DNA Sequence Specificity of the Pyrrolo[1,4]benzodiazepine Antitumor Antibiotics. Methidiumpropyl-EDTA–Iron(II) Footprinting Analysis of DNA Binding Sites for Anthramycin and Related Drugs†

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ABSTRACT: Anthramycin, tomaymycin, and sibiromycin are members of the pyrrolo[1,4]benzodiazepine [P(1,4)B] antitumor antibiotic group. These drugs bind covalently through N^2 of guanine and lie within the minor groove of DNA [Petrusek, R. L., Anderson, G. L., Garner, T. F., Fannin, Q. L., Kaplan, D. J., Zimmer, S. G., & Hurley, L. H. (1981) *Biochemistry 20*, 1111–1119]. The DNA sequence specificity of the P(1,4)B antibiotics has been determined by a footprinting method using methidiumpropyl-EDTA–iron(II) [MPE·Fe(II)], and the results show that each of the drugs has a two to three base pair sequence specificity that includes the covalently modified guanine residue. While 5′PuGPu is the most preferred binding sequence for the P(1,4)Bs, 5′PyGPy is the least preferred sequence. Footprinting analysis by MPE·Fe(II) reveals a minimum of a three to four base pair footprint size for each of the drugs on DNA with a larger than expected offset (two to three base pairs) on opposite strands to that observed in previous analyses of noncovalently bound small molecules. There is an extremely large enhancement of MPE·Fe(II) cleavage between drug binding sites in AT rich regions, probably indicating a drug-induced change in the conformational features of DNA which encourages interaction with MPE·Fe(II). In the presence of sibiromycin or tomaymycin the normally guanine-specific methylene blue reaction used in Maxam and Gilbert sequencing cleaves at other bases in defined positions relative to the drug binding sites. Finally, modeling studies are used to rationalize the differences and similarities in sequence specificities between the various drugs in the P(1,4)B group and their reactions with DNA.

Anthramycin, tomaymycin, and sibiromycin (see Figure 1) are members of the pyrrolo[1,4]benzodiazepine [P(1,4)B][1] class of antitumor antibiotics [for reviews, see Hurley (1977) and Thurston & Hurley (1983)]. The potent antitumor activity of these agents is believed to be due to their ability to react covalently with N^2 of guanine (see Figure 2) in double-stranded DNA (Hurley & Petrusek, 1979; Graves et al., 1984). The resultant drug adducts lie snugly cradled within the minor groove of DNA (Petrusek et al., 1981), with the right-handed twists of the drug molecules (Mostad et al., 1978; Arora, 1979, 1981) tightly following the curvature of the double helix in B-form DNA. The close match between the twists of the drugs and of DNA led us to hypothesize that, in their reaction with DNA, these agents might be sensitive to sequence-dependent variations in DNA helical structure and that alterations in the twists of the drug molecules might also result in alteration of DNA binding selectivity [for reviews of sequence specificity in drug–DNA interactions, see Dabrowiak (1983) and Wilkins (1984)].

Several lines of evidence support the suggestion that the P(1,4)Bs react with DNA in a sequence-specific fashion. First, competitive binding experiments have demonstrated incomplete competition for binding sites between anthramycin and tomaymycin (Hurley et al., 1977) and between anthramycin and CC-1065 (Swenson et al., 1982), indicating that members of the P(1,4)B class bind preferentially to specific regions on the DNA. Second, a direct but imprecise demonstration of sequence dependence in the reaction of anthramycin with DNA has been obtained from studies that established that poly(dG)·poly(dC) is far more receptive to anthramycin binding than is poly(dG-dC)·poly(dG-dC) (Petrusek et al., 1981). This finding is supported by the results of studies (Sumner & Bennett, 1981; Kaplan, 1982) that revealed that anthramycin inhibits the action of restriction enzymes whose recognition sequences include 5′GG to a greater extent than those whose recognition sequences include 5′GC. Additional indications that members of the P(1,4)B class are sensitive to structural variations in the DNA helix are to be found in the reduced reaction kinetics of anthramycin (Reynolds & Hurley, 1982) and sibiromycin (Kozmyan et al., 1978) toward chromatin relative to naked DNA and in the preference of anthramycin for linker DNA rather than nucleosome core DNA (Reynolds & Hurley, 1982). However, steric constraints due to the presence of histone protein may also contribute to the reduced preference for nucleosome core DNA.

The experiments described in this paper were designed to test the hypothesis that members of the P(1,4)B class react with DNA in a sequence-selective fashion. Two experimental strategies were adopted. The more general method, MPE·Fe(II) footprinting [for example, see Van Dyke et al., (1982), Van Dyke & Dervan (1983a–c), and Van Dyke & Dervan (1984)] was used to demonstrate that the most preferred

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[1] Abbreviations: bp, base pair; CPK, Corey–Pauling–Koltun; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MPE, methidiumpropyl-EDTA; P(1,4)B, pyrrolo[1,4]benzodiazepine; Pu, purine; Py, pyrimidine; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; TBE, Tris–borate–EDTA (Maxam & Gilbert, 1980); HPLC, high-performance liquid chromatography.

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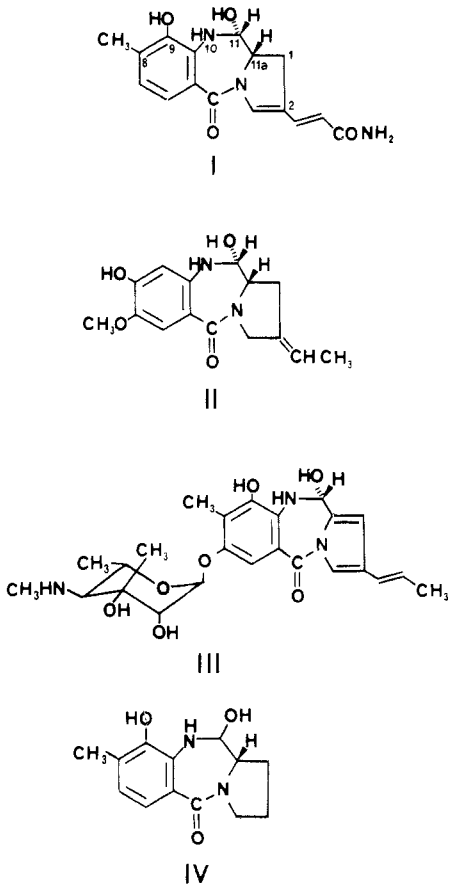

FIGURE 1: Structures of anthramycin (I), tomaymycin (II), sibiromycin (III), and 8-methyl-9-hydroxy-P(1,4)B (IV).

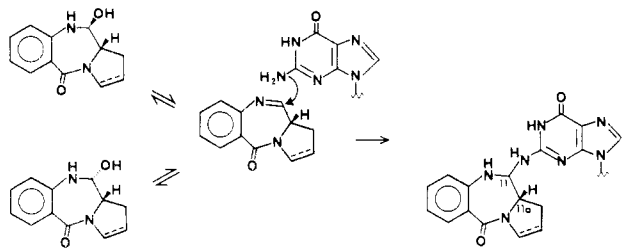

FIGURE 2: Plausible mechanism for the reaction of the P(1,4)Bs with DNA to form the P(1,4)B-(N^2-guanine)-DNA adducts (Petrusek et al., 1981; Graves et al., 1984).

binding sequences for the drugs are 5′PuGPu and the least preferred at 5′PyGPy. Since, to our knowledge, this is the first example of a covalent DNA binder that has been subjected to footprinting analysis using MPE·Fe(II), a comparison of the characteristics of the footprints of the P(1,4)B antibiotics to those of noncovalent minor groove binders and intercalating agents is presented. Portions of this work have appeared in preliminary form (Reynolds et al., 1983; Hurley et al., 1984a).

Materials and Methods

Drugs, Enzymes, and Chemicals. Tomaymycin and sibiromycin were supplied by Fujisawa Pharmaceutical Co. (Osaka, Japan) and by Bristol Laboratories (Syracuse, NY), respectively, and were checked for purity by TLC (Hurley, 1977) prior to use. The synthetic derivative 8-methyl-9-hydroxy-P(1,4)B (IV) was synthesized in our laboratories (Thurston et al., 1984). Polynucleotide kinase from bacteriophage T4 infected *Escherichia coli* was isolated and purified according to published procedures (Richardson, 1971) or purchased from Boehringer Mannheim (Indianapolis, IN). Bovine alkaline phosphatase was purchased from Boehringer Mannheim. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). All chemicals used were reagent grade or better. Chemicals used to prepare polyacrylamide gels were electrophoresis grade. [γ-^{32}P]ATP was synthesized according to published procedures (Walseth & Johnson, 1979) or purchased from New England Nuclear. [α-^{32}P]dNTP's were purchased from New England Nuclear. Bacteriophage T7 was grown and purified using established procedures (Studier, 1969). pBR322 and SV40 DNA were purchased from BRL (Bethesda, MD). MPE was a generous gift of Dr. Peter B. Dervan, California Institute of Technology, Pasadena, CA.

Spectrophotometric Methods. Concentrations of the following solutions were determined spectrophotometrically by using the indicated extinction coefficients: anthramycin in methanol, E_{333} = 36 800 (Kaplan & Hurley, 1981); tomaymycin in methanol, E_{320} = 3600 (Hurley, 1977); sibiromycin in methanol, E_{310} = 21 800 (Hurley, 1977); MPE in deionized water, E_{488} = 5994 or E_{286} = 54 725 (Hertzberg & Dervan, 1984).

DNA Sequencing. DNA sequencing was carried out by using the methods of Maxam & Gilbert (1977, 1980). The restriction fragments employed were as follows (the first position given is that of the single radioactive label): from T7 (Dunn & Studier, 1981) 1–68 and 8315–7914, both 5′ end labeled; from pBR322 (Sutcliffe, 1978) 380–654 and 375 (through 1)–4361, both 3′ end labeled; from SV40 (Tooze, 1980) 1494–1641, 1712–1509, 2774–2533, 4740–4843, all 5′ end labeled and 3′-end-labeled fragments were 2770–2528, 1782–1509, 1787–2260, and 346 (through 1)–5119. Subsequent analysis demonstrated that the sequences of DNA restriction fragments used in this study exactly matched the published sequences of bacteriophage T7 (Dunn & Studier, 1981), SV40 (Tooze, 1980), and pBR322 (Sutcliffe, 1978).

Preparation and Determination of Sequence Specificity of the P(1,4)B–DNA Adducts. Two methods were used in the preparation and analysis by MPE·Fe(II) footprinting of P-(1,4)B–DNA adducts. Additionally, methylene blue was used to determine the binding sites of sibiromycin and tomaymycin. The most important difference between methods 1 and 2 described below was the drug to nucleotide ratios in the drug incubation mixtures. Only in the case of method 2 was carrier calf thymus DNA used in the incubation mixtures. While higher concentrations of drug were used where carrier DNA was present, it is likely that the final drug to nucleotide ratios differed markedly between the two methods. These differences would be expected to effect the kinetics of drug binding to DNA but not the final DNA sequence specificity.

Method 1. MPE·Fe(II) footprinting reactions (data for Figures 3 and 5 and portions of Table I). Each reaction (10 μL) contained 50 or 200 μM (bp) calf thymus DNA and singly ^{32}P-end-labeled DNA restriction fragments in 10 mM Tris-HCl, pH 7.4, and 50 mM NaCl. The P(1,4)B was dissolved in ethanol and added to the reaction at the indicated concentrations (final ethanol concentrations was <10%). After 4 h at 37 °C (shorter times are indicated in the figure legends), MPE·Fe(II) was added to a final concentration of 5 μM. The reaction was incubated at room temperature for 15 min, and cleavage was initiated by the addition of DTT (final concentration 2 mM). The cleavage was allowed to proceed for 15 min and was terminated by precipitation of the DNA in ethanol. The samples were then electrophoresed as will be described.

Method 2. The MPE·Fe(II) footprinting procedure described here was used to acquire data for Figure 4 and portions

of Table I, while the methylene blue procedure was used to acquire the data in Figure 6A. Solutions of anthramycin, tomaymycin, and sibiromycin were prepared in methanol (HPLC grade) immediately prior to use. Drug concentrations were determined spectrophotometrically as described earlier. Aliquots of these solutions containing 1.5, 0.15, 0.075, or 0.015 nmol of drug were dried under vacuum, combined with singly ^{32}P-end-labeled DNA in 1 mM NaCl (8 μL final volume), and incubated (3 h for anthramycin and tomaymycin samples; 1 h for sibiromycin samples) at room temperature. For samples that were to be subjected to the methylene blue reaction, unreacted drug was removed by extraction with water-saturated 1-butanol (2 × 100 μL). The samples were then lyophilized, resuspended in 20 μL of deionized water, subjected to reaction with methylene blue as described (Friedmann & Brown, 1978), and electrophoresed as will be described. For samples that were to be treated to MPE·Fe(II) digestion, the reaction mixtures were adjusted to 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 90 μM calf thymus DNA (bp), and 10 μM MPE·Fe(II) in a final volume of 10 μL as described (Van Dyke & Dervan, 1983a). Following incubation for 30 min at room temperature, 1 μL of 40 mM DTT was added, and digestion was carried out for 15 min at 37 °C as described (Van Dyke & Dervan, 1983a). In order to alleviate P(1,4)-B-related smearing of bands upon subsequent electrophoresis (Sumner & Bennett, 1981), bound drug was removed from the DNA by heating (90 °C, 5 min) followed by either extraction with water-saturated 1-butanol or precipitation of the DNA in ethanol (Hurley et al., 1979). The samples were then dried under vacuum and electrophoresed as will be described.

Electrophoretic Analysis of P(1,4)B Binding Sites on DNA. DNA samples that had been either treated to base-specific sequencing reactions or analyzed for P(1,4)B binding sites as described earlier were resuspended in 4–10 μL tracking dye (50% deionized formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol in TBE), heated (90 °C, 5 min) to denature the DNA and release covalently bound P(1,4)B (Hurley et al., 1979), and quickly chilled on ice–water. Equal amounts of radioactivity from the DNA samples were loaded onto thin denaturing polyacrylamide gels (8%; 1:20 acrylamide–bisacrylamide; 50% urea) and electrophoresed at 1200–1800 V for 2–4 h. Autoradiograms were developed by using Kodak XRP-1 or XAR-2 film with the aid of intensifying screens.

Densitometric Analysis of Sequencing Gels. Autoradiograms were scanned with an LKB 2202 laser densitometer interfaced with an HP3390 integrator. Background absorbance due to the film density was subtracted from the scans. The data are reported as percentage cleavage, $P = 100(A_p - A_c)$, where A_p is the area of a peak corresponding to an MPE·Fe(II) cleavage band in the presence of P(1,4)B, and A_c is the area of the peak corresponding to the same MPE·Fe(II) cleavage band for unmodified DNA. Values of P greater than 100% indicate cleavage enhancement, while values less than 100% indicate cleavage inhibition.

Results

Our initial approach to determine the DNA sequence specificity of the P(1,4)Bs was based upon the assumption that because these drugs bind to DNA covalently through N^2 of guanine, they should inhibit a guanine-specific methylene blue reaction (Friedmann & Brown, 1978). Experiments were carried out with the expectation that the binding site of the drug molecule could be revealed as a diminution of band intensity on autoradiographs of sequencing gels in which singly ^{32}P-end-labeled DNA was bound with drug and then treated with methylene blue. To our surprise, it was found that sibiromycin- and tomaymycin-treated DNA samples exhibited new bands at sites other than guanine upon treatment with methylene blue. This interesting observation points to a potential problem inherent in experiments where DNA binding ligands can alter the normal specificity of DNA cleavage reagents, presumably through ligand-induced conformational changes in DNA. Although we were able to identify the binding sites on DNA of tomaymycin and sibiromycin from the methylene blue experiments this technique was unfortunately not general for all members of the P(1,4)B class and therefore an alternative, more generally applicable method was sought. The results to be presented in this report are largely derived from the MPE·Fe(II) footprinting method. A comparison of the results of an experiment with sibiromycin using the methylene blue and MPE·Fe(II) methods is also presented.

Time Course Binding of Anthramycin to DNA As Revealed by MPE·Fe(II) Footprinting Analysis. MPE·Fe(II) cleavage of a 123 base pair restriction enzyme fragment labeled at the 5′ end with ^{32}P affords a relatively uniform DNA cleavage pattern on a Maxam–Gilbert sequencing gel (Figure 3A, lane 2). In contrast, pretreatment of the DNA with anthramycin leads to nonuniform pattern that is due to the inhibition of MPE·Fe(II) cleavage at sites where anthramycin is bound superimposed on an enhanced degree of cleavage between binding sites. Enhancement of MPE·Fe(II) cleavage has also been observed by Dervan and co-workers (Van Dyke & Dervan, 1983c). The DNA fragments pretreated with anthramycin for incremental amounts of time (Figure 3A, lanes 3–6) revealed three major MPE·Fe(II) footprints (sites A–C in Figure 3A). The arrows in lane 7 identify the locations of guanines on the unlabeled DNA strand, while the bands in lane 7 identify the guanines on the labeled DNA strand. Therefore, collectively the arrows and bands in lane 7 identify all the possible anthramycin binding sites on this fragment that are found up to footprint C. Significantly even at the longest incubation time not all of the possible guanines are modified by anthramycin (see, for example, the guanine between sites A and B), and some sites (sites A and B) are filled before others (site C). A discussion of the relative affinities of anthramycin for these sites is given below. The footprint at site A increased in size from the 15-min to the 270-min exposure (Figure 3A, lanes 3 and 6), most likely due to the inclusion of a lower affinity binding site at the longer incubation times. In contrast, the footprint at site B appeared to decrease in size from lanes 3 to 5. This appeared to be related to the considerable enhancement of MPE·Fe(II) cleavage at sequences between drug binding sites. The enhancement between sites A and B was most pronounced for the 270-min incubation time where a 12-fold increase was evident (see Figure 3B). Figure 3B shows histogram plots derived from densitometric tracings of footprinting lanes 3 and 6 from Figure 3A, and clearly reveals both the MPE·Fe(II) footprints of anthramycin and the enhancement of cleavage outside drug binding sites.

Comparison of MPE·Fe(II) Footprinting of Anthramycin, Tomaymycin, and Sibiromycin. While MPE·Fe(II) cleavage of the unmodified 204 bp fragment shown in Figure 4A afforded a relatively uniform DNA cleavage pattern on a Maxam–Gilbert sequencing gel (lane 10), the cleavage of drug-treated fragments (lanes 1–9) again produced a much more uneven pattern. The histogram plots derived from the densitometric tracings of lanes 2, 6, and 8 in Figure 4A provide an improved visual perception of the MPE·Fe(II) cleavage inhibition by sibiromycin, tomaymycin, and anthramycin (Figure 4B). The histograms show the relative cleavage by MPE·Fe(II) of drug-treated vs. control DNA. The 100% value

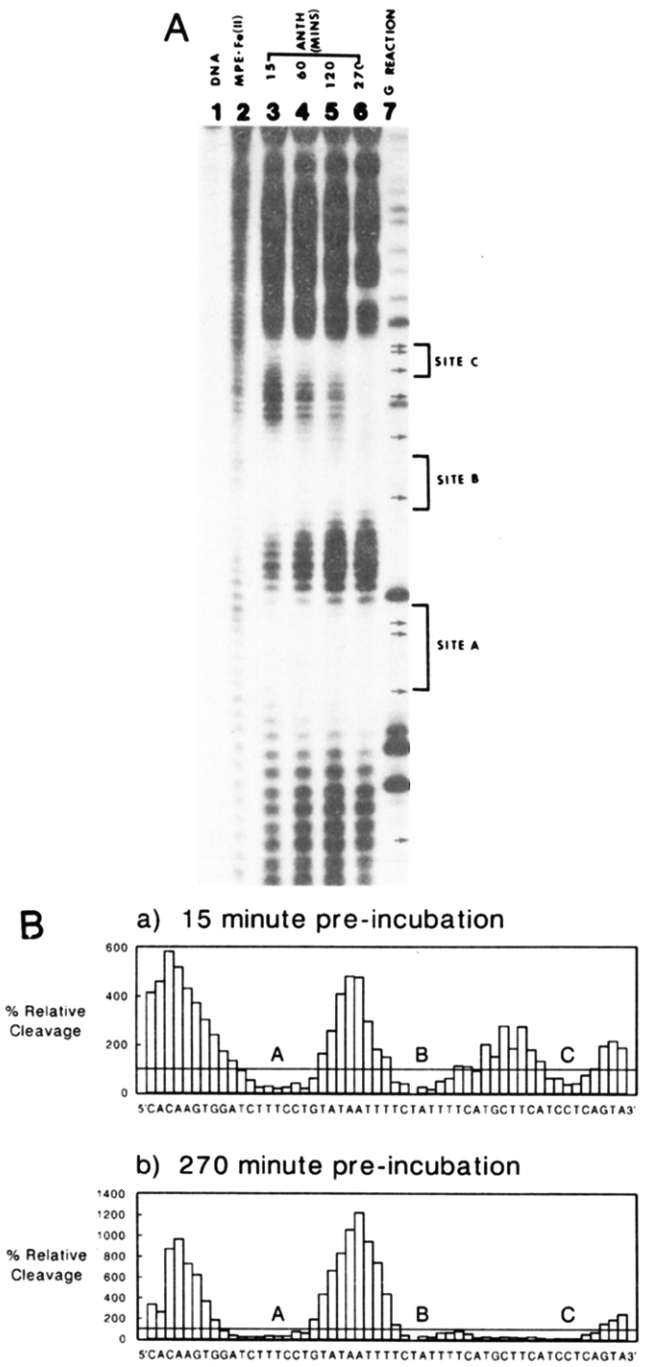

FIGURE 3: (A) Time course autoradiogram of DNA cleavage inhibition by anthramycin. A 5′-^{32}P-end-labeled 123 bp fragment was reacted with anthramycin (100 μM) and subjected to MPE·Fe(II) footprinting as described in method 1 (Materials and Methods). Lane 1, intact DNA; lane 2, MPE·Fe(II) cleavage of unprotected DNA; lanes 3–6, MPE·Fe(II) cleavage inhibition by anthramycin at 100 μM preincubated for 15, 60, 120, and 270 min (lanes 3–6, respectively) at 37 °C; lane 7, G-specific reaction. The DNA concentration was 50 μM (bp). Arrows in lane 7 indicate positions of the guanines on the opposite (unlabeled) strand. Brackets labeled A–C are anthramycin footprints. (B) Histograms of anthramycin-induced differences in the susceptibility of DNA to MPE·Fe(II) cleavage. The data are from lanes 3 and 6 in (A) and represent 15- and 270-min preincubation with anthramycin. Footprints A–C are the same as those depicted in (A). Values over 100% indicate percentage enhancement relative to control (see Materials and Methods), whereas values of 0–100 indicate percentage inhibition relative to the control.

shown in Figure 4B therefore represents zero change in the amount of cleavage in drug-treated vs. control lanes. A comparison of the three histograms in Figure 4B substantiates the observed enhancement of MPE·Fe(II) cleavage of flanking sequences adjacent to drug binding sites shown in Figure 3 although the effect was much less pronounced under these particular experimental conditions. The two most prominent binding sites were almost the same for all three drugs, maximum inhibition of MPE·Fe(II) cleavage occurring one base removed on the 3′ side of a common sequence 5′AGGA. While sibiromycin and anthramycin produced pronounced inhibition of MPE·Fe(II) cleavage (maximum 70–80% and 30–40%, respectively), tomaymycin cleavage inhibition was much less pronounced and was only visible relative to the enhanced cleavage on either side of the binding sites. Sibiromycin had additional binding sequences (i.e., to the 3′ side of B and the 5′ side of A in Figure 4B) not evident in tomaymycin or anthramycin treatment lanes.

Opposite Strand Analysis for Anthramycin (I), Tomaymycin (II), and 8-Methyl-9-hydroxy-P(1,4)B (IV). On the basis of the results of published experiments with noncovalent minor groove binding agents using the MPE·Fe(II) cleavage technique, it was expected that an asymmetric DNA inhibition pattern would be found with the P(1,4)Bs in accord with the model proposed by Van Dyke & Dervan (1983b). Both the model and experimental results show that the maximum inhibition of DNA cleavage is shifted one to two base pairs to the 3′ side of the drug binding site. In order to determine whether the same observations apply to the P(1,4)Bs, MPE·Fe(II) was allowed to cleave a 241 bp fragment which was either 5′ or 3′ end-labeled at the same molecular end and preincubated with anthramycin, IV, or tomaymycin. The autoradiogram of the 5′-end-labeled fragment is shown in Figure 5A, and the histograms of the opposite strand footprints derived from this autoradiogram and its 3′-end-labeled counterpart are shown in Figure 5B.

A composite comparison of the opposite strand footprints revealed by MPE·Fe(II) of anthramycin, IV, and tomaymycin is shown in Figure 5C. Five major footprints occurred within this 57 bp region, located around positions 8, 19, 30, 42, and 53 on both strands. All three drugs exhibited footprints at four of the five sites, while anthramycin alone had an additional strong footprint site around position 19. Without exception the protected zones were staggered one to three bases to the 3′ side of the presumed covalently modified guanine, as anticipated from previous studies (Van Dyke & Dervan, 1983b; Low et al., 1984a,b).

In accord with the data in Figures 3A and 4A, there was considerably enhanced MPE·Fe(II)-induced cleavage adjacent to certain binding sequences, e.g., around position 25 in Figure 5B, panels a and c. The average size of the footprints was about three to four base pairs. The seven to eight base pair footprint for anthramycin in the region around position 43 was most probably due to two adjacent binding sites in this GC-rich region.

While four of the five binding sites were found in *approximately* the same location for each of the drugs tested, the precise sites of covalent attachment, the intensity of the footprints, and thus the rank order of binding site preference were quite different between the three drugs. For example, the individual footprints around position 8 differed in location by as much as three base pairs (Figure 5C), and while this was a prime binding site of IV and tomaymycin, it was a lower ranking binding site for anthramycin (Figure 5B). These differences are presumably a reflection of the structural variation between the various P(1,4)Bs (vide infra).

Consensus Sequence Analysis for Anthramycin, Tomaymycin, and Sibiromycin. We utilized the cleavage inhibition patterns from the opposite strand analysis (see Figure 5B,C)

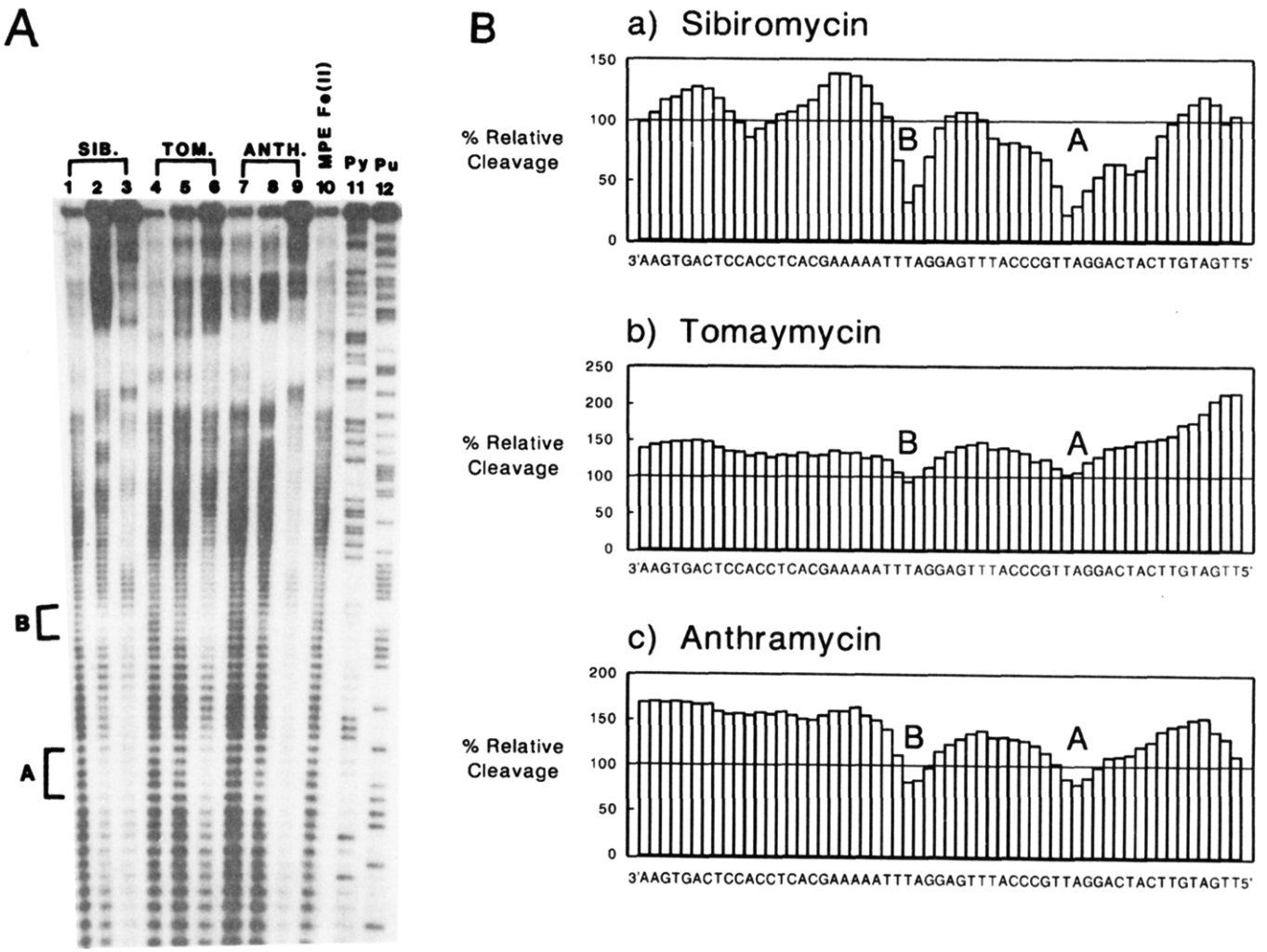

FIGURE 4: (A) Autoradiograms of the DNA cleavage inhibition produced by the P(1,4)B antibiotics, sibiromycin, tomaymycin, and anthramycin. A 5′-end-labeled 204 bp fragment was prepared and reacted with the P(1,4)Bs as described under Materials and Methods (method 2). Lanes 1–9, P(1,4)B treatments. DNA was incubated with 0.015, 0.15, or 1.5 nmol of sibiromycin (lanes 1–3), tomaymycin (lanes 4–6), or anthramycin (lanes 7–9), respectively, and then digested with MPE·Fe(II). Lane 10 is MPE·Fe(II), control (no drug treatment). Lanes 11 and 12 are the Maxam–Gilbert T+C and A+G reactions, respectively. Brackets labeled A and B indicate footprints common to all three drugs. (B) Histograms of MPE·Fe(II) footprints of sibiromycin, tomaymycin and anthramycin. Data are taken from (A), lanes 2, 5, and 8. For explanation of percent relative cleavage see legend for Figure 3B. Footprints A and B are the same as those depicted in (A).

for members of the P(1,4)B antibiotic group to predict with some confidence the location of the covalently modified guanine within each footprint. By use of the data derived from a variety of restriction enzyme fragments which had been modified by either anthramycin, tomaymycin, or sibiromycin and then subjected to MPE·Fe(II) cleavage, the sequences flanking the guanines that bound these drugs were determined (see Table I). For comparison, the sequences flanking guanines that did not bind drug molecules were also scored. The data in Table I show that for anthramycin and sibiromycin slightly less than 50% of the total guanines actually footprinted as binding sites, whereas only about one quarter of the potentially available guanines formed adducts with tomaymycin. There was no significant preference for a particular base or base type >1 base removed from either side of the covalently modified guanine. While a preference for a defined base was not obvious for the positions directly flanking gunine, there was a significant preference for the trimer 5′PuGPu as a binding site for anthramycin or sibiromycin, and to a lesser extent for tomaymycin. The least favored trimer for all these drugs was 5′PyGPy, with 5′PyGPu slightly favored over 5′PuGPy.

Sibiromycin- and Tomaymycin-Induced Alterations in the Normally Guanine-Specific Methylene Blue Reaction. The methylene blue sensitized photooxidation of DNA (Simon & Van Vunakis, 1962, 1964; Sastry & Gordon, 1966) has been adapted for use in DNA sequencing as a guanine-specific reagent (Friedmann & Brown, 1978) although in the earlier work a low reactivity with thymine compounds was noted (Simon & Van Vunakis, 1964) and extensive irradiation of

Table I: Consensus Sequence Analysis of Footprinting Data for Anthramycin, Tomaymycin, and Sibiromycin[a]

	anthramycin[b]		tomaymycin[b]		sibiromycin[b]	
	+[c] (%)	−[c] (%)	+[c] (%)	−[c] (%)	+[c] (%)	−[c] (%)
5′PuGPu	54 (83)	11 (17)	22 (45)	27 (55)	16 (67)	8 (33)
5′PyGPy	11 (26)	32 (74)	3 (10)	28 (90)	8 (36)	14 (64)
5′PyGPu	21 (38)	34 (62)	9 (20)	35 (80)	12 (40)	18 (60)
5′PuGPy	16 (26)	46 (74)	15 (32)	32 (68)	11 (41)	16 (59)
5′NGN	102 (45)	123 (55)	49 (29)	122 (71)	47 (45)	56 (55)

[a] All guanines were scored for the presence or absence of drug binding on the basis of their ability to significantly inhibit MPE·Fe(II) cleavage. There was no significant preference for a base or base type >1 base pair to either side of the covalently modified guanine. Likewise, individual base analysis (G, C, A, T) at positions adjacent to the covalently modified guanine did not reveal any significant preferences. [b] A total of approximately 515, 363, and 194 bp were analyzed for anthramycin, tomaymycin, or sibiromycin binding sites. [c] Plus (+) and minus (−) refer to the presence or absence of a footprint as determined by inhibition of MPE·Fe(II) cleavage.

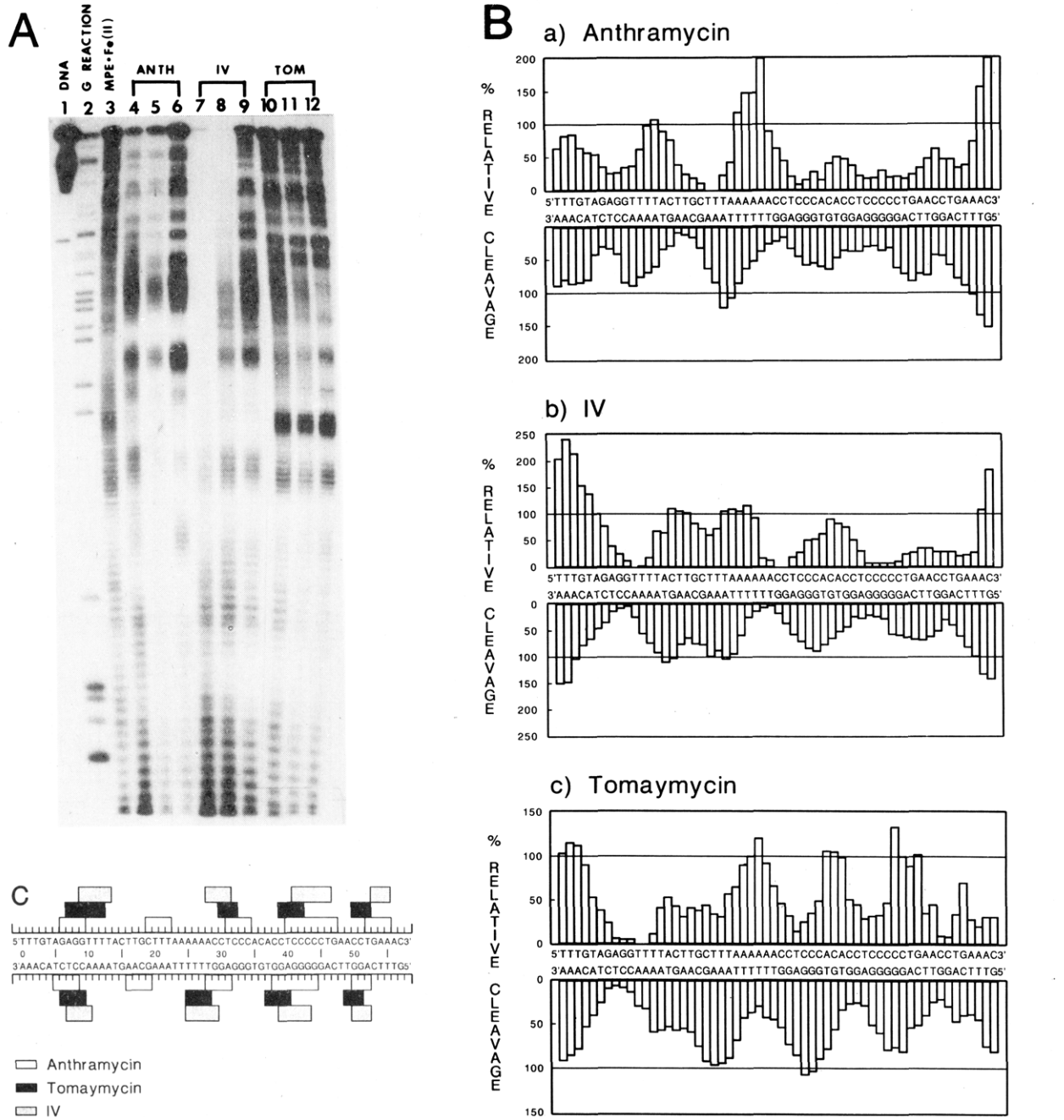

FIGURE 5: (A) Autoradiogram of the DNA cleavage inhibition by anthramycin, IV, and tomaymycin on a 5′-^{32}P-end-labeled 241 base pair fragment. Drug treatment and MPE·Fe(II) footprinting was carried out by using method 1 (see Materials and Methods). Lane 1, intact DNA; lane 2, Maxam–Gilbert chemical sequencing G reaction; lane 3, MPE·Fe(II) cleavage of unprotected DNA; lanes 4–6, MPE·Fe(II) cleavage of anthramycin-bound DNA at 20, 50, and 200 μM; lanes 7–9, MPE·Fe(II) cleavage of IV-bound DNA at 200 μM, 1 mM, and 4 mM; lanes 10–12, MPE·Fe(II) cleavage of tomaymycin-bound DNA at 20, 50, and 200 μM. Concentrations refer to inhibiting drug during the preincubation reaction. The DNA concentration was 200 μM (bp). (B) Histograms of MPE·Fe(II) footprints of anthramycin (a), IV (b), and tomaymycin (c) on opposite strands of the same fragment shown in panel A. The 5′-^{32}P-end-labeled data for anthramycin, tomaymycin, and IV are taken from lanes 6, 9, and 12 in panel A. (C) Composite of opposite strand footprinting results for anthramycin (unfilled bars), IV (shaded bars), and tomaymycin (filled bars). Bars indicate protected regions. These maps were compiled from densitometric measurements of 3′- and 5′-^{32}P-labeled fragments of DNA shown in (B).

DNA also caused destruction of the sugar moiety (Sastry & Gordon, 1966). Since the P(1,4)B derivatives are also guanine specific, it was thought that they may interfere with the methylene blue DNA sequencing reaction and that the latter may provide a means of detecting the site of binding of DNA-binding ligands. Indeed, P(1,4)B binding sites can be observed as an inhibition of the methylene blue reaction (Figure 6A) though the technique is less sensitive than that employing MPE-Fe(II), and we have found it difficult to reproduce reliably.

Of more general interest, and of potential concern for chemical footprinting methods, is the fact that additional bands, at non-guanine residues, were observed when methylene blue was used in the presence of sibiromycin or tomaymycin. We have not, however, observed these "artifactual" bands in the presence of anthramycin. An example of sibiromycin-induced changes in the methylene blue reaction is shown in Figure 6. The fragment of T7 DNA used in this experiment contains only two potential P(1,4)B binding sites (PuGPu) on the labeled DNA strand whose sequence can be resolved in this gel. Both sites, indicated in Figure 6A, showed a partial inhibition of the G-specific methylene blue reaction. However,

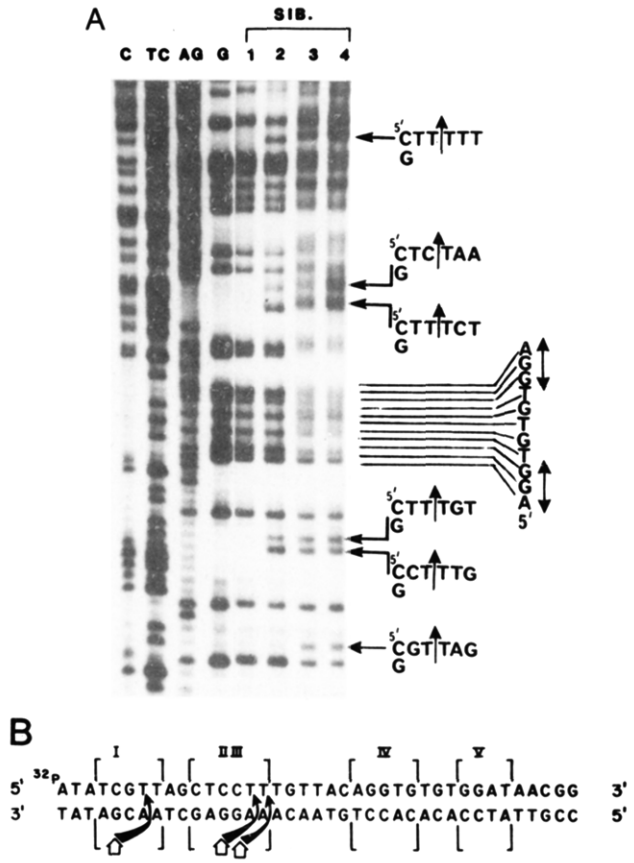

FIGURE 6: (A) Sibiromycin binding sites on DNA revealed by the methylene blue reaction. A 401 bp DNA restriction fragment was 5′-end-labeled as described under Materials and Methods. Left side lanes are base-specific chemical cleavage reactions for T+C, C, A+G, and G; lanes labeled 1–4 are sibiromycin-plus-methylene blue treaments. In lanes 1–4, DNA was reacted with 0, 0.075, 0.015, or 1.5 nmol, respectively, of sibiromycin, butanol extracted, and treated with methylene blue as described under Materials and Methods. Double-ended arrows indicate PuGPu sequences on the labeled strand, whereas the single-ended arrows indicate the position of artifactual bands within the sequence of the labeled strand. (B) Schematic representation depicting the agreement of results between the two methodologies used to determine the DNA sequence specificity of sibiromycin. The same fragment was used as described in Figure 5A. Sites of sibiromycin binding were then determined by either the methylene blue reaction or MPE·FeII) footprinting (method 2), followed by electrophoresis as described under Materials and Methods. The data for the methylene blue experiment are taken from (A). Open arrows point to putative sibiromycin-bound guanines. Solid arrows point to sites of drug-induced strand scission in the methylene blue reaction. Brackets enclose MPE·Fe(II) footprints of sibiromycin (data not shown). Binding sites IV and V, detected by MPE·Fe(II) footprinting, are only revealed by a diminution in the methylene blue reaction since the strand break resulting from sibiromycin adducts at these sites would occur on the opposite (nonisotopically labeled) strand. The three binding sites revealed by methylene blue at the 3′ end of the gel (see panel A) were not included in this figure, since they were too high in the gel to be clearly footprinted by MPE·Fe(II).

a number of prominent methylene blue induced bands that do not correspond to guanine residues were also apparent when the reaction was carried out in the presence of sibiromycin (similar results have also been obtained with tomaymycin; data not shown). These bands occurred at positions corresponding to thymine or cytosine residues, and when other DNAs were used at adenine residues. These artifactual bands invariably occurred on the 5′ side of, and two bases away from, a potential sibiromycin binding site (PuGPu), but on the opposite strand. The altered methylene blue reaction required the presence of the P(1,4)B compound (cf, lane 1 with lanes 2–4 of Figure 6A).[2] It is possible that sibiromycin and tomaymycin cause a conformational change in the structure of the DNA helix and in doing so may allow methylene blue to catalyze a modification of non-guanine bases such that they become sensitive to a subsequent reaction with piperidine. It is, however, important to note that some DNA-binding ligands have the capacity to alter the mode of action, and apparent sequence specificity, of other reagents that react with DNA.

A comparison of the results from the methylene blue and MPE·FE(II) methods using the same 5′-end-labeled strand is shown in Figure 6B. The covalent binding sites for sibiromycin that are on the opposite strand were revealed by artifactual bands (sites I–III in Figure 6B), and the additional binding sites on the labeled strand revealed by MPE·FE(II) (sites IV and V in Figure 6B) were observed directly in the methylene blue method by the diminution of the G-specific methylene blue reaction (see Figure 6A). The two methods therefore illuminate the same binding sites for sibiromycin and tomaymycin; however, anthramycin failed to elicit the artifactual bands, and thus, sites I–III of Figure 6B cannot be detected by the methylene blue procedure.

Discussion

The P(1,4)B antitumor antibiotics are a structurally unique group of drugs that bind quite specifically through N^2 of guanine and lie within the minor groove of DNA overlapping with two to three base pairs (Petrusek et al., 1981; Graves et al., 1984). CPK and molecular graphics modeling studies predict close van der Waals contacts between the floor of the minor groove of DNA and that part of the drug molecule which hugs the curvature of the minor groove of DNA. Therefore, the two to three base pair sequence specificity of the P(1,4)Bs demonstrated in this investigation was anticipated. In addition to the P(1,4)Bs, a number of other potent antitumor agents such as the saframycins (Lown et al., 1982) and naphthridinomycin (Zmijewski et al., 1985) also bind covalently to DNA through N^2 of guanine. However, little is known about the three-dimensional aspects of their binding to DNA. In general noncovalent minor groove DNA binding agents such as the distamycins, netropsin, SN 18071, berenil, and stilbamidine (Zimmer, 1984) recognize AT- rather than GC-rich regions of DNA. CC-1065 is the only present example of an AT-specific drug that binds covalently in the minor groove of DNA. This drug binds through N^3 of adenine and has four to five base pair DNA sequence specificity (Hurley et al., 1984b; Reynolds et al., 1985). While the subject of AT sequence specificity has received considerable discussion (Baguley, 1982; Zimmer, 1975, 1984), little is known about the molecular interactions which give rise to GC sequence specificity; therefore, these studies with the P(1,4)Bs have added importance because this class of compounds is one of a select group which bind covalently in the minor groove and recognize GC rather than AT base pairs.

The results presented here demonstrate that MPE·Fe(II) footprinting can be used to locate covalent adducts that bind within the minor groove of DNA. A special property of the P(1,4)B–DNA adducts is that the covalent attachment is reversible, because of the inherently labile animal (N–C–N) linkage which connects these drugs to N^2 of guanine (see Figure 2). Therefore, while the footprinting is carried out on

[2] Although we think it is unlikely, we cannot eliminate the possibility that the P(1,4)Bs produce a staggered double-strand break in the presence of methylene blue. The staggered double-strand break would occur at the P(1,4)B-modified guanine and at the "artifactual" position as defined in the text. The observed inhibition of the methylene blue reaction by P(1,4)B would tend to argue against this possibility.

a double-stranded DNA template where the adduct is stabilized by noncovalent interactions (Petrusek et al., 1981; Graves, 1984), upon denaturation of the duplex these secondary interactions are lost and the drug is released from the template (Hurley et al., 1979), leaving an unmodifed strand for electrophoretic sequencing. This point is important, because it avoids problems in the analysis of the DNA binding sites that may be evident if a drug-modified template were subjected to gel electrophoresis.

MPE·Fe(II) and DNase I have been used extensively to footprint ligand binding sites on DNA [for review, see Dabrowiak (1983)]. While the synthetic reagent MPE·Fe(II), which was modeled on bleomycin by Hertzberg and Dervan (1982), provides the more precise binding site location, DNase I is more sensitive to DNA structure and consequently reports on the extent and sequence dependency of altered DNA structure (Van Dyke & Dervan, 1983c). Unfortunately, neither reagent provides information on the orientation of a covalently bound ligand in the minor groove of DNA, unless the length of the drug molecule is asymmetrically distributed around the covalent binding site. Since at the present time the size and precise location of the binding sites were of paramount importance to us, we chose MPE·Fe(II) as the reagent of choice for our studies.

Since the P(1,4)Bs are the first ligands that bind covalently to DNA to be subjected to the footprinting method using MPE·Fe(II), it was important to compare the characteristics of cleavage inhibition by these agents to those already established for intercalating agents, such as the quinoxaline antibiotics, and noncovalent binders, such as distamycin and netropsin. MPE·Fe(II) reported a minimum three to four base pair footprint for the P(1,4)Bs which was about the same or slightly larger than the protection site predicted by CPK models. This compared well with the minimum footprint size, relative to model building studies, for drugs such as actinomycin D and distamycin (Van Dyke & Dervan, 1983c). The DNA cleavage inhibition pattern was shifted by the P(1,4)Bs to the 3′ side by about two to three bases and was underprotected on the 5′ side by the same amount. The direction of the shifts are in accord with the model proposed by the Dervan group (Van Dyke & Dervan, 1983b) but quantitatively appeared one more base displaced to each side of the binding site than previously observed.

Although MPE·Fe(II) may not be the reagent of choice for examination of the extent or sequence dependency of altered DNA structure or conformation (Van Dyke & Dervan, 1983c), the enhanced cleavage of AT-rich regions of DNA adjacent to P(1,4)B binding sites was remarkable nonetheless (see Figures 3B, 4B, and 5B). In one case, a 12-fold enhancement of cleavage occurred between two anthramycin binding sites in an AT-rich region (Figure 4B, 270-min preincubation). The observed enhancement for this particular sequence is severalfold higher than we have noted in any other segment of DNA that we have examined, and may be peculiar to this sequence and others having similar properties. While enhanced cleavage has been observed previously for both noncovalent minor groove binding drugs such as distamycin (Van Dyke & Dervan, 1983c) and intercalating agents such as the quinoxaline antibiotics (Low et al., 1984a,b), it has been given the most attention by the Waring group (Low et al., 1984a). This group has suggested that the enhanced cleavage by DNase I, effected by the quinoxaline antibiotics, is caused by a local distortion of the helix due to unwinding of adjacent regions of DNA by intercalation of the antibiotic. While this seems a reasonable explanation to rationalize the enhancement of cleavage by DNase I for the quinoxalines and other intercalating agents such as actinomycin D, this reasoning cannot be extended to the P(1,4)Bs or distamycin since these drugs bind without intercalation into DNA (Glaubiger et al., 1974; Petrusek et al., 1981). Nevertheless, it seems probable from our data and from those of Van Dyke & Dervan (1983c) that both distamycin and the P(1,4)Bs bind to DNA in a fashion that produces conformational changes which result in enhanced cleavage by MPE·Fe(II). In this regard, Kopka et al. (1985), in an insightful paper, have demonstrated that the distamycin analogue netropsin, produces a "Koshland induced type of fit" when this drug binds to a dodecamer, resulting in a 0.5–2.0 Å widening of the minor groove of DNA. Such an effect on the immediate DNA structure is likely to extend some distance from the actual drug binding site and produce regional disturbances in DNA structure and dynamics. Since methidium intercalates in the minor groove of DNA, the widening of this groove by drugs may be responsible for the enhanced binding and cleavage by MPE·Fe(II) adjacent to drug binding sites.

We interpret the sibiromycin- and tomaymycin-induced alterations in the normally guanine-specific methylene blue reaction as further evidence that the P(1,4)Bs produce conformational changes in DNA in proximity to the drug binding site. While only a small fraction of the total bound molecules produced the additional DNA strand breakages, each of the drugs was consistent in its site of breakage, relative to the presumed covalently modified guanine on the opposite strand. Not unexpectedly, both the propensity to induce new strand breakage sites and the location relative to the drug binding sites differed between each of the drug molecules (i.e., anthramycin did not show the additional strand breakage sites, while sibiromycin and tomaymycin produced strand breakage on the opposite strand but two or three bases removed from the covalently modified guanine, respectively). To account for these quantitative and qualitative differences, we suggest that there are subtle differences in the way these drugs interact with DNA and thereby cause variable conformational changes in DNA at adjacent sites.

We have previously compared the relative DNA binding properties of anthramycin, tomaymycin, and sibiromycin (Hurley et al., 1977) and determined that sibiromycin, at saturation binding conditions, produced the greatest degree of DNA modification, while tomaymycin produced the least. These results are substantiated by the present footprinting data, where both the degree of cleavage inhibition (see Figure 4B) and relative number of guanines which bind these drugs (see Table I) are in good agreement.

The sequence specificity data shown in Table I demonstrate that while guanines flanked by purines are preferred binding sites, guanine flanked by pyrimidines can also accommodate all three drugs. It is possible that the 5′PuGPu sequences are kinetically favored sites and are filled first, while guanines flanked by pyrimidines on one or both sides are less favored and filled at a slower rate. This hypothesis is supported by the results of the time course binding experiment shown in Figure 3. Guanines flanked by purines are the only sites filled during the first 120 min (lanes 3–6 in Figure 3A). Other sites in which a pyrimidine occurs on one or both adjacent sides of the guanine either remain unfilled (e.g., see guanines on the 3′ and 5′ sides of site A) or are only filled at the longest incubation time (e.g., guanines between binding sites B and C). Again consistent with our hypothesis is the results of time course kinetic experiments of covalent drug binding to DNA (Hurley et al., 1977). These experiments reveal a rapid phase of drug binding which occurs in the first 5–15 min followed

by a much slower phase of binding taking between 15 min and 1 h, dependent upon the particular drug. Since these kinetics are dependent upon the relative concentrations of drug and DNA and the time course binding of anthramycin to DNA, shown in Figure 3A, was carried out under different conditions to those reported previously (Hurley et al., 1977), the kinetics are not directly comparable.

CPK models of the anthramycin–, tomaymycin–, and sibiromycin–DNA adducts (Petrusek et al., 1981) predict close van der Waals contacts between both the floor and sides of the minor groove of DNA and the inside edge of the drug molecules on both sides of the covalent linkage bond. In fact, structure–activity relationships can be predicted rather precisely from these models (Thurston & Hurley, 1983). Since the concave side of the drug molecules (i.e., positions 9, 10, 11, 11a, and 1 in structure I of Figure 1) follows the floor of the minor groove of DNA, the overall characteristic twist of each molecule probably is of major importance in the determination of sequence recognition. Both the dihedral angle, between the aromatic ring the five-membered ring, and the pucker of the pyrrole ring contribute to the overall twist and therefore to the sequence recognition properties of each drug. A comparison of molecular models of anthramycin, IV, and tomaymycin reveal major differences in the overall twists which may explain the individual preferences for guanines in different sequence environments shown in Figure 5C. Anthramycin and tomaymycin have been shown to have 35° and 9° twists, respectively, between the aromatic ring and the five-membered ring (Mostad et al., 1978; Arora 1979, 1981) with little (tomaymycin) or no pucker (anthramycin) in the five-membered ring. On the other hand, we have shown by energy minimization that while IV has about an equivalent dihedral angle to anthramycin between the aromatic ring and the five-membered ring, in IV the saturated five-membered ring is puckered, which results in an extension of the overall twist of this molecule. It is likely that a Koshland-like-induced fit occurs upon drug binding to DNA, and because of the large variation in overall twists of the P(1,4)Bs, drugs such as anthramycin and IV having the larger overall twists may "open up" adjacent regions on the helix and facilitate intercalation and/or cleavage of the deoxyribose by MPE·Fe(II) (see Figure 5A,B). Last, the substituents of C-8 and C-9 of the aromatic ring and C-2 of the pyrrole ring point into (C-9) or along the minor groove of DNA (C-8 and C-2) and may also contribute to sequence recognition.

Primarily on the basis of CPK model building studies, we have previously proposed that when the P(1,4)Bs bind to DNA, the stereochemistry at C-11 is *S* (Petrusek et al., 1981). The stereochemistry at C-11 determines the orientation of the drug molecule in the minor groove of DNA. If the configuration at C-11 is *S*, then the aromatic ring of the P(1,4)Bs lies to the 3′ side of the covalently modified guanine strand. One- and two-dimensional ^{1}H NMR techniques have been used to examine the structure of the anthramycin–d(ATGCAT)$_2$ adduct. Although this duplex belongs to the least preferred binding sequence (5′PyGPy), saturation binding was observed but only after prolonged incubation times (48 h) using a large excess of anthramycin-11-methyl ether. The data from a one-dimensional difference nuclear Overhauser effect experiment prove that the aromatic ring of anthramycin lies to the 3′ side of the covalently modified guanine, and therefore, the stereochemistry at C-11 is *S* (Graves et al., 1985). Fluorescent decay measurements on tomaymycin bound to DNA demonstrate that there are two forms of tomaymycin bound to calf thymus DNA (Barkley et al., 1986). These are likely to be the two C-11 diasteromeric forms of tomaymycin bound in different orientations on DNA. Models show that the length of the P(1,4)B molecule in the case of anthramycin, tomaymycin, and IV is almost equally distributed to the 3′ and 5′ side of the covalently bound guanine. This fact precludes the determination of binding orientation by the use of MPE·Fe(II) footprinting. For sibiromycin the amino sugar should theoretically produce an asymmetric footprint, but there is no evidence of this from our footprinting data (see Figure 4B). In order to answer the question of drug orientation in the minor groove of DNA, some other technique will have to be applied. One possibility would be the attachment of a DNA affinity cleaving molecule such as EDTA·Fe(II) (Taylor et al., 1984) to these molecules.

In summary, we have demonstrated using MPE·Fe(II) footprinting that the P(1,4)Bs bind to DNA in a sequence-selective manner. There is a marked preference for covalent binding of the P(1,4)Bs to sequences flanked by purines rather than pyrimidines. Evidence was also presented for drug-induced conformational changes in DNA at sequences in proximity to the drug binding sites.

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