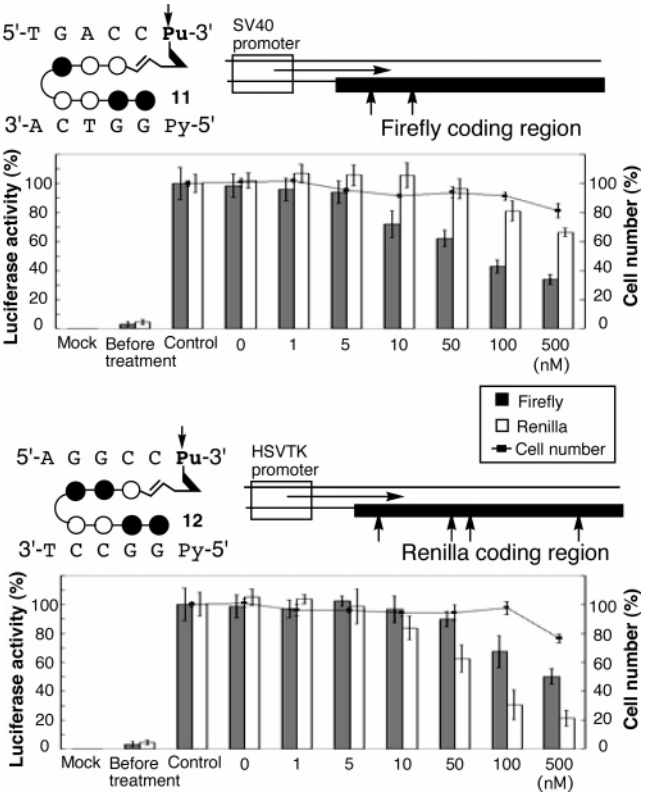


FIGURE 14. Schematic presentation of the sites of alkylation in firefly and renilla luciferase vectors and selective inhibition of luciferase translation by sequence-specific alkylation with conjugates **11** and **12**.

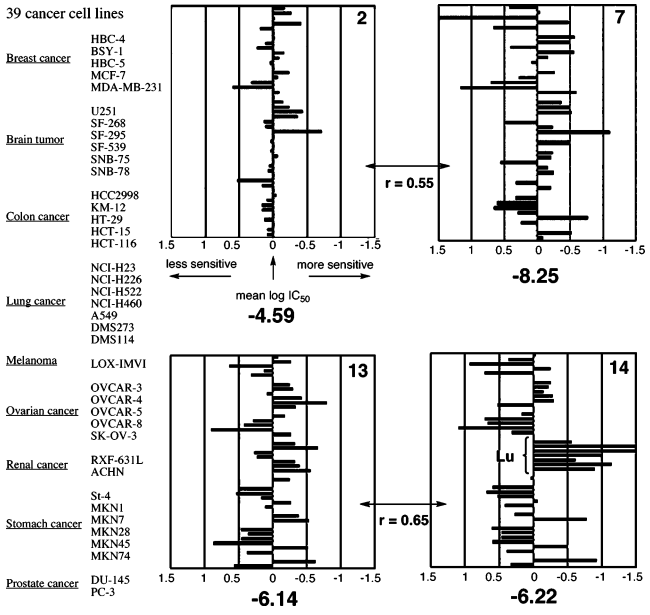


FIGURE 15. IC_{50} profiles by sequence-specific alkylating Py−Im polyamides **2**, **7**, **13**, and **14** and the correlation coefficients for the mean IC_{50} values.

can tailor the binding preference of DNA-binding agents to particular sequences and thereby create a tailor-made antitumor agent.

The progress in our study of DNA alkylation has led to the methodology for the development of novel antitumor agents with sequence recognition ability. DNA sequence specificity is one important component contributing to the cytotoxic potency of several alkylating Py−Im polyamides.[49] We examined in detail comparative studies of DNA sequence-specific alkylation and the antitumor activity of the alkylating ImPy diamide conjugates **2** and **7** using high-resolution denaturing gel electrophoresis and the panel of 39 human cancer cell lines.[50] The mean log IC_{50} values of **2** and **7** were −4.59 (25.7 μM) and −8.25 (5.62 nM). Importantly, we observed a substantial difference in cytotoxicity between these structural analogue conjugates (r = 0.55), despite them having a common DNA-alkylating mechanism (purine N3 alkylation). Recently, we found that alkylating Py−Im polyamides **13** and **14**, which differ only in that the C−H atoms are substituted by an N atom in the second ring, showed significantly different cytotoxicity in the 39 human cancer cell line panel (Figure 15).[51] The mean log IC_{50} values of **13** and **14** were −6.14 (0.72 μM) and −6.22 (0.60 μM), respectively, which are better than the values for mitomycin C (−6.0) and cisplatin (−5.2). A graph of the means showed that the Py−Im conjugates **13** and **14** did not correlate well with each other (r = 0.65), confirming the notion that sequence specificity may correlate with their cytotoxicity.

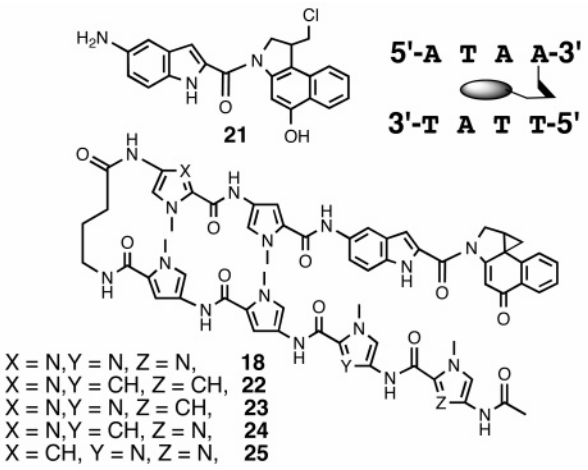
Moreover, the growth inhibitory effects of Py−Im indole−*seco*-CBI conjugates, such as **18** and **22**−**25**, are dramatically different from indole−*seco*-CBI **21** conjugated with various Py−Im polyamides. These results further confirmed that differences in sequence specificity might affect the pattern of cytotoxicity (Figure 16).[52]

Summary and Outlook

Py−Im polyamides are attractive artificial molecules developed from detailed analysis of the DNA recognition mechanisms of Dist and netropsin binding in the minor groove. They are expected to be useful tools for gene regulation or functional analysis of genes because of their high sequence specificity and binding ability equal to transcription factors. Gene-expression control by Py−Im polyamides has thus been achieved by competitive binding with a transcription factor in a promoter region. RNAi technology has also been developed as a useful gene-regulation tool, targeting specific mRNAs, large numbers of copies of which occur in the cytoplasm. Therefore, complete inhibition of expression by this method over the long term is difficult, and several hurdles need to be cleared before their therapeutic use.

Our alkylating Py−Im conjugates, which have little restriction on their targeting of DNA sequences, make possible the molecular regulation of the expression of specific genes. In addition, we have successfully developed Py−Im hairpin polyamide conjugates that precisely alkylate DNA at specific matching sequences at nanomolar concentrations. The selectivity and efficiency of DNA alkylation were higher than those of DNA-alkylating antibiotics. The alkylating moiety CBI can be synthesized from commercially available 1,3-naphthalenediol, and the

A)



B)

22	23	24	25	18	
-0.026	-0.316	-0.314	-0.272	-0.016	**21**
	0.714	0.709	0.701	0.438	**22**
		0.639	0.840	0.394	**23**
			0.515	0.633	**24**
				0.470	**25**

FIGURE 16. (A) Chemical structures of the conjugates **18** and **21**−**25** and (B) correlation coefficients for the mean IC_{50} values of **18** and **21**−**25**.

DNA-binding moiety of Py−Im polyamides can be made by solid-phase synthesis. These two functional moieties are then linked with a chemically stable indole linker. The present alkylating Py−Im polyamides can be synthesized on a large scale, which would allow for future animal studies for the development of antitumor agents targeting the expression of specific genes responsible for cancer cell growth. Preliminary examination of cytotoxicity using a nude mouse xenograft model revealed that conjugate **18** inhibits the growth of the estrogen receptor positive human breast cancer cell Br 10, suggesting that Py−Im polyamides targeting specific sequences in individual cancer cell lines will provide a promising methodology for the development of tailor-made antitumor drugs. Future studies in our laboratory will involve knowledge-based design as well as a combinatorial approach to identifying effective Py−Im polyamides that target the Achilles' heel of cancer cells.

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