

Modulation of Sequence Specificity of Duocarmycin-Dependent DNA Alkylation by Pyrrole–Imidazole Triamides

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The ability of small molecules to modify nucleic acids irreversibly has generated considerable current interest.¹ Many anticancer drugs interact with DNA, although most have little sequence specificity and often exhibit severe toxicity to normal tissues. Rational design of alkylating agents targeting specific sequences in the human genome may provide useful molecules for various applications, including better anticancer drugs.²

Anticancer antibiotic duocarmycin A (Duo) normally alkylates duplex DNA at the A-N3 site on the 3' side of 3–4 consecutive A·T base pairs.³ Previously, we found that the addition of distamycin A (Dist) markedly modulates the sequence specificity of Duo, causing the alkylation to shift primarily to the G residues in GC-rich sequences.⁴ Our 2D-NMR analysis⁵ revealed that the molecular mechanism of modulation of sequence specificity involves a cooperative heterodimer formation of Duo and Dist in the minor groove. In the ternary complex, Dist recognizes the DNA strand (complementary to the alkylated strand) using a binding mode similar to that of the dimers of *N*-methylimidazole-(Im)-*N*-methylpyrrole(Py) polyamides.⁶ These results suggest an intriguing possibility that the sequence specificity of Duo can be controlled in a predictable manner by pairing with Py/Im

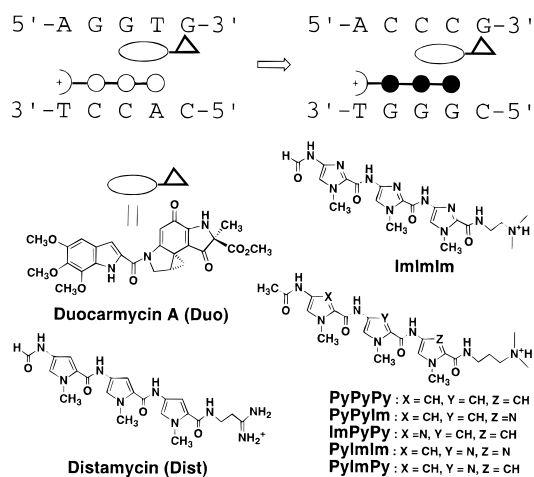


Figure 1. Schematic representation of the recognition of 5'-GGTG-3' by the Duo-Dist heterodimer and the proposed recognition of 5'-CCCG-3' by the Duo-ImImIm heterodimer. The Im and Py are represented by black and open circles, respectively. Chemical structures of duocarmycin A, distamycin A, and Py/Im triamides.

triamides.⁷ For example, DNA alkylation by Duo in the presence of ImImIm is expected to occur at 5'-CCCG-3' sequence according to the pairing rule⁶ for Im/Py polyamides. To test the above hypothesis, we synthesized six sets of Im/Py triamides and analyzed the site of DNA alkylation by Duo in the presence of these Im/Py triamides (Figure 1).

Figure 2 shows the polyacrylamide gel electrophoresis (PAGE) of a 427-bp Duo-treated DNA fragment in the absence and presence of ImImIm after heat treatment, in which all sites alkylated at purine N3 gave cleavage bands.⁸ In the presence of ImImIm, alkylation specifically occurred at 5'-CCA-3' and 5'-CCG-3' sequences, different from that of Duo alone.² Further support for the dramatic modulation of Duo alkylation by ImImIm was obtained by the efficient alkylation of d(ATACCAGG)/d(CCTGGTAT), d(TTCCAGTC)/d(GACTGGAA), and d(TGC-CAGCT)/d(AGCTGGCA) at A-N3 of the 5'-CCA-3' site.⁹ HPLC analysis of the reaction mixture of d(ATACCAGG)/d(CCTGGTAT) incubated with Duo alone over 30 min showed no appreciable alkylation products. In contrast, Duo selectively alkylated the target A6 site efficiently (90% complete after 1-min incubation) in the presence of ImImIm (Figure 1S, Supporting Information). These results clearly indicate that ImImIm dramatically modulates the sequence specificity of Duo and that the two N-terminal Im residues determine the sequence-specificity of 5'-CCA or 5'-CCG.

To investigate the generality of the modulation of Duo-mediated DNA alkylation by Py/Im triamides, we examined the DNA alkylation by Duo in the presence of various Py/Im triamides. To achieve an optimal cooperative alkylation, various concentra-

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(9) A reaction mixture (50 μ L) containing Duo (75 μ M), ImImIm (125 μ M), and the DNA octamer (1 mM base concentration) in 50 mM ammonium formate (pH 6.5) was incubated at 0 $^{\circ}$ C for 1 min and then subjected to HPLC (254 nm). The HPLC profile of the experiment using d(ATACCAGG)/d(CCTGGTAT) is shown in Figure 1S (b). The covalent adducts were collected, and their complementary strands were added. The electrospray mass spectra of the reconstruction complexes are shown in Figures 2S–4S (Supporting Information). Similarly, d(TTCCAGTC)/d(GACTGGAA) and d(TGCCAGCT)/d(AGCTGGCA) were alkylated at the A5 site by Duo only in the presence of ImImIm. The sites of alkylation were determined according to the previous reported procedures.^{3a}

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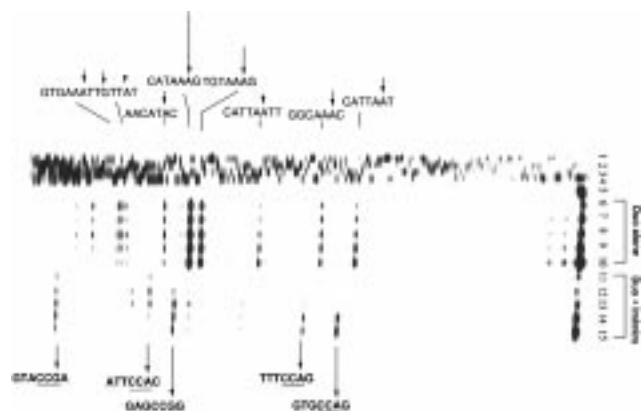


Figure 2. Thermally induced strand cleavage of 5' TexasRed-labeled pUC 18 forward 352–778 DNA fragment by Duo in the presence and absence of ImImIm. Lanes 1–4, Sanger G, C, T, and A sequencing standard; lane 5, DNA control; lanes 6–10, 5 μ M Duo and 0, 0.125, 0.25, 0.50, and 1.0 mM calf thymus DNA, respectively; lanes 11–15, 5 mM Duo and 10 μ M ImImIm and 0, 0.125, 0.25, 0.50, and 1.0 mM calf thymus DNA, respectively. The 5'-TexasRed-labeled 426-base pair fragment was prepared by PCR using 5'-end TexasRed-modified 5'-CGCCAGGGTTTCCAGTCACGA-3' (pUC 18 forward 352–373) and 5'-TGGATAACCGTATTACCGCC-3' (pUC 18 reverse 1910–1929) as primers. The 5'-TexasRed-labeled DNA fragment (60 nM) was alkylated in 10 μ L of 10 mM Na phosphate buffer (pH 7.0) at room temperature for 1 h. The reaction was quenched by heating at 90 $^{\circ}$ C for 5 min. DNA was collected by ethanol precipitation. The pellet was resolved in 8 μ L of loading dye (formamide with fushin red), heated at 94 $^{\circ}$ C for 20 min, and immediately cooled at 0 $^{\circ}$ C. Two microliters of aliquot was electrophoresed on 6% denaturing polyacrylamide gel using a 5500-S DNA sequencer.

Table 1. Consensus DNA Alkylation Site by Duo in the Presence of Various Triamides

triamide	site of alkylation	triamide	site of alkylation
none	5'-(A/T)(A/T)A-3'	ImPyPy	5'-TCG-3'/5'-TCA-3' (2:1)
dist	5'-PuTG-3'	PyImIm	5'-CAG-3'
PyPyPy	5'-PuTG-3'	PyImPy	5'-CAG-3'/5'-CAA-3' (5:1)
PyPyIm	5'-PuTG-3'	ImImIm	5'-CCA-3'/5'-CCG-3' (2:1)

tions of Duo (1–50 μ M) and triamide (2–100 μ M) were tested, because these systems contain a very complex equilibrium of the binding of the monomer, homodimer and heterodimer. Figure 3 shows the PAGE of Duo-treated DNA, indicating that, in the presence of PyPyIm, major alkylation occurred at G of 5'-GTG-3' (lanes 9 and 10), similar to that with Dist (lanes 7 and 8) or PyPyPy, which has a different amino group at the C-terminus, confirming that only N-terminal residues determine the sequence specificity. In the presence of PyImPy and ImPyIm, alkylation by Duo occurred at G of 5'-CAG-3' and A of 5'-TCA-3', respectively. The consensus alkylation sequences by Duo in the presence of each of six sets of Im/Py triamides for several DNA fragments are summarized in Table 1. The results demonstrated that two N-terminal Py or Im residues of the Py/Im triamides minimumly are required in order to define the selectivity of DNA alkylation.

Our NMR-refined structure of a Duo–Dist–DNA octamer complex⁵ indicated that two N-terminal Py residues of Dist have van der Waals interaction with the B unit of Duo, whereas the C-terminal Py of Dist is located in the relatively open minor groove (Figure 3, inset). Therefore only a well-stacked Py (or Im) in the minor groove would recognize the base pair precisely, similar to the proposed rules, i.e., an Im/Py pair recognizing G•C and a Py/Py pair recognizing A•T or T•A.⁶

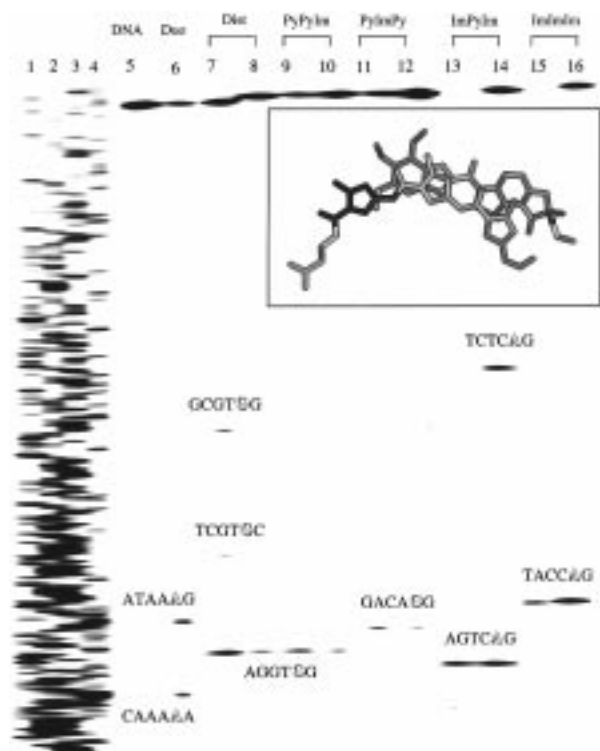


Figure 3. Thermally induced strand cleavage of 5'-TexasRed-labeled pUC 18 forward 780–1229 DNA fragment by Duo in the presence and absence of indicated triamide. Lanes 1–4, Sanger G, C, T, and A sequencing; lane 5, DNA control; lane 6, 0.05 μ M Duo; lanes 7 and 8, 20 mM Dist and 1 and 0.2 μ M Duo, respectively; lanes 9 and 10, 85 μ M PyPyIm and 2, 0.4 μ M of Duo, respectively; lanes 11 and 12, 85 μ M PyImPy and 1.0, 0.2 μ M Duo, respectively; lanes 13 and 14, 20 μ M ImPyIm and 0.25, 0.05 μ M Duo, respectively; lanes 15 and 16, 20 μ M ImImIm and 0.25, 0.05 μ M Duo, respectively. The alkylated bases are outlined. 5'-TexasRed-modified oligonucleotides 5'-AGAATCAGGG-GATAACGCAG-3' (pUC 18 forward 780–799) and 5'-TTACCAGTGG CTGCTGCCAG-3' (pUC 18 reverse 1459–1478) were used as primers to prepare DNA fragment by PCR. (Inset) Overlap of Dist and reacted Duo in the minor groove of the NMR-refined structure of Duo/Dist/d(CAGGGGT)d(ACCACCTG).⁵ For simplicity, DNA octamers are not shown. The third Py moiety of Dist is shown in dark.

In conclusion, we showed that only the Py (or Im) unit of the (Py/Im)–Duo dimer is needed to fulfill the DNA base pair recognition code when the minor groove is filled with an appropriate aromatic ligand, such as the B unit of Duo. Py/Im triamides can effectively modulate the site of alkylation by Duo in a predictable manner. Our results suggest a promising combinational approach for developing a new type of sequence-specific DNA alkylating agent. Further studies on the generality and optimization of this new class of alkylation systems are currently in progress.

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Supporting Information Available: HPLC profile of Duo-treated d(ATACCAGG)/d(CCTGGTAT) in the presence of ImImIm and ESMS of Duo/Ligand complex of d(ATACCAGG)/d(CCTGGTAT), d(CAG-GTGGT)/d(ACCACCTG), and d(GTATCAGC)/d(GCTGATAC) (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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