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Biochemical Pharmacology

Biochemical Pharmacology 61 (2001) 351–356

Short communication

Amplification of bleomycin-induced DNA cleavage at cytosine residues 3′ to GGG sequences by pyrrole triamide

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Received 29 February 2000; accepted 19 July 2000

Abstract

We investigated the amplification of bleomycin-induced DNA cleavage by synthetic triamides containing *N*-methylpyrrole (Py) and/or *N*-methylimidazole (Im), PyPyPy, PyPyIm, PyImPy, and PyImIm, using ^{32}P-labeled DNA fragments obtained from the human c-Ha-*ras*-1 and *p53* genes. Peplomycin, a bleomycin analog, plus Fe(II) caused DNA cleavage at the 5′-G<u>C</u>-3′ and 5′-G<u>T</u>-3′ sequences (damaged bases are underlined). The addition of PyPyPy dramatically enhanced the cleavage, particularly at cytosine residues 3′ to consecutive guanines. Alteration in the site specificity was not observed with other triamides (PyPyIm, PyImPy, and PyImIm). DNase I footprinting revealed that PyPyPy bound to the sites adjacent to the sites where DNA cleavage was enhanced by PyPyPy, and that PyPyPy enhanced DNase I-induced cleavage in GC-rich regions. These findings suggest that binding of PyPyPy to the DNA minor groove changes the DNA conformation to allow peplomycin to cleave DNA more efficiently at GC-rich sequences, resulting in intensive site-specific DNA cleavage particularly at cytosines at the 3′-side of polyguanines. The present study on amplifiers of antitumor drugs would appear to offer a novel approach to the establishment of more effective chemotherapy.

Keywords: DNA cleavage; Amplification; Bleomycin; Pyrrole triamide; DNase I footprinting; Minor groove

1. Introduction

Bleomycins are antineoplastic antibiotics that have been used for the treatment of tumors of head and neck, lungs, and testes. Bleomycins are activated in the presence of Fe(II) and O_2 through the formation of the bleomycin–iron–oxygen complex, which induces DNA cleavage preferably at the 5′-G<u>C</u>-3′ and 5′-G<u>T</u>-3′ sequences (damaged bases are underlined) by abstracting hydrogen from deoxyribose [1–6]. Bleomycins are known to cause severe side effects, such as pulmonary fibrosis [7,8]. Non-toxic amplification of the DNA-cleaving activity of bleomycins and other antitumor drugs would reduce drug dose and side effects that are not associated with DNA cleavage, leading to development of effective chemotherapy. It has been reported that certain DNA-binding molecules (DNA-binding ligands) enhance DNA cleavage by antitumor drugs and change its site specificity [9–14].

Polyamides containing *N*-methylpyrrole (Py) and/or *N*-methylimidazole (Im) have been reported to bind to the DNA helix in sequence-specific manners [15–19]. These polyamides can be combined in antiparallel side-by-side dimeric complexes in the minor groove for sequence-specific DNA recognition [20]. However, no attempt has been made to use polyamides in combination with antitumor drugs for treatment of cancer. In this study, we examined the effects of synthetic triamides containing pyrrole and/or imidazole (PyPyPy, PyPyIm, PyImPy, and PyImIm, Fig. 1) on DNA cleavage induced by peplomycin, a bleomycin analog (Fig. 1), using ^{32}P-5′ end-labeled DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene and the *p53* tumor suppressor gene. Particularly, we investigated the effect of PyPyPy on the site specificity of peplomycin-induced DNA cleavage with a modified method of the Maxam–Gilbert procedure [21]. In addition, the DNase I

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Abbreviations: Py, *N*-methylpyrrole; and Im, *N*-methylimidazole.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00563-3

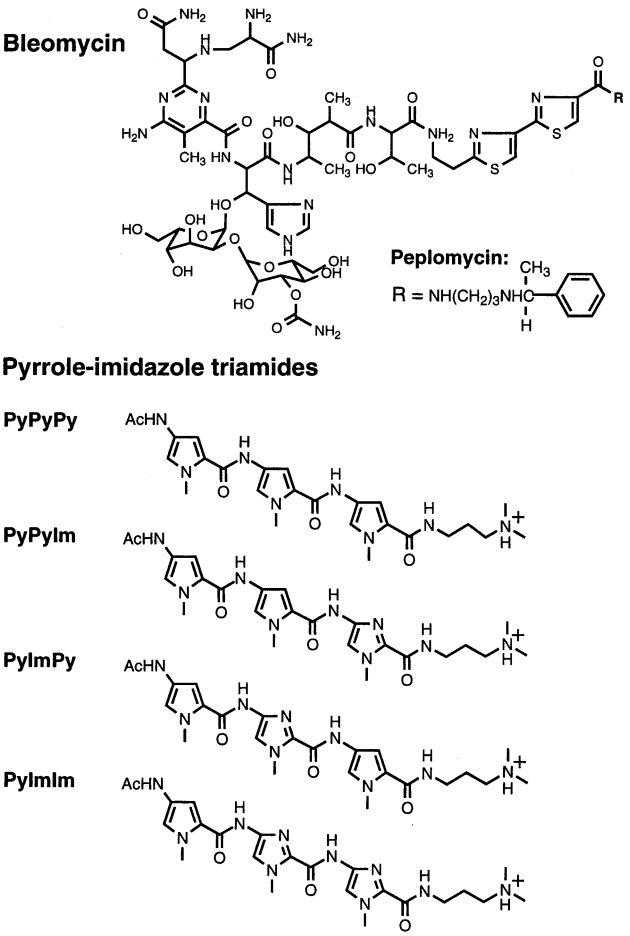

Fig. 1. Chemical structures of bleomycin (peplomycin) and synthetic pyrrole–imidazole triamides.

footprinting assay was performed to examine the DNA-binding sites of PyPyPy.

2. Materials and Methods

2.1. Materials

Restriction enzymes (AvaI, PstI, and HindIII) and T_4 polynucleotide kinase were purchased from New England Biolabs. Restriction enzymes (EcoRI and ApaI) were from Boehringer Mannheim GmbH. [γ-^{32}P]ATP was from New England Nuclear. Peplomycin and ferrous ammonium sulfate were obtained from Wako Pure Chemical Industries. Synthetic triamides containing Py and/or Im (PyPyPy, PyPyIm, PyImPy, and PyImIm) were synthesized by a method reported previously [22]. DNase I (2900 units/mg from bovine pancreas) was from Sigma Chemical Co.

*2.2. Preparation of ^{32}P-labeled DNA fragments from the c-Ha-*ras*-1 and the* p53 *genes*

DNA fragments were prepared from plasmid pbcNI, which carries a 6.6-kb BamHI chromosomal DNA segment containing the human c-Ha-*ras*-1 proto-oncogene [23,24]. The DNA fragments were labeled at 5′ end with [γ-^{32}P]ATP, and the singly labeled 337-bp fragment (*Pst*I 2345–*Ava*I* 2681) was obtained as described previously [23,24]. The asterisk indicates ^{32}P-labeling and nucleotide numbering starts with the *Bam*HI site [25]. DNA fragments were also obtained from the human *p53* tumor suppressor gene [26]. The ^{32}P-5′ end-labeled 650-bp (*Hind*III* 13972–*Eco*RI* 14621) fragment was obtained as described previously [27], and digested with ApaI to obtain the singly labeled 211-bp (*Hind*III* 13972–*Apa*I 14182) fragment.

2.3. Examination of the effect of PyPyPy on the site specificity of peplomycin-induced DNA cleavage

The standard reaction mixture in a microtube (1.5-mL Eppendorf) contained [^{32}P]DNA fragment, sonicated calf thymus DNA, and PyPyPy in 10 mM sodium phosphate buffer (pH 7.8). After the mixture was incubated for 5 min at 37°, peplomycin and ferrous ammonium sulfate were added to the mixture followed by incubation for 5 min at 37°. The DNA fragments were treated and electrophoresed as described previously [23,24]. The autoradiogram was obtained and the site specificity of DNA cleavage was analyzed as described previously [14].

2.4. Determination of DNA binding sites of PyPyPy by DNase I footprinting

DNase I footprinting was carried out according to the method reported previously [13,28]. The reaction mixture containing [^{32}P]DNA fragment, 50 μM/base sonicated calf thymus DNA, 50 μM PyPyPy, 10 mM NaCl, 0.4 mM $MgCl_2$, and 0.4 mM $MnCl_2$ in 10 mM Tris–HCl buffer (pH 7.0) was incubated for 5 min at 37°. Peplomycin and $CoCl_2$ were then added to the mixture, followed by a 5-min incubation at 37°. Co(II) was used instead of Fe(II) to prevent DNase I-independent DNA cleavage and to form the peplomycin–Co(II) complex, which interacts with DNA in a similar manner to the peplomycin-Fe(II) complex [29,30]. Subsequently, the DNA fragment was digested with 0.05 u/mL of DNase I for 10 min at 20°. The DNA fragments were treated and electrophoresed as described above. The autoradiogram was analyzed with a laser densitometer (LKB 2222 UltroScan XL) to determine the DNA-binding sites of PyPyPy.

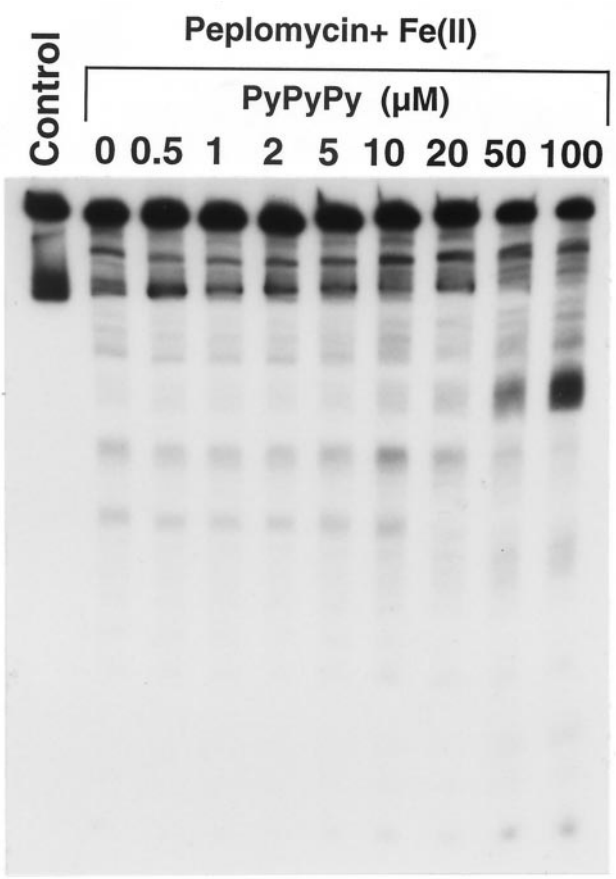

Fig. 2. Autoradiogram of ^{32}P-labeled DNA fragments incubated with peplomycin plus PyPyPy. The standard reaction mixture contained the ^{32}P-5′ end-labeled 337-bp fragment (*Pst*I 2345–*Ava*I* 2681), 50 μM/base of sonicated calf thymus DNA, and the indicated concentration of PyPyPy in 10 mM sodium phosphate buffer (pH 7.8). After the mixture was incubated for 5 min at 37°, 0.5 μM peplomycin and 0.5 μM ferrous ammonium sulfate were added, followed by incubation for 5 min at 37°. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an x-ray film to the gel. Control contained none of peplomycin, ferrous ammonium sulfate, or PyPyPy.

3. Results

3.1. Effects of synthetic triamides on peplomycin-induced DNA cleavage

We examined the effects of synthetic triamides (PyPyPy, PyPyIm, PyImPy, and PyImIm) on DNA cleavage induced by peplomycin. Among these triamides, only PyPyPy enhanced peplomycin-induced DNA cleavage and changed the cleavage pattern at 50–100 μM (Fig. 2). The other triamides did not show the alteration in the cleavage pattern (data not shown). PyPyIm enhanced peplomycin-induced DNA cleavage, whereas PyImPy had no effect on the intensity of DNA cleavage. PyImIm slightly inhibited the DNA cleavage (data not shown).

3.2. Alteration in the site specificity of peplomycin-induced DNA cleavage by PyPyPy

Fig. 3 shows PyPyPy-mediated alteration in the site specificity of peplomycin-induced DNA cleavage. Peplomycin plus Fe(II) caused DNA cleavage at the 5′-G<u>C</u>-3′ and 5′-G<u>T</u>-3′ sequences (damaged bases are underlined in the text both here and in what follows). The addition of PyPyPy dramatically enhanced the DNA cleavage at cytosines at the 3′-side of consecutive guanines (5′-GG<u>C</u>-3′, 5′-GGG<u>C</u>-3′, and 5′-GGGG<u>C</u>-3′) and to a lesser extent, at thymines in the 5′-GGGG<u>T</u>-3′ sequence and guanines in the 5′-G<u>G</u>-3′ sequence. On the other hand, PyPyPy inhibited the cleavage at certain thymines in the 5′-<u>T</u>A-3′ sequence.

3.3. DNA-binding sites of PyPyPy determined by DNase I footprinting

Fig. 4 shows an autoradiogram presenting the cleavage patterns of DNA fragments treated with peplomycin plus Co(II) and/or PyPyPy, followed by digestion with DNase I. Peplomycin plus Co(II) did not affect the pattern of DNase I-induced DNA cleavage under the condition used (lanes 1 and 2), whereas the addition of PyPyPy changed the DNA cleavage pattern (lane 3). Peplomycin plus Co(II) did not change the DNA cleavage pattern even in the presence of PyPyPy (lanes 3 and 4). Peplomycin did not cause DNA cleavage in the presence of Co(II) (lane 5). Fig. 5 shows DNA-binding sites of PyPyPy and peplomycin-induced DNA cleavage sites enhanced by PyPyPy. PyPyPy bound to DNA at the sequences that contain adenines and thymines, adjacent to the sites where peplomycin-induced DNA cleavage was enhanced by PyPyPy. On the other hand, PyPyPy enhanced DNase I-induced DNA cleavage at GC-rich regions. The site specificity of the enhancement of DNase I-induced cleavage was similar to that of peplomycin-induced DNA cleavage. Similar results were obtained with other DNA fragments (data not shown).

4. Discussion

In the present study, we examined the effects of synthetic triamides on peplomycin-induced DNA cleavage. We showed that peplomycin plus Fe(II) induced DNA cleavage at the 5′-G<u>C</u>-3′ and 5′-G<u>T</u>-3′ sequences as reported previously [1,5,6]. It is noteworthy that the addition of PyPyPy enhanced DNA cleavage, particularly at cytosines 3′ to consecutive guanines. The mechanism of the site-specific amplification of peplomycin-induced DNA cleavage by PyPyPy could be explained by assuming that PyPyPy binds to DNA at the sequences containing adenines and thymines, resulting in the DNA conformational change, which allows peplomycin to access easily to GC-rich regions adjacent to the DNA-binding sites of PyPyPy. It is unlikely that the alteration in the site specificity of DNA cleavage is simply

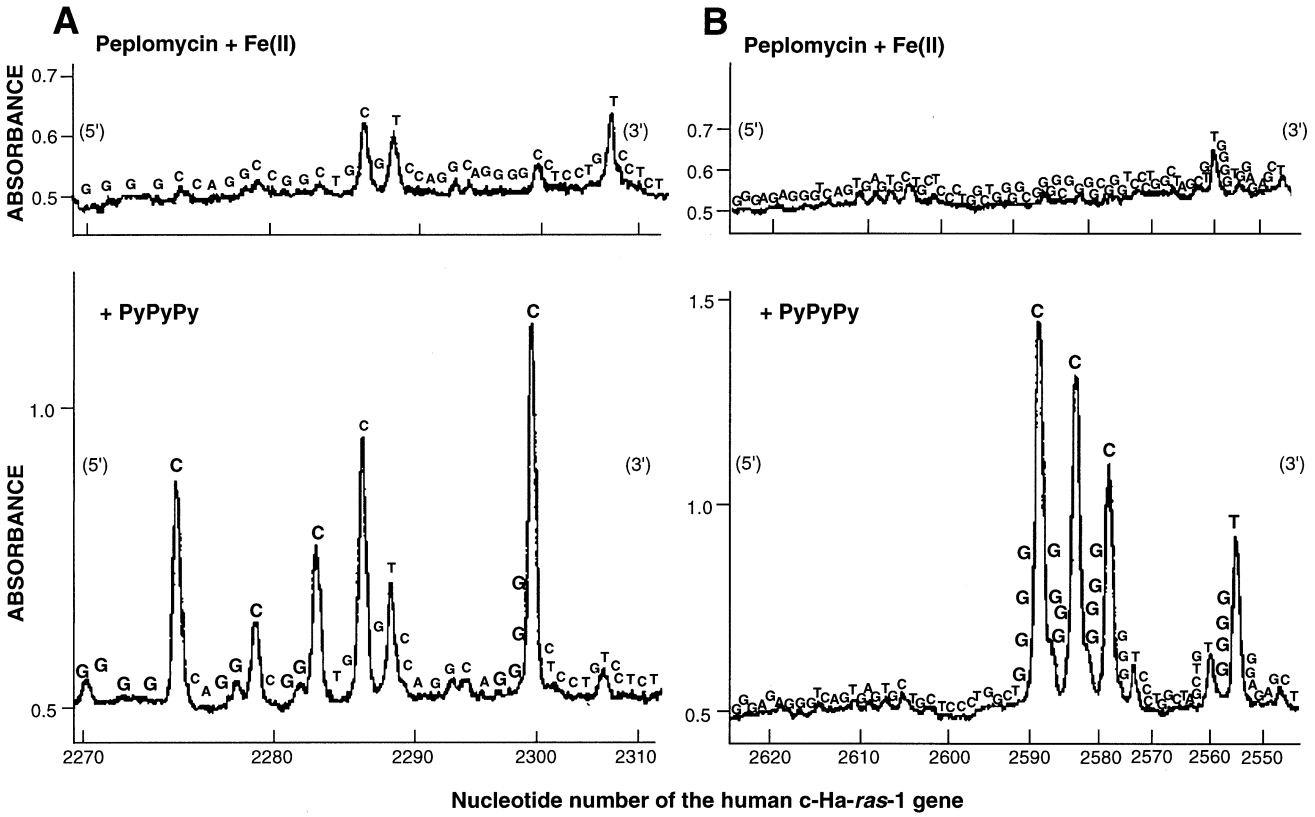

Fig. 3. Alteration in the site specificity of peplomycin-induced DNA cleavage by PyPyPy. The standard reaction mixture contained the ^{32}P-5′ end-labeled 98-bp (*Ava*I* 2247–*Pst*I 2344, A) or 337-bp (*Pst*I 2345–*Ava*I* 2681, B) DNA fragment, 50 μM/base of sonicated calf thymus DNA, and 50 μM PyPyPy in 10 mM sodium phosphate buffer (pH 7.8). After the reaction mixture was incubated for 5 min at 37°, 0.5 μM peplomycin and 0.5 μM ferrous ammonium sulfate were added to the mixture, followed by incubation for 5 min at 37°. The DNA fragments were electrophoresed and the autoradiogram was obtained by the method described in the legend to Fig. 2. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). *Abscissa*, nucleotide numbers of the c-Ha-*ras*-1 proto-oncogene starting with the *Bam*HI site [25].

due to displacement of peplomycin from the PyPyPy-binding sites to GC-rich regions, because PyPyPy enhanced peplomycin-induced DNA cleavage.

Pyrrole–imidazole triamides have been reported to alter the site specificity of DNA cleavage induced by duocarmycin A, and in particular, PyPyPy and PyPyIm changed the site specificity in a similar manner to distamycin A, which has three *N*-methylpyrrole rings as well as PyPyPy [31]. Distamycin A and duocarmycin A form the ternary complex with DNA by forming a cooperative heterodimer in the minor groove [13,32]. On the contrary, in this study, the DNase I footprinting experiment revealed that peplomycin and PyPyPy bound to DNA at adjacent but distinct sites, suggesting that these drugs do not co-occupy the common sequences. In addition, PyPyPy enhanced DNase I-induced DNA cleavage in GC-rich regions. The enhancing pattern of the DNA cleavage was similar to peplomycin-induced DNA cleavage, suggesting that these cleavages are amplified by PyPyPy in similar manners. It has been reported that DNase I-induced DNA cleavage is enhanced by certain DNA-binding molecules [33,34]. Our observation and these reports support the idea that the alteration in the cleavage pattern is due to changes in local DNA structure.

Triamides containing imidazole did not alter the site specificity of peplomycin-induced DNA cleavage, although PyPyPy amplified the cleavage at cytosines 3′ to polyguanines. This observation could be explained by the difference in the DNA-binding modes of these polyamides [35]. Imidazole-containing polyamides bind to DNA at sequences containing guanines and cytosines. On the other hand, PyPyPy, which is structurally similar to distamycin A, appears to bind to AT-rich regions.

It is well known that a number of chemotherapeutic drugs cause severe side effects. The effects of DNA ligands on DNA cleavage by antitumor drugs have been investigated in an attempt to establish more effective cancer chemotherapy and reduction of side effects. It has been reported that certain DNA-binding molecules enhance DNA cleavage caused by antitumor drugs and change the site specificity. Bleomycin-induced DNA cleavage can be amplified by DNA-binding molecules, actinomycin D [9], distamycin A [10], polyamines [11], and polyaromatic compounds [12] as well as PyPyPy. In this study, PyPyPy enhanced bleomycin-induced DNA cleavage at the 5′-GG<u>C</u>-3′, 5′-GGG<u>C</u>-3′, 5′-GGGG<u>C</u>-3′, 5′-GGGG<u>T</u>-3′, and 5′-G<u>G</u>-3′ sequences. Previous studies demonstrated that distamycin A enhanced

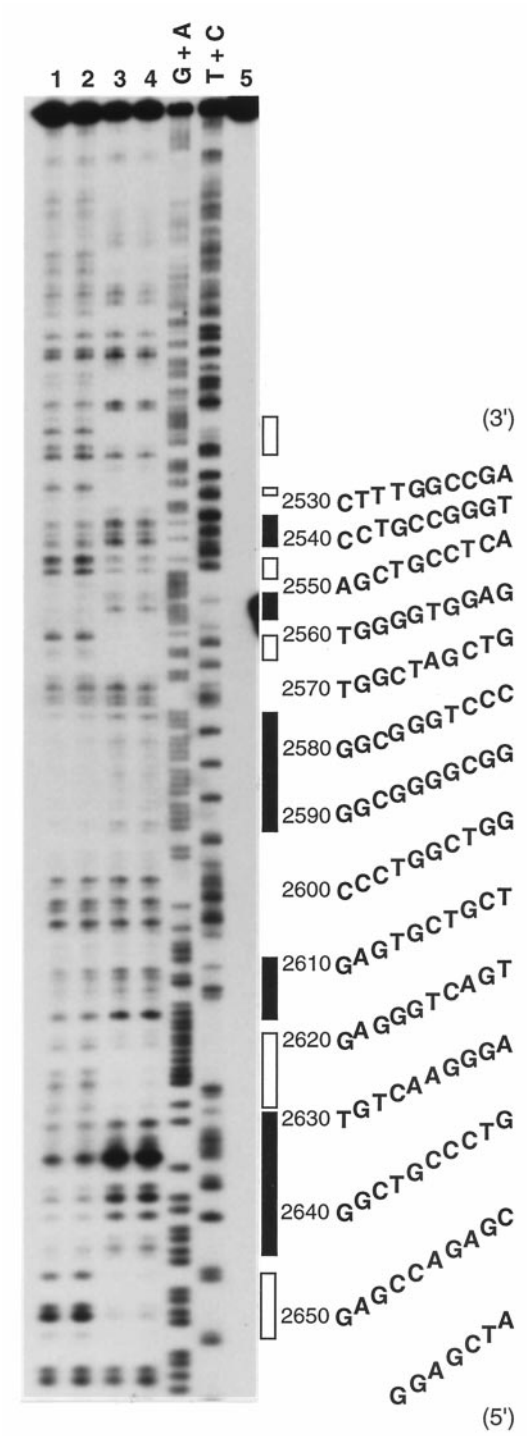

Fig. 4. DNase I footprinting of peplomycin and PyPyPy bound to the ^{32}P-labeled DNA fragment. G + A and T + C lanes are for the fragments cleaved by the chemical methods of Maxam and Gilbert [21]. The ^{32}P-5′ end-labeled 337-bp DNA fragment (*Pst*I 2345–*Ava*I* 2681) was treated with 50 μM PyPyPy and/or 0.5 μM peplomycin plus 0.5 μM $CoCl_2$, followed by digestion with 0.05 u/mL of DNase I as described in Materials and Methods. *Lane 1*, control (in the absence of PyPyPy, peplomycin, and $CoCl_2$); *lane 2*, peplomycin plus $CoCl_2$; *lane 3*, PyPyPy alone; *lane 4*, PyPyPy and peplomycin plus $CoCl_2$; *lane 5*, DNA fragments treated with PyPyPy and peplomycin plus $CoCl_2$ without DNase I digestion. *Open bars*, binding sites of PyPyPy. *Closed bars*, DNase I-induced cleavage sites enhanced by PyPyPy.

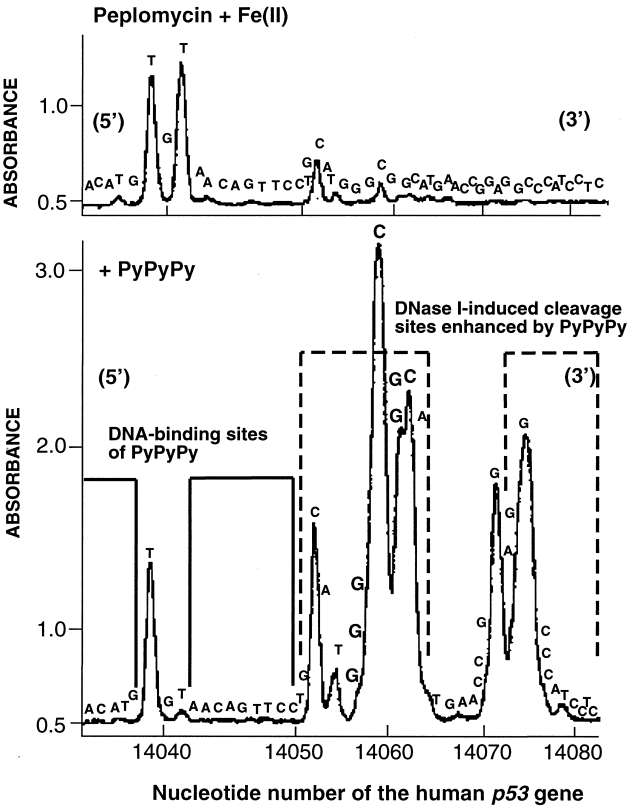

Fig. 5. Comparison of DNA-binding sites of PyPyPy and peplomycin-induced DNA cleavage sites enhanced by PyPyPy. The ^{32}P-5′ end-labeled 211-bp (*Hind*III*13972–*Apa*I 14182) DNA fragment was treated with PyPyPy and peplomycin plus ferrous ammonium sulfate, and the autoradiogram was obtained and analyzed by the method described in the legend to Fig. 2. The DNA-binding sites (solid lines) and DNase I-induced DNA cleavage sites amplified by PyPyPy (dashed lines) were determined as described in Fig. 4. *Abscissa*, nucleotide numbers of the human *p53* gene.

bleomycin-induced DNA cleavage at the 5′-GGGGC-3′ sequence [10], whereas actinomycin D, which intercalates between base pairs, amplified the cleavage at the 5′-GA-3′ and 5′-AT-3′ sequences [9]. These studies indicate that different DNA-binding molecules show different patterns of alteration in the site specificity of DNA cleavage by antitumor drugs. Further investigation on the mechanism of amplification of DNA cleavage by DNA-binding molecules would be useful for development of new DNA-binding compounds and establishment of more effective chemotherapy.

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