Molecular Basis for the DNA Sequence Specificity of the Pluramycins. A Novel Mechanism Involving Groove Interactions Transmitted through the Helix via Intercalation To Achieve Sequence Selectivity at the Covalent Bonding Step[1]

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Abstract: The pluramycin antitumor antibiotics, which include the altromycins, pluramycin, hedamycin, and rubiflavin, are a group of highly evolved DNA-reactive compounds that have structural features reminiscent of both nogalamycin and the aflatoxins. As such, they are characterized as "threading intercalators" with the added ability to alkylate N7 of guanine (see preceding article in this issue). In this article we have demonstrated that different members of this group of antibiotics have sequence specificities that differ for the base pair to the 5′ side of the alkylated guanine and also have a range of reactivities with susceptible sequences. Subsequent experiments were designed to determine the molecular origin for both these observed contrasting sequence specificities and covalent reactivities. First, neopluramycin, an analog of pluramycin that lacks the epoxide, and thus is unable to covalently modify DNA, but is in other respects structurally similar, exhibits no discernible sequence selectivity. This suggests that the sequence selectivity of the pluramycins is determined at the covalent bonding step rather than the precovalent binding interactions. Second, using A_n tracts of varying length ($n = 1-5$) to modulate the minor groove geometry to the 5′ side of the covalent alkylation site, this structural parameter has been shown to have a major effect on both sequence specificity and alkylation reactivity. Last, the electronegativity of the N7 position of the alkylated base can also affect reactivity and, to a lesser extent, sequence specificity. In order to determine the molecular details of the interactions in the minor and major grooves, which could give rise to the different sequence specificities of the nonclassical (typified by altromycin B) and classical (typified by hedamycin) pluramycins, we have used molecular models of the altromycin B and hedamycin-DNA adducts that are derived from high-field NMR data of their 10-mer duplex diadducts. These studies demonstrate that it is likely that the sequence-dependent reactivities of the epoxide of the pluramycin to N7 of guanine are dependent upon the relative extent of a "proximity effect". The magnitude of the proximity effect is determined by a "steering reaction", which takes place in the minor and major grooves due to the different placement of the carbohydrate substituents on the pluramycins and their hydrogen bonding and van der Waals interactions with the base pairs to the 5′ side of the alkylation site. This is proposed to be a novel mechanism for sequence recognition, where cooperative interactions in the minor and major grooves transmitted via the intercalation moiety dictate the positioning of the epoxide in the major groove and, thus, sequence reactivity. Finally, we propose that the increased reactivity of the classical pluramycins in contrast to the altromycins is at least partially determined by the "reach" of the reactive epoxide in the major groove, which varies from one group to another. The molecular mechanisms for sequence recognition described here provide a new paradigm for sequence recognition by minor and major groove interactions mediated by intercalactive binding but achieved at the covalent bonding step.

Introduction

The pluramycin group of antibiotics consists of a family of antitumor compounds that are characterized by a planar 4*H*-anthra[1,2-*b*]pyran-4,7,12-trione moiety, from which a variety of substituents are extended from two or more corners (Figure 1).[2] The presence of an epoxide at position two is necessary for the potent cytotoxicity and antitumor activity.[2c] In a previous study, using gel electrophoresis methods in combination with NMR and mass spectroscopy, we have demonstrated that the pluramycins intercalate between base pairs and, where an epoxide is present, alkylate N7 of guanine (Figure 2).[3] In the present study, we have examined, using a thermal strand breakage assay (Figure 2), the sequence specificity of select antibiotics within this group and also determined their relative reactivity in the alkylation of DNA. An analysis of the sequence selectivity results, using molecular models of the altromycin B

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(1) Abbreviations: bp, base pair; DDW, double-distilled water; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

(2) (a) Uosaki, Y.; Yasuzawa, T.; Hara, M.; Saitoh, Y. *J. Antibiot.* **1991**, *44*, 40-44. (b) Gonda, S. K.; Byrne, K. M.; Herver, P. K.; Tondeur, Y.; Leverato, D.; Hilton, B. D. *J. Antibiot.* **1984**, *37*, 1344-1356. (c) Yasuzawa, T.; Saitoh, Y.; Sano, H. *J. Antibiot.* **1990**, *43*, 485-491. (c) Sequin, U. *Fortschr. Chem. Org. Naturst.* **1986**, *50*, 57-122. (d) Sato, Y.; Watabe, H.; Nakazawa, T.; Shomura, T.; Yamamoto, H.; Sezaki, M.; Kondo, S. *J. Antibiot.* **1989**, *42*, 149-152. (e) Itoh, J.; Shomura, T.; Tsuyuki, T.; Yoshida, J.; Ito, M.; Sezaki, M.; Kojima, M. *J. Antibiot.* **1986**, *39*, 773-779. (f) Nadig, H.; Sequin, U. *Helv. Chim. Acta* **1987**, *70*, 1217-1288. (g) Nadig, H.; Sequin, U. *Helv. Chim. Acta* **1987**, *68*, 953-957. (h) Abe, N.; Enoki, N.; Nakakita, Y.; Uchida, H.; Nakamura, T.; Munekata, M. *J. Antibiot.* **1993**, *46*, 1536-1549. (h) Jackson, M.; Karwoski, J. P.; Theriault, R. J.; Hardy, D. J.; Swanson, S. J.; Barlow, G. J.; Tillis, P. M.; McAlpine, J. B. *J. Antibiot.* **1990**, *43*, 223-228. (i) Brill, G. M.; McAlpine, J. B.; Whittern, D. N.; Buko, A. M. *J. Antibiot.* **1990**, *43*, 229-237. (j) McAlpine, J. B.; Karwoski, J. P.; Jackson, M.; Brill, G. M.; Kadam, S.; Shen, L.; Clement, J. J.; Alder, J.; Burres, N. S. In *Antitumor Drug Discovery and Development*; Valeriote, F. A., Crobett, T. H., Baker, L. H., Eds.; Kluwer Academic Publishers: Boston, in press. (k) Byrne, K.; Gonda, S. K.; Hilton, B. D. *J. Antibiot.* **1985**, *38*, 1040-1049. (l) Hara, M.; Takiguchi, T.; Ashizawa, T.; Gomi, K.; Nakano, H. *J. Antibiot.* **1991**, *44*, 33-39.

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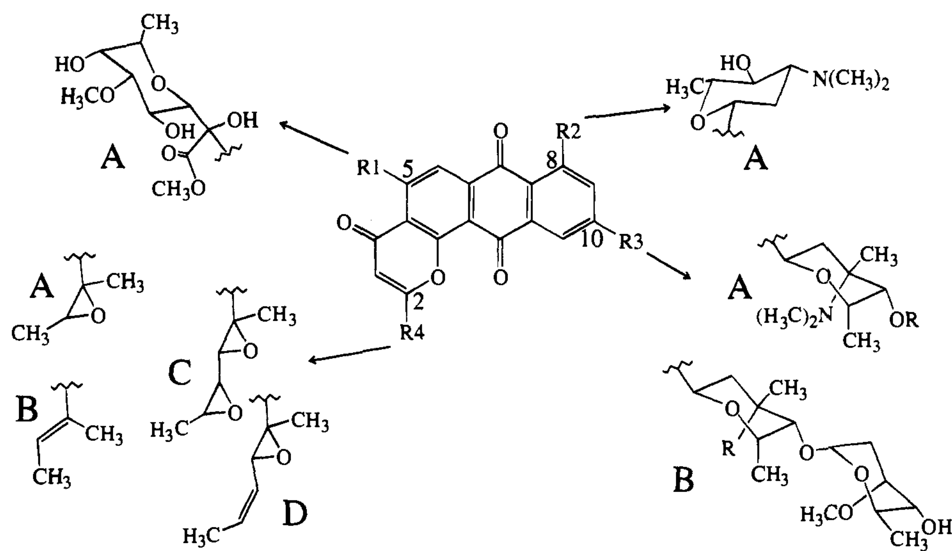

Compound	R1	R2	R3	R4	R'
altromycin B	A	H	B	A	$N(CH_3)_2$
altromycin H	OH	H	B	A	$N(CH_3)_2$
altromycin I	OH	H	B	A	$NHCH_3$
akinomycin	CH_3	H	A	C	H
pluramycin	CH_3	A	A	D	acetyl
neopluramycin	CH_3	A	A	B	acetyl
hedamycin	CH_3	A	A	C	H
rubiflavin A	CH_3	A	A	D	H

Figure 1. Structures of altromycins B, H, and I, akinomycin, pluramycin, neopluramycin, hedamycin, and rubiflavin A.[2]

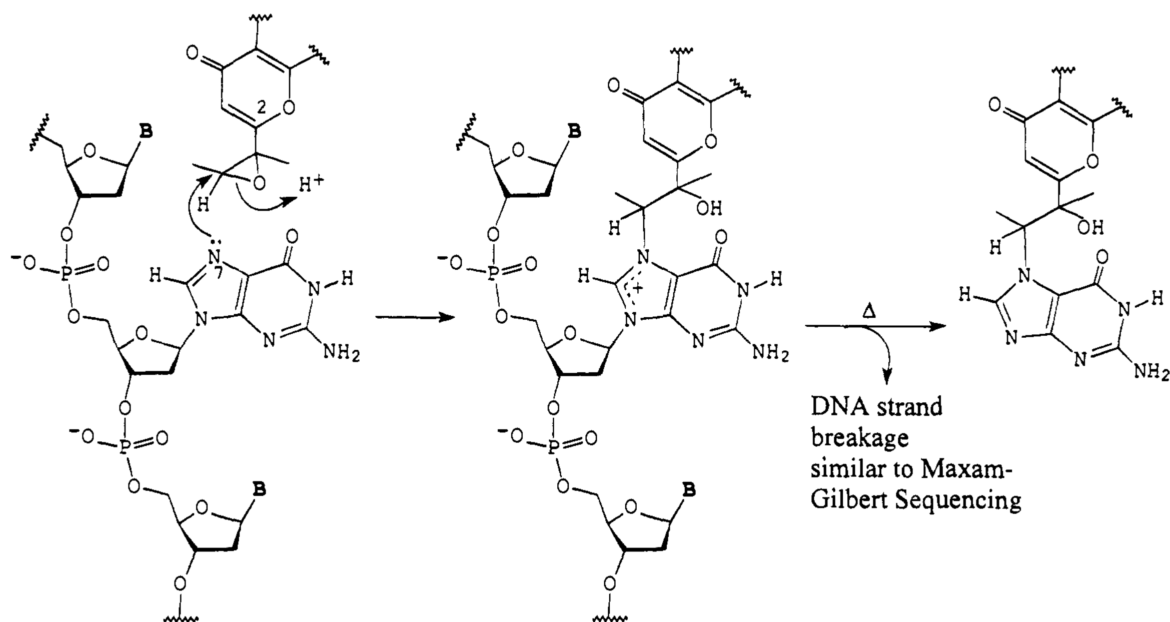

Figure 2. Reaction of altromycin B with DNA to form the altromycin B−(N7-guanine)−DNA adduct and products of thermal and piperidine cleavage of the DNA adduct.[3,6] Analogous products are produced from the other pluramycin antibiotics.

and hedamycin−DNA adducts derived from high-field NMR studies[4] of 10-mer duplex diadducts, provides valuable information concerning the ways in which both the position and character of the substituents on the 4*H*-anthra[1,2-*b*]pyran ring system can dramatically affect the sequence selectivity and alkylation reactivity of the pluramycin family of antibiotics. In turn, the results provide important additional insights into the potential design of new sequence-selective compounds based on the pluramycin group of compounds.

(3) Sun, D.; Hansen, M.; Clement, J. J.; Hurley, L. H. *Biochemistry* **1993**, *32*, 8068−8074.

(4) (a) Hansen, M.; Hurley, L. H. *J. Am. Chem. Soc.* **1995**, *117*, 2421−2429. (b) Hansen, M.; Hurley, L. H. Manuscript in preparation.

Results

Determination of the Comparative Reactivity and Sequence Specificity of the Pluramycins. A. Concentration Dependency of Sequence Alkylation by Altromycin B. A heat-induced strand breakage assay following altromycin B treatment of the + and − strands of a 64-bp oligomer DNA (64M in Table 1) was carried out to reveal the drug's alkylation sequence selectivity. To determine in a quantitative manner the hierarchy of altromycin B alkylation sites, six different concentrations of altromycin B solutions were reacted individually with aliquots of the 5′-labeled 64-bp restriction enzyme fragment. The alkylation sites for altromycin B at each concentration are shown in Figure 3, and inspection of the

Table 1. Sequence of Oligomer DNA Molecules Used in This Study

64M	5´-CGATCTTTGTCAAGCATCCCAGAAGGTATAAAAACGCCCTTGGGACCAGGCAGCCTCAAAC 3´-GCTAGAAACAGTTCGTAGGGTCTTCCATATTTTTGCGGGAACCCTGGTCCGTCGGAGTTTG
42A	5´-GCTCATCAAGACAAAAAGACAGACAAAGTACAAAAGCAATCA-3´ 3´-CGAGTAGTTCTGTTTTTCTGTCTGTTTCATGTTTTCGTTAGT-5´
23G	5´-AAAAGTTGATCGTAAGTACAATC-3´ 3´-CAACTAGCATTCATGTTAGTTTT-5´
23I	5´-AAAAITTIATCITAAITACAATC-3´ 3´-CAACTAGCATTCATGTTAGTTTT-5´
77M	5´-CGCAGCGCGCGATCGGGCATTATAAAAAAGCATTGCTTATCAATTTGTTGAATTAGATTTTGCACGCAACGCGCTGT-3´ 3´-TCGCGCGCTAGCCCGTAATATTTTTTCGTAACGAATAGTTAAACAACTTAATCTAAAACGTGCGTTGCGCGACAGCG-5´

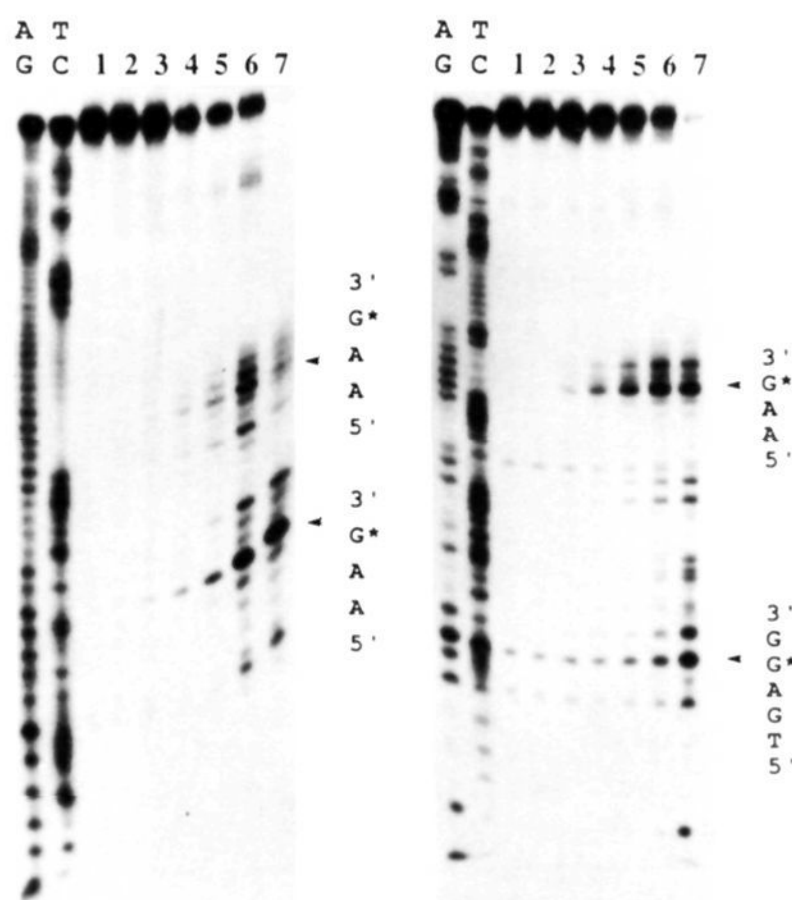

Figure 3. Concentration dependency of altromycin B alkylation sites on the + (right panel) and − (left panel) strands of the 64-bp oligomer duplex (Table 1) following thermal treatment of the altromycin B−DNA adduct. For both the right and left panels, 5′ end-labeled oligomer DNA was used in this experiment. The reaction mixtures (20 μL) consisted of 10 mM NaCl, 10 mM Tris-HCl (pH 7.6), about 10 ng of DNA, and the indicated amount of drug (see below), and these were incubated at room temperature for 1 h. AG and TC represent the purine- and pyrimidine-specific cleavage reactions. Lanes 1−7 in each case contain 0, 2, 5, 10, 20, 40, and 100 ng of drug, respectively. Arrowheads point to the alkylation sites (G*) in altromycin-modified sequences. The multiplicity of bands at higher molecular weight than the major cleavage site is due to incomplete degradation of the initial depurination product.[3]

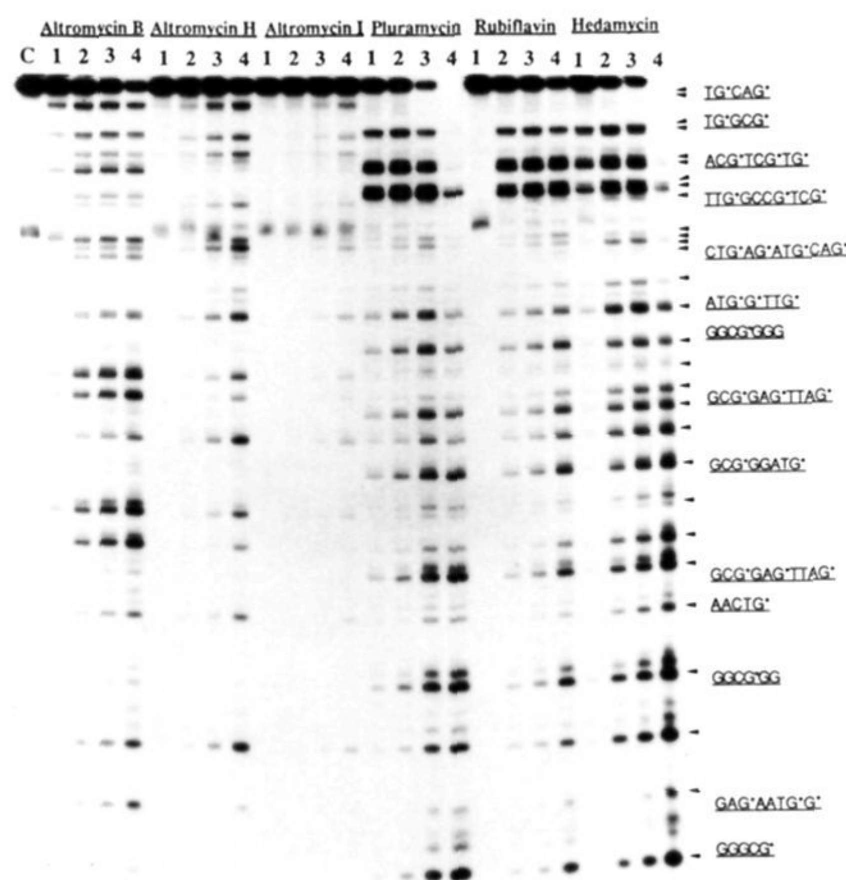

Figure 4. Comparison of the DNA sequence selectivity and alkylation reactivity of altromycins B, H, and I and pluramycin, rubiflavin, and hedamycin. The DNA in this experiment is an NcoI−PvuII fragment (189 bp) from plasmid pCAT that was 5′ end-labeled at the NcoI site. The reaction mixture (20 μL), consisting of 10 mM NaCl, 10 mM Tris-HCl (pH 7.6), about 5 ng of DNA, and the indicated amount of drug (see below), was incubated at room temperature for 1 h. Arrowheads point to the alkylation sites, and the drug-modified bases are indicated with an asterisk. Lanes 1−4 contain 2, 10, 20, and 50 ng of drug molecules for altromycin B; 10, 20, 50, and 100 ng for altromycins H and I; 0.5, 1, 2, and 5 ng for pluramycin and rubiflavin; and 0.1, 0.5, 1, and 2 ng for hedamycin. The complete DNA sequence is given in Figure 5.

autoradiogram shows a remarkable degree of sequence discrimination at the lowest concentrations of drug, especially for a 926 Da molecule. By using Maxam−Gilbert chemically produced markers as controls, the 5′ AAG* sequence (* indicates the covalent reactivity site) was shown to be the most sensitive cleavage site, although other sequences were also cleaved, but at higher concentrations. A preliminary analysis suggested that the nucleotides to the 5′ side of the alkylation site are the primary influence on the sequence specificity, and conversely, the alkylation appeared to be little effected by the identity of the 3′ neighboring nucleotide. A more detailed analysis, in which other members of the pluramycin family were compared to altromycin B, was then carried out.

B. Comparison of the Alkylation Reactivity of Altromycin B and Its Pluramycin Analogs. Upon the basis of the analysis of the results of the previous experiment, in which the 64-bp oligomer DNA was used, a second DNA fragment (189 bp) was selected, in which clustered GC base pairs were found (for sequence, see Figure 5). Using this restriction fragment, the relative reactivities of altromycin B with other epoxide-containing pluramycins were determined; an autoradiogram of the results is shown in Figure 4. In general, the classical pluramycin antibiotics (pluramycin, rubiflavin, and hedamycin) showed at least a 5-fold higher reactivity in the alkylation of DNA than altromycins B, H, and I (see Figure 4). This result demonstrates that the arrangement and character of the sub-

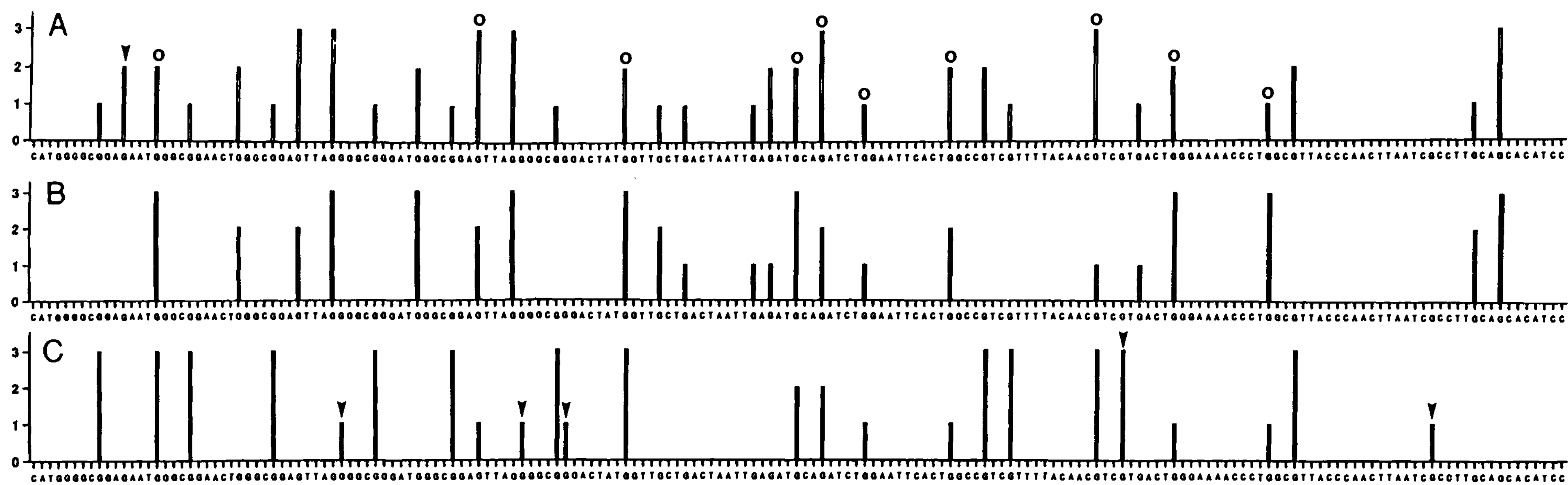

Figure 5. Summary of observed alkylation sites of altromycins B, H, and I, pluramycin, rubiflavin, and hedamycin from the data in Figure 4. The drugs were classified (see Table 2) into groups A, B, and C, corresponding to panels A, B, and C. The alkylation sites for each group were classified into high-, medium-, and low-reactivity sites, based upon the amount of strand breakage at the modified base from the data in Figure 4. Densitometric measurements were made using an LKB 2202 Ultrascan LX laser densitometer to compute the percentage of strand breakage at a given site relative to the total strand breakage. High-, medium-, and low-reactivity sites are represented by three-, two-, and one-unit bars, respectively. Open circles indicate common alkylation sites for groups A, B, and C, and arrow heads represent unique alkylation sites for individual groups.

Table 2. List of Individual High- and Medium-reactivity Sites and Consensus Sequences for the Pluramycin Antitumor Antibiotics, Including the Covalently Modified Base (G*) and Bases That Are Two and One Nucleotides to the 5′ and 3′ Sides of the Modification Site, Respectively

	altromycin B (group A)	altromycins H and I (group B)	pluramycin, hedamycin, and rubiflavin (group C)
high-reactivity sites (HR)	5′ CAG*C	5′ ATG*G	5′ CCG*T
	5′ TAG*G	5′ ATG*G	5′ ACG*T
	5′ TAG*G	5′ ATG*G	5′ GCG*T
	5′ GAG*T	5′ CAG*C	5′ CTG*G
	5′ GAG*T	5′ CTG*G	5′ TCG*T
	5′ CAG*A	5′ CTG*G	5′ GCG*G
	5′ ACG*T	5′ ATG*C	5′ GCG*G
		5′ CTG*C	5′ GCG*G
		5′ TAG*G	5′ GCG*G
			5′ GCG*G
			5′ GCG*G
consensus sequence (HR)	5′ NAG*N	5′ NTG*N	5′ NCG*G
medium-reactivity sites (MR)	5′ GCG*T	5′ TTG*C	5′ ATG*G
	5′ GAG*A	5′ TAG*G	5′ ATG*G
	5′ ATG*C	5′ CTG*G	5′ ATG*G
	5′ ATG*G	5′ GAG*T	5′ TTG*A
	5′ ATG*G	5′ GAG*T	5′ GAG*A
	5′ CTG*G	5′ TTG*C	
	5′ CTG*G	5′ CAG*A	
	5′ CTG*G		
	5′ TCG*T		
consensus sequence (MR)	5′ NTG*N	5′ N(A/T)G*N	5′ ATG*(A/G)

stituents at each corner (R1 to R3 in Figure 1) of the anthrapyran ring and/or comparative reactivity of the epoxide(s) at R4 significantly affect the relative DNA reactivity of different members of the pluramycin family of antitumor antibiotics. Furthermore, altromycin B shows greater alkylation reactivity than altromycins H and I, presumably because of the presence of an additional substituent (R1) at C5 in altromycin B. Last, altromycin H is about 5-fold more reactive than altromycin I, due to the presence of a dimethylamino group rather than the monomethylamino in the amino sugar in the disaccharide at C10.

Among the non-altromycin analogs, hedamycin showed the highest reactivity to DNA, which may be due to the diepoxide group present at C2, in contrast to the olefinic monoepoxide in pluramycin and rubiflavin (see later). Pluramycin is more reactive to DNA than rubiflavin, which was not completely unexpected, since neopluramycin, the noncovalent binding analog of pluramycin, showed better relative binding affinities for DNA than kidamycin, the noncovalent binding analog of rubiflavin (Sun and Hurley, unpublished results).

C. Comparison of the DNA Sequence Specificity of the Pluramycin Antitumor Antibiotics. A quantitative comparison of the sequence selectivity of the pluramycins, derived from the results shown in Figure 4, is presented in Figure 5. A cursory examination of Figure 4 shows that while altromycin B shows many sites in common to altromycins H and I, which are themselves very similar, they are all three different from the group representing pluramycin, rubiflavin, and hedamycin. For this reason, in the bar size comparison in Figure 5 and in Table 2, the antibiotics are pooled together in three main groups. In Figure 5, panel A represents altromycin B, panel B represents both altromycins H and I, and panel C is collectively pluramycin, hedamycin, and rubiflavin. Table 2 follows the same pattern. Of the total of 37 sites of alkylation on just one strand of the 189-mer, 10 are common alkylation sites for all three groups [5′ TG* (seven sites)/5′ AG* (two sites)/5′ CG* (one site); * denotes site of alkylation] and six sites are unique to just one of the groups [group C (five sites)/group A (one site)]. These sites are denoted in Figure 5.

If the high-reactivity alkylation sites for all three groups are compared, those in group A clearly prefer a 5′ AG* (seven out of eight sites) sequence, while those in group B prefer 5′ TG* (seven out of nine sites) and those in group C prefer 5′ CG* (10 out of 11 sites). In group C, there may be a preference for the 5′ GCG* sequence (seven out of 11 sites). For medium reactivity sites, the preference to the 5′ side is different: those in group A prefer 5′ TG* (six out of nine sites); those in group B, 5′ (A/T)G* (seven out of seven sites); and those in group C, 5′ TG* (four out of five sites). Only in one group is there a possible preference for a nucleotide to the 3′ side, i.e., those in group C have a preference for G to the 3′ side of G* (seven out of 11 sites) for high reactivity sites and 5′ G*Pu (five out of five sites) for medium reactivity sites. It is quite striking that, while altromycin B (group A) prefers 5′ AG* (high reactivity) and 5′ TG* (medium reactivity) sites, altromycins H and I (group B) prefer 5′ TG* (high and medium) and pluramycin, hedamycin, and rubiflavin (group C) prefer 5′ CG* (high) and 5′ TG* (medium). Only in one case (altromycin B) is a purine preferred at the 5′ side of the alkylation site; in all the other cases, a pyrimidine, usually T, is preferred. An alternative way of summarizing the data is that in only one case (group C) is a non-AT base pair preferred at the 5′ side of the alkylation site, although, in this case, the medium reactivity site includes T. In summary, it appears that the nature and pattern of substituents (R1 to R4) on a 4*H*-anthra[1,2-*b*]pyran ring system determine quite significantly the sequence selectivity, as well as reactivity, of the pluramycin family of antibiotics.

Determination of Factors That Affect Sequence Specificity of the Pluramycin Antitumor Antibiotics. The origin of the sequence specificity could lie in the precovalent binding interactions and/or the bonding reactivity of alkylated sequences. A precedent exists for both cases, perhaps most clearly demonstrated for the cyclopropapyrroloindoles, where for the + series (e.g., (+)-CC-1065) bonding reactivity predominates, while for the − series (e.g., (−)-CC-1065) precovalent binding interactions predominate.[5] In order to shed some light on this problem, non-alkylating and alkylating drugs were compared for sequence specificity, and specific sequences were synthesized in which either minor groove geometry or the electronegativity of the alkylated base was varied and then used in a comparison of the alkylation reactivity.

A. Comparison of Alkylation Sites of Pluramycin with the Noncovalent Binding Sites of Neopluramycin. In order to determine whether the noncovalent binding sites of the pluramycin antitumor antibiotics correspond to the favored sequences for alkylation of N7 of guanine, the two structurally similar, naturally occurring anthrapyran antibiotics pluramycin and neopluramycin were compared for sequence specificity. Structurally, these drugs differ only in that neopluramycin has a single olefin in place of the olefinic epoxide of pluramycin (see Figure 1). In parallel experiments, the sequence specificity of the noncovalent neopluramycin was determined by DNase I footprinting, and the sequence specificity of the alkylating pluramycin was determined using the thermal cleavage assay.[3,6] The results in the left and right panels of Figure 6 show that, while neopluramycin does not show defined DNase I footprints (i.e., no favored binding sequences), pluramycin produced the

(5) (a) Warpehoski, M. A.; Hurley, L. H. *Chem. Res. Toxicol.* **1988**, *1*, 315−333. (b) Hurley, L. H.; Warpehoski, M. A.; Lee, C.-S.; McGovren, J. P.; Scahill, T. A.; Kelly, K. C.; Wicnienski, N. A.; Gebhard, I.; Bradford, V. S. *J. Am. Chem. Soc.* **1990**, *112*, 4633−4649.
(6) Bennett, G. N. *Nucleic Acids Res.* **1982**, *10*, 4581−4594.

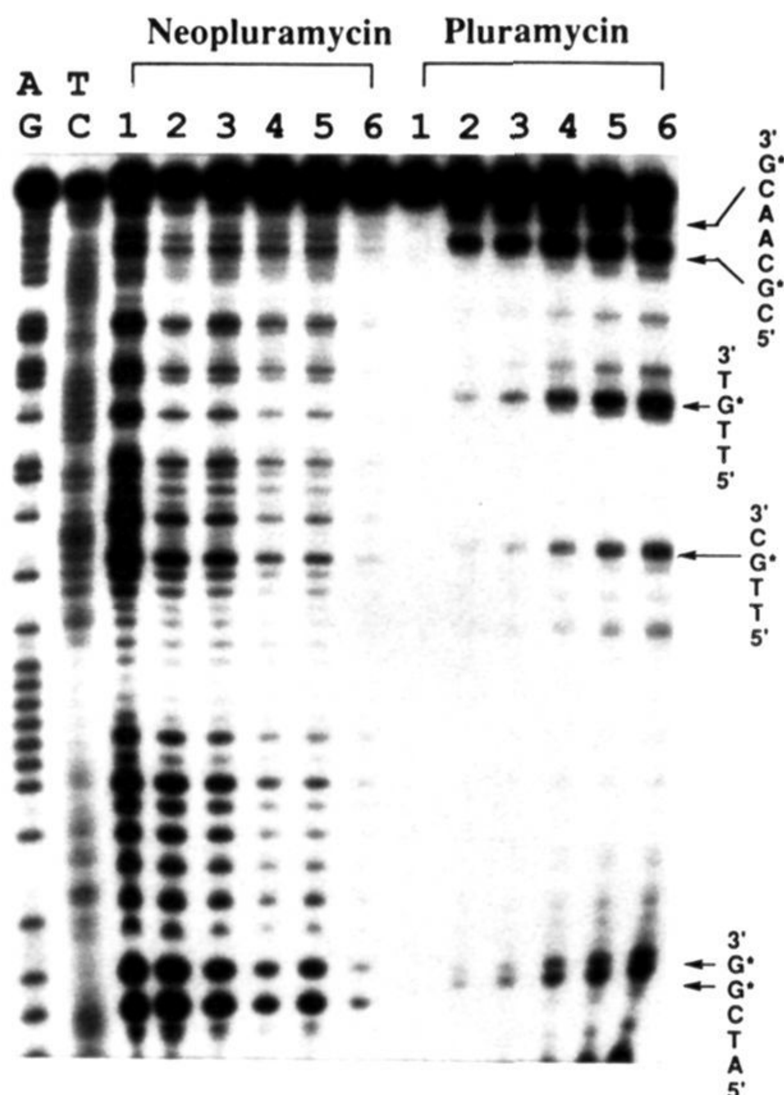

Figure 6. Comparison of the pluramycin alkylation sites (right) with the neopluramycin DNase I footprinting sites (left). Neopluramycin was incubated with the 77-mer DNA for 10 min, and the drug–DNA complex was digested with DNase I (0.2 U) for 1 min. At the same time, DNA was incubated with pluramycin for 10 min and samples were heated at 95 °C for 15 min to induce strand breakage. Lanes 1–6 contain 0, 5, 10, 20, 50, and 100 ng of drug molecules for neopluramycin and 0, 0.5, 2.5, 5, 7.5, and 15 ng for pluramycin with 5 ng of DNA molecules in a 20 μL reaction. AG and TC represent the purine- and pyrimidine-specific cleavage reactions. Arrows point to the alkylated guanines, which are marked by an asterisk in the sequences to the right.

expected 5′ PyG* sequence specificity. This result suggests that the precovalent binding interactions are not primarily responsible for the sequence selectivity of the pluramycins.

B. Effect of Minor Groove Geometry on Alkylation by the Pluramycins of N7 of Guanine in the Major Groove. The consensus sequence analysis of the pluramycin family of antibiotics shown in Table 2 revealed that, except for perhaps group C, the determinant of sequence specificity lies exclusively with the base to the 5′ side of the covalently modified guanine. Substituents at C8 and C10 of the pluramycins occupy the minor groove of DNA, with the C10 substituent to the 5′ side of the alkylated guanine and the C8 substituent across from the modified base.[4] Since both the sequence context (AT vs GC) and order (ATA vs AAA) can have a profound effect on minor groove geometry, a 42-mer oligomer was designed and synthesized (42A in Table 1) in which the effect of sequence and associated minor groove geometry to the 5′ side of the covalently modified guanine on pluramycin alkylation reactivity could be evaluated. Therefore, an oligomeric DNA sequence containing an incremental number ($n = 1-5$) of adenines to the 5′ side of the targeted guanines (see Figure 7) was designed in order to determine the effect of local *minor* groove geometry on the covalent reactivity of DNA in the *major* groove, specifically at N7 of guanine, of those compounds. As shown in Figure 7, the precise length of the A tracts dramatically influences the alkylation reactivity of altromycin B, altromycin H, and pluramycin with N7 of the guanine to the 3′ side of the A_n tract.

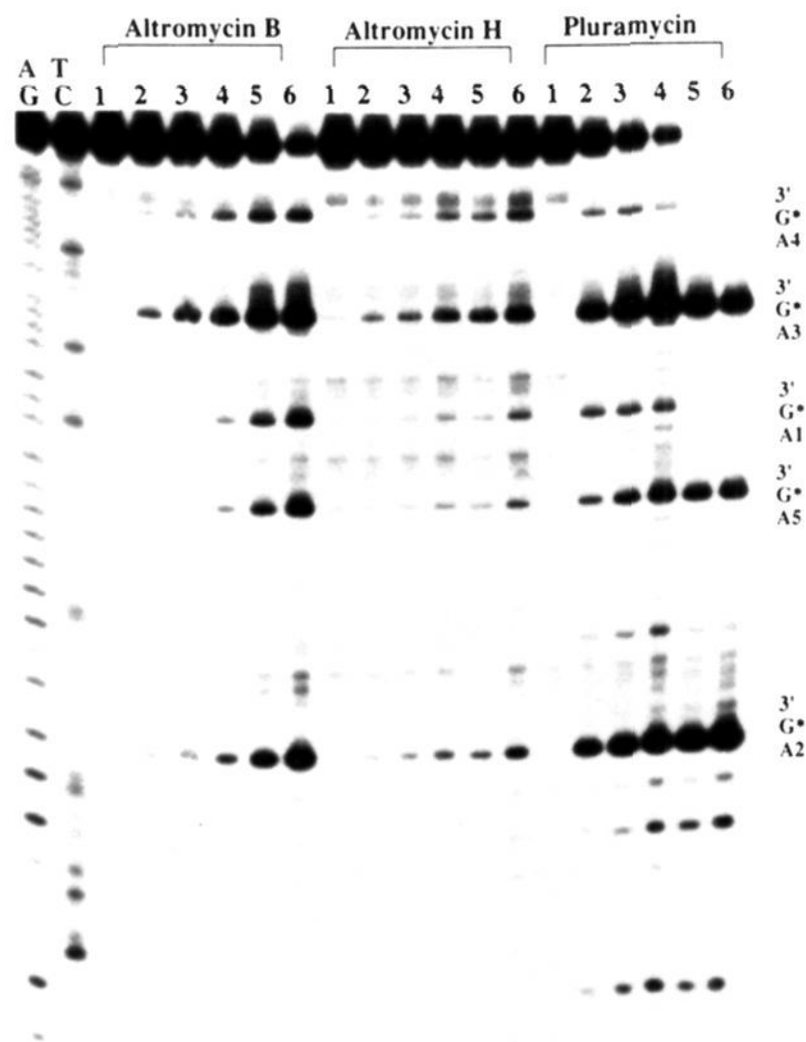

Figure 7. Effect of the length of A tracts on the alkylation reactivity of the guanine base located at the 3′ side of A tracts. About 10 ng of oligomer DNA (42A in Table 1) was treated with drug molecules for 1 h at room temperature. Lanes 1–6 contain 0, 5, 10, 20, 50, and 100 ng of drug molecules for altromycin B; 0, 10, 20, 50, 100, and 200 ng for altromycin H; and 0, 1, 2, 5, 10, and 20 ng for pluramycin. AG and TC represent the purine- and pyrimidine-specific cleavage reactions. The A1 to A5 tracts and associated 3′ guanines are marked by an asterisk to the right of the gel.

The results shown for altromycin B in lanes 3 and 4 of Figure 7 provide the optimum data set to determine the precise order of the reactivity of the pluramycins with the guanine flanked on the 5′ side with A tracts (where $n = 1-5$), although the other two drugs appear to show similar patterns. In order of decreasing reactivity, $A_3 > A_2 = A_4 > A_1 = A_5$. The maximum reactivity of altromycin B with guanine was achieved when $n = 3$, while reactivity decreased by about the same amount when n was changed to 2 or 4, and even further when n was 1 or 5. In previous studies,[7] it was demonstrated that A tracts, when $n \leq 3$, produce only slight electrophoretic anomalies and bending in DNA; however, when $n \geq 4$, the effect is substantial. In conclusion, modulation of A-tract geometry and/or minor groove width dramatically affects the reactivity of pluramycin with guanines to the 5′ side of A tracts such that, when groove width is decreased or increased from the optimum size (i.e., when $n = 3$), drug reactivity decreases.

C. Effect of Replacing Guanine Residues in DNA with Inosine on Alkylation Reactivity and Sequence Selectivity of the Pluramycins. In previous studies, only guanine residues were found to be alkylation sites for the pluramycin family of antibiotics.[3,6] To evaluate how substitution of inosine for guanine affects the reactivity and sequence specificity of the pluramycins, a pair of oligomers was synthesized in which one duplex contained guanines on both strands, while the other duplex contained guanines on only one strand, with inosine in place of guanine on the complementary strand (see 23G and

(7) (a) Koo, H. S.; Wu, H. M.; Crothers, D. M. *Nature* **1986**, *320*, 501–506. (b) Koo, H. S.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1763–1767. (c) Hagerman, P. J. *Annu. Rev. Biochem.* **1990**, *59*, 755–781.

23I in Table 1). The effect of replacing guanine residues in DNA with inosine is to remove one hydrogen bond from the normal G•C base pair and the steric bulk associated with the exocyclic 2-amino group of guanine. In addition, removal of the exocyclic 2-amino group from guanine is expected to decrease the nucleophilicity of the N7 position.[8]

A comparison of the reactivity of the upper strand (containing I) vs the equivalent strand (containing G) to altromycin B, altromycin H, and pluramycin is shown in Figure 8 (top). Replacement of guanine by inosine dramatically affects the reactivity of all three antibiotics to the extent that a greater than 10-fold loss of alkylation reactivity is found. Furthermore, it is apparent that replacing the guanine residue with inosine also modulates the sequence selectivity of pluramycin. For this drug, the modulating effect of nucleotides to the 5′ side of modified inosine becomes significantly decreased, so that there is only a slight difference in the extent of alkylation between the sequences 5′ AAI*, 5′ TCI*, and 5′ TTI* (see Figure 8 (top)). The effect of replacing guanine residues with inosine on the non-alkylating strand of the duplex is to reduce the reactivity of these antibiotics to guanine residues on the other strand, but to a much lesser extent than replacement at the covalent reaction site (see Figure 8 (bottom)). For example, only in the case of altromycin H does the sequence 5′ ACG* become more reactive to altromycin H when the guanine of the complementary strand was replaced with the inosine. This result indicates that the minor groove substituent of the base pair adjacent to the 5′ side of modified guanine also affects the reactivity of these antibiotics, but this effect is much less than the change in alkylation reactivity at inosine.

Discussion

The pluramycin antitumor antibiotics represent a group of highly structurally evolved DNA-reactive drugs. It can be imagined that the simplest DNA interactive compound would have been the intercalative anthraquinone moiety. Addition of a pyran ring system and an epoxide at C2 would create the first pluramycin compounds, perhaps similar in structure to sapurimycin,[21] which would have the ability to modify DNA by alkylation through N7 of guanine. Continued evolution to compounds such as akinomycin, altromycin H, and hedamycin was made by adding substituents at the C8 and C10 positions, giving rise to the stabilizing and potentially sequence-selective interactions in the minor groove. Finally, the evolution of compounds like altromycin B that have both minor and major groove binding interactions was achieved through the addition of major groove binding sugar moieties to the C5 position.

This structurally evolved complexity is also predictive of the potential complexity in the molecular mechanisms, giving rise to sequence specificity. In principle, the intermolecular interactions that give rise to the sequence selectivity may occur through intercalative and/or minor/major groove binding forces such as stacking interactions, H-bonding, van der Waals contacts, or electrostatic interactions. In the simplest case, the sequence selectivity could arise from the noncovalent binding interactions, which would preferentially stabilize the bound complex at certain sequences, thereby increasing the chance of productive covalent reactivity. This situation is perhaps best exemplified by (−)-CC-1065, where, in contrast to (+)-CC-1065, the covalent alkylation sites are dictated by the binding interactions.[5b] If this were the case for the pluramycins, then the optimum *binding sites* for a drug such as neopluramycin, which lacks the epoxide but in most other structural respects is similar to pluramycin, should be predictive of the covalent bonding sites of pluramycin revealed by the thermal cleavage reaction. The results in Figure 6 show that neopluramycin does not bind specifically to the same sequences alkylated by pluramycin, and furthermore, this antibiotic shows little if any DNA sequence selectivity. A separate NMR study on an oligomer with kidamycin, which also lacks an epoxide, also failed to show selective binding to a particular sequence (Hansen and Hurley, unpublished results). These results are in contrast to nogalamycin, which, through binding interactions, does attain sequence selectivity.[9] Therefore, while we do not rule out some minor contribution to the overall sequence selectivity from the precovalent binding interactions (k_b in eq 1), we favor the covalent bonding step (k_r in eq 1) as the primary step at which sequence selectivity is achieved by the pluramycins.

$$\text{drug} + \text{DNA} \overset{k_b}{\rightleftharpoons} [\text{drug·DNA}]_{\text{noncov}} \xrightarrow{k_r} [\text{drug}-\text{DNA}]_{\text{cov}} \quad (1)$$

Importance of the Covalent Reactivity Step in Determining Sequence Selectivity of the Pluramycins. An alternate source of sequence selectivity to the noncovalent binding interactions is the alkylation reaction (k_r in eq 1). The bonding step is sequence selective if the rate constant (k_r) for covalent bond formation is different for different sequences, i.e., if the free energy of activation to the transition state leading to the covalent adduct depends upon the nucleotide sequence around it. For sequence selectivity to be achieved at this step, it is necessary that the dissociation rate from DNA be rapid at both cognate and noncognate sequences. Only in this case can selective reactivity at cognate sequences be expressed because, at the noncognate sequences, dissociation takes place before covalent reactivity occurs.

A second requirement is that the inherent covalent reactivity of the epoxide be low so that productive alkylation can only occur when a "proximity effect" is achieved by the sequence-dependent "steering effect" of minor and major groove interactions. As emphasized before, slow dissociation rates lead to indiscriminate reactivity at many sites almost irrespective of the magnitude of the proximity effect. In addition, the sum total of the electrophilicity of the substrate (drug) and nucleophilicity of the receptor (N7 of guanine) needs to be in a narrow window, where indiscriminate alkylation does not occur (i.e., like the highly reactive electrophilic reagent dimethyl sulfate) or the sum of the reactivity is too small, such that other less discriminating factors, such as individual site-binding times, play a major role. The results in Figure 8 (top), where there is substitution of inosine for guanine, which causes a decrease in the nucleophilicity of N7, producing a loss of hierarchy of sequence selectivity, may result from this effect.

Since the epoxide is not intrinsically a highly reactive electrophilic agent, this leads us to propose that a proximity effect is required for productive alkylation of N7 of guanine. This proximity effect enables juxtaposition of N7 of guanine and the epoxide for in-line S_N2 attack. Thus, both the positioning of the epoxide of pluramycin in the major groove, which is achieved through minor and major groove interactions, and the drug-induced repositioning of N7 of the targeted guanine are critical factors in determining if this reaction will take place. There are at least two independent mechanisms by which sequence selectivity can be achieved at the alkylation step. First, binding interactions in one or both of the grooves could "steer"

(8) In previous experiments (Yuan and Hurley, unpublished results) where we have substituted inosine for adenine, this has resulted in lower reactivity of N3 with (+)-CC-1065 consistent with the reduction of N7 reactivity with the pluramycins.

(9) (a) Zhang, X.; Patel, D. J. *Biochemistry* **1990**, *29*, 9451−9466. (b) Searle, M. S.; Hall, J. G.; Denny, W. A.; Wakelin, L. P. *Biochemistry* **1988**, *27*, 4340−4349.

(10) Lin, C. H.; Beale, J.; Hurley, L. H. *Biochemistry* **1991**, *30*, 3597−3602.

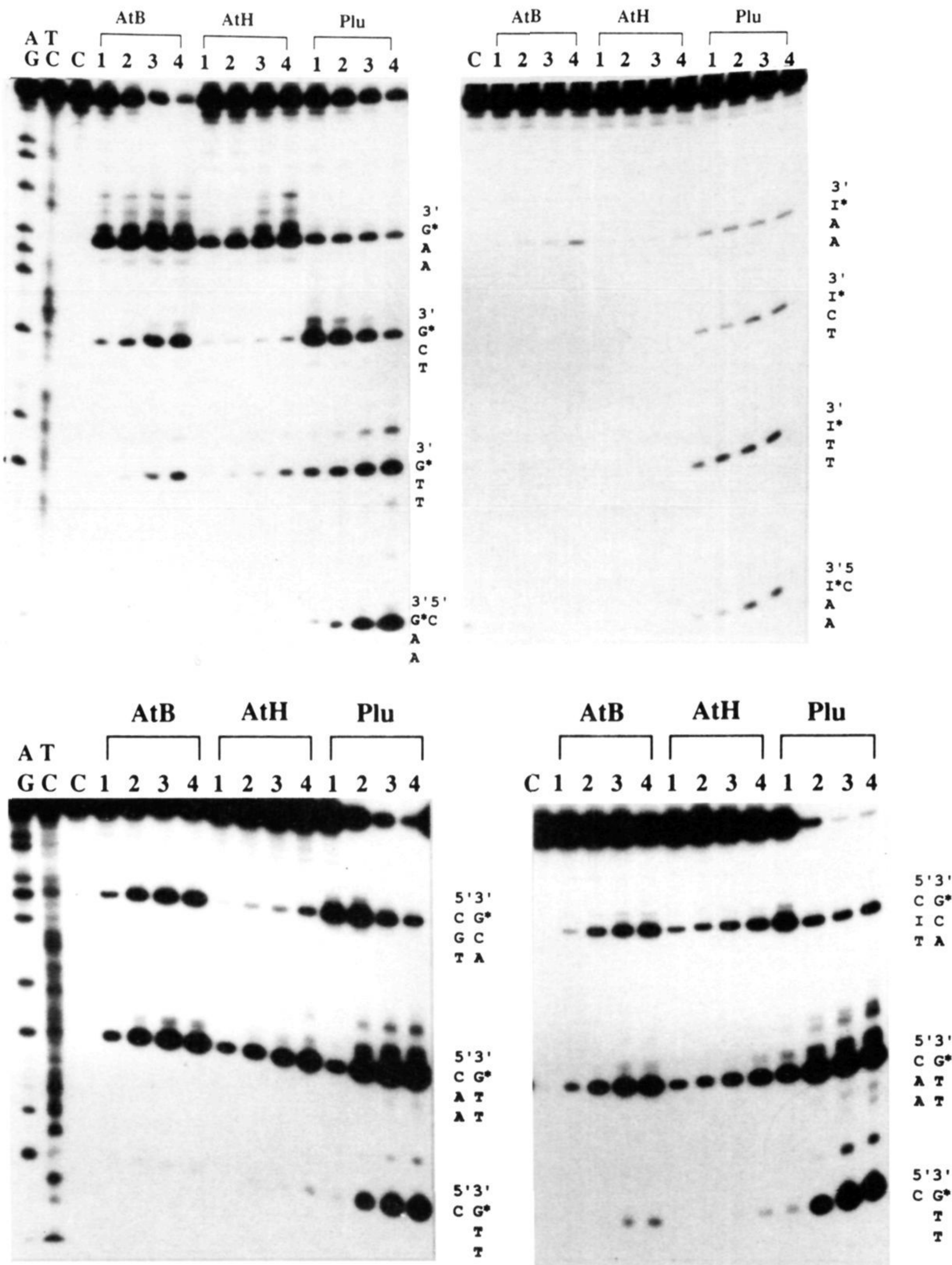

Figure 8. Effect of replacing the guanine residues in DNA with inosine on the alkylation of DNA by the pluramycins. (top) and (bottom) represent the autoradiogram of pairs of the + and − strand-labeled oligomers from 23G and 23I in Table 1 modified with drug molecules. For both A and B, the left and right panels correspond to 23G and 23I, respectively. In both cases (top and bottom), lanes 1−4 contain 10, 20, 50, and 100 ng of drug molecules for altromycin B (at B); 20, 50, 100, and 200 ng of drug molecules for altromycin H (at H); and 5, 10, 20, and 50 ng of drug molecules for pluramycin (plu). The alkylated sequences with covalently modified guanines (G*) or inosines (I*) are shown to the right of each panel.

the epoxide into proximity to N7 of guanine. Experiments in which minor groove geometry has been modulated by varying the length of the A tract (Figure 7) clearly point to an important role for minor groove geometry in "steering" reactivity in the major groove of DNA. This has a precedent in the proposed sequence-specific interactions of mitomycin C with DNA, in that a specific hydrogen bond in the precovalent state positions mitomycin C to alkylate CG* preferentially over NG*.[11] Second, in a number of other cases (reviewed in ref 5a), catalytic activation by DNA has been proposed to be a mechanism for

(11) Kumar, S.; Lipman, R.; Tomasz, M. *Biochemistry* **1992**, *31*, 1399−1407.

sequence recognition. For example, a specific phosphate catalysis of the reaction between the cyclopropapyrroloindole (+)-CC-1065 and N3 of adenine in the minor groove of DNA has been proposed.[10] In the case of the pluramycins, an acid catalysis involving water-bridged phosphates in the major groove might constitute a parallel mechanism; however, as yet no evidence exists for this mechanism. Moreover, it may not be necessary to invoke an acid catalysis mechanism involving a specific phosphate because computations by Lamm and Peck[12] have highlighted the intrinsically high acidity associated with the grooves of DNA. While the pluramycins are not DNA–DNA cross-linkers in the strict sense of forming two covalent attachments on one or both strands, they do have features that mimic this type of lesion on DNA. In particular, in order for the "cross-linking" reaction to occur at N7 of guanine in the major groove, the epoxide must be able to "reach" from its attachment site on the intercalated moiety, which is itself tethered in the minor groove by binding interactions. Since the reach of the epoxide is strictly controlled by the binding interactions, the ability of DNA, and in particular deoxyguanosine, to distort to meet the positioned epoxide is an important factor in determining both site reactivity and sequence specificity. Therefore, sequence-dependent conformational flexibility and the principles proposed by Hopkins,[13] in which "minimal reorganization" is proposed to be an important factor in sequence recognition of cross-linkers, may also apply here.

Importance of Steering by Minor and Major Groove Binding Interactions in Achieving a Proximity Effect in the Major Groove. The importance of molecular interactions in the minor and major grooves, which give rise to the proximity effect, can be addressed by experimental data (two-dimensional high-field NMR) and molecular modeling.[4] The results of DNA sequence specificity studies reported here are quite clear and show that for high reactivity sites group A compounds prefer 5′ AG*, group B compounds prefer 5′ (A/T)G*, and group C compounds prefer 5′ CG*. Molecular modeling of the bis-(altromycin B) 10-mer 5′(GAAG*TACTTC)$_2$ (5′-AG*-) and bis-(hedamycin) 10-mer 5′(GATG*TACATC)$_2$ (5′-TG*-) duplex diadducts, based upon two-dimensional ^{1}H-NMR results, provides important insights into how this sequence selectivity mediated through binding interactions may be achieved.

These ^{1}H-NMR studies show that, upon intercalation of the planar drug chromophore into the DNA, the C5, C8 and C10 glycoside binding interactions function to steer their reactive epoxide into proximity with the nucleophilic N7 of guanine in the major groove, thereby increasing DNA reactivity. One key interaction in sequence recognition made by pluramycins with DNA is through the C10 amino sugar. The NMR studies involving altromycin B and hedamycin covalently bound to their respective highly reactive sequences reveal a preference for the dimethylamino substituents on these glycosides to interact directly with pyrimidines on the *noncovalently* (altromycin B) or the *covalently* (hedamycin) modified strands in the minor groove,[4] presumably through hydrogen bonding in the protonated state with the negatively charged O2 carbonyl of the pyrimidine.

In modeling studies of altromycin B covalently bound to its most preferred sequence (5′ AG*), the amino sugar shows a preference to interact with the nucleotide (8T) on the noncovalent strand associated with the base pair on the 5′ side of the covalently modified guanine. The thymine at this position, which is found in the most reactive 5′ AG* sequence, offers an optimal hydrogen bond acceptor, the O2 carbonyl, which is highly negative and protrudes slightly from the floor of the minor groove, to interact with the C10 amino sugar (see Figure 9 (top)). Sequence selective effects of this interaction arise from the association of the amino sugar with this *noncovalent* strand to create a minor groove pocket for the terminal neutral 6-deoxy-*O*-3-methylaltrose to fit and to favorably align the C5 substituent for interaction in the major groove. In the less reactive case, the 5′ TG* bonding site, the amino sugar is limited either to hydrogen bonding to the thymine carbonyl located on the covalently modified strand, which would sterically crowd both the terminal neutral 6-deoxy-3-*O*-methylaltrose in the minor groove and the C5 2,6-dideoxy-3-*O*-methylaltrose against the floor of the major groove, *or* to forming a less optimal hydrogen bond with N3 of the base-paired adenine on the floor of the minor groove.

In addition to the sequence-selective effects of intermolecular minor groove interactions, the neutral C5 sugar substituent, characteristic of altromycin B, exerts a selective influence through interactions in the DNA major groove.[4] Altromycins lacking the C5 neutral sugar substituent, such as altromycin H, demonstrate decreased reactivity with DNA and a modified sequence selectivity compared to that of altromycin B. The key interaction of the C5 substituent is between the hydrophobic face of this sugar and the hydrophobic pocket formed in the major groove by the two pyrimidines (7C and 8T) adjoining the intercalation site on the nonmodified strand in the preferred sequence (5′-AG*-). The net effect of this interaction in the major groove is to shift the drug functionalities in the major groove, namely the C2 epoxide group, toward the strand to be modified. As a result, the class A compounds have increased their reactivity toward DNA over the class B agents by increasing the proximity effect between the epoxide and N7 of guanine.

In the case of the C5 altrose-deficient altromycins (i.e., class B compounds), these agents demonstrate diminished selectivity for 5′ AG* over 5′ TG*. The loss of steric interactions in the major groove potentially allows for rotation of the intercalation chromophore toward the covalently modified strand (see Figure 9 (top)) in the minor groove to permit hydrogen bonding by the C10 amino sugar to 5′ thymine O2 on the covalently modified strand. The class B compounds therefore have less steric conflicts in reactions with 5′ TG* than class A agents, but they have an overall lower reactivity to DNA.

In the case of the classical pluramycin subfamily, or group C (e.g., hedamycin), modeling of the medium reactivity 5′ TG sequences[13] reveals a requirement for the C10 amino sugar to interact with the nucleotide (3T) on the *covalently* modified strand, 5′ to the modified guanine (Figure 9 (bottom)). Association of the C10 sugar with the covalent strand allows sufficient room in the minor groove for the C8 amino sugar to interact with the cytosine base-paired to the modified guanine. In the low reactivity case, 5′ AG*, the C10 amino sugar is limited to interaction with the 5′ adenine. Interactions with the noncovalent strand, specifically the pyrimidine, would sterically crowd the C8 amino saccharide. Therefore, these interactions lead to a 5′ (Py)G* selectivity, which positions a pyrimidine to the 5′ side of the modified guanine on the drug-modified strand for interaction with the C10 amino sugar and a pyrimidine on the noncovalent strand base-paired to the modified guanine for interaction with the C8 amino sugar.

Analysis of altromycin B and hedamycin interactions in the minor groove provides insight into the reasons for the low tolerance of C·G base pairs on the 5′ side of the modified guanine demonstrated by the altromycins, in contrast to the tolerance exhibited by the classical pluramycins. It is likely

(12) Lamm, G.; Peck, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9033–9036.

(13) Hopkins, P. B.; Millard, J. T.; Woo, J.; Wiedner, M. F.; Kirchner, J. J.; Sigurdsson, S. Th.; Raucher, S. *Tetrahedron* **1991**, *47*, 2475–2489.

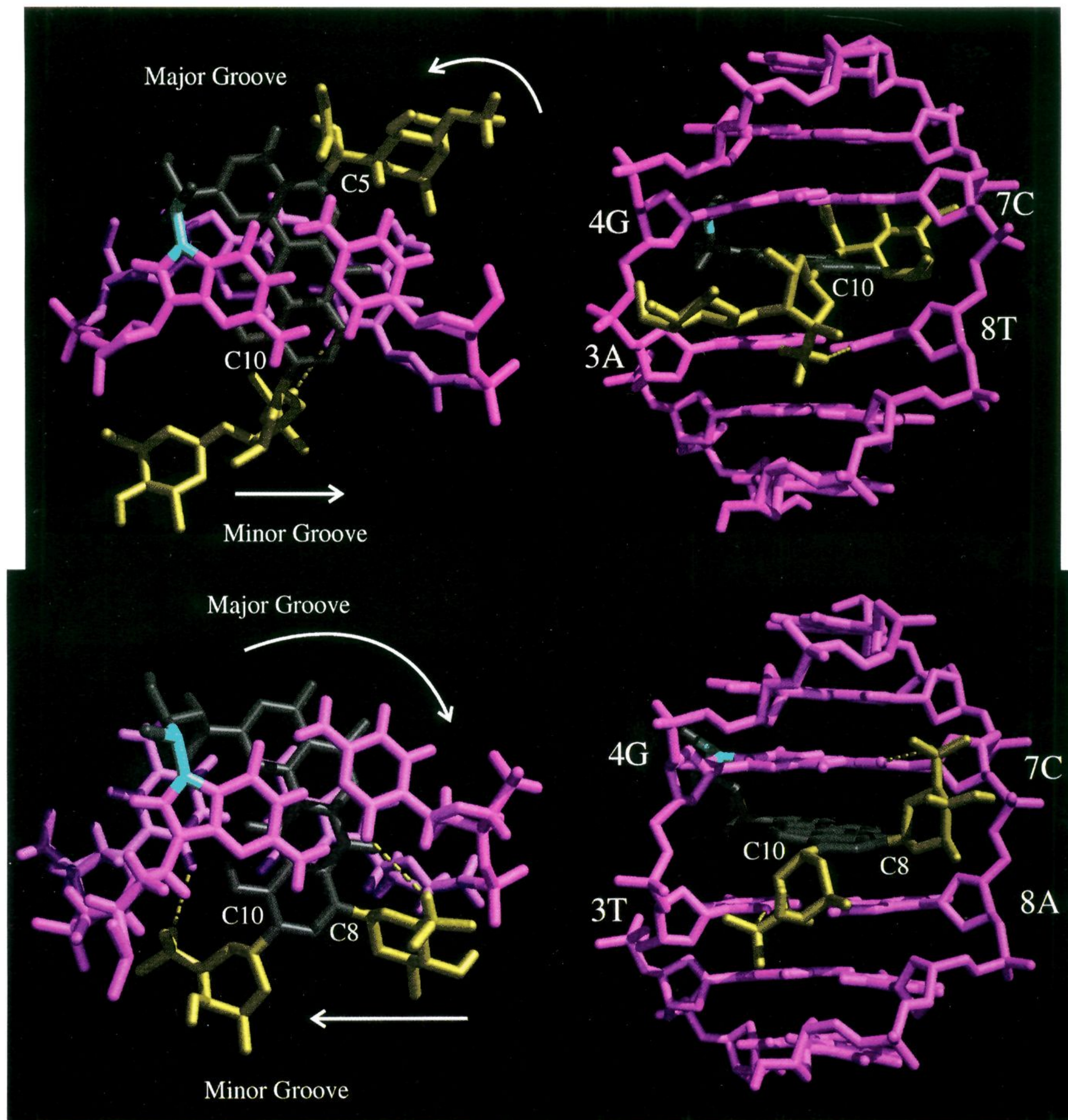

Figure 9. Molecular models[18] of the bis(altromycin B)−[d(GAAGTACTTC)]$_2$ and bis(hedamycin)−[d(GATGTACATC)]$_2$ diadducts. Shown in the top panel is the interaction made by altromycin B with its most-preferred sequence, 5′-AG*-. On the right is the interaction of the altromycin B amino disaccharide (yellow) in the minor groove, and on the left is the proposed steering of the altromycin chromophore (gray) by the C5 and C10 glycosides (yellow) to perpetuate alkylation to N7 of guanine (cyan). Shown in the bottom panel is the parallel interaction made by hedamycin with its highly reactive 5′-TG*- sequence. Shown on the right is the interaction of the C8 anglosamine and the C10 *N*,*N*-dimethylvancosamine (yellow) in the minor groove, and shown on the left is the steering of the drug chromophore (gray) by the C8 and C10 substituents (yellow) for alkylation of N7 of guanine (cyan).

that interaction of the guanine exocyclic N2 amino substituent in the minor groove provides steric and electrostatic hindrance to the interaction of the C10 disaccharide to both sides of the minor groove. The classical pluramycins avoid this steric interference by positioning each amino saccharide in the minor groove to either side of the exocyclic amino group, never directly interacting with it, as in the case of the altromycin C10 amino disaccharide.

Modulation of Reactivity Is Also Dependent upon Multiple Flanking Base Pairs. In addition to the observed local two-base-pair sequence selectivity, there is an observed modulation of this two-base-pair selectivity as a function of flanking DNA sequences. The most striking examples of these fluctuations are the enhanced hedamycin reactivity with 5′ TG* sequences set in (TG)$_n$ runs over those set in seemingly random sequences[6] and the observed modulation of altromycin B reactivity shown in Figure 7, which is induced by flanking A tracts. This second level of sequence selectivity is probably induced by the local helical parameters of the DNA molecule dictated by sequences greater than two base pairs flanking each side of the intercalation site.

As with the two-base-pair selectivity, pluramycin sugar substituents govern this mode of sequence recognition as well. The C10 amino sugar interacts in the minor groove of the DNA

molecule with the 5′ base pair on either the covalently modified strand, as in the case of pluramycin, which has a C8 substitution, or the noncovalently modified strand, as in the case of the altromycin, which has a C5 substitution. Around this point of intermolecular interaction, the conjugated chromophore will rotate clockwise when viewed from the 3′ side of the modified guanine when there exists a C8 amino sugar and counterclockwise when there exists a C5 neutral sugar substituent to steer the reactive epoxide moiety toward N7 of guanine in the major groove. The resulting binding orientation between the two base pairs will determine the proximity of the epoxide to the reactive guanine N7 and, consequently, its reactivity. It therefore follows that, in addition to recognizing two base pairs through intermolecular contacts, the pluramycins also recognize local DNA helical parameters dictated by flanking sequences, which ultimately affect the position of the epoxide in the major groove relative to guanine N7.

Importance of "Epoxide Reach" in Determining Alkylation Reactivity and Minimizing Distortion of the Alkylated Base. While binding interactions that steer the epoxide into proximity of N7 of guanine affect covalent reactivity and sequence specificity, it is also apparent that the overall level of alkylation reactivity differs markedly from groups A and B to group C (see Figure 3). The classical pluramycins hedamycin and rubiflavin have a 5-fold greater reactivity than altromycin B (group A), which is more reactive than altromycins H and I (group B). We propose that the origin of the different reactivities of group C and groups A and B is in the variable reach and/or flexibility of the alkylating substituent (R4) at C2 of the anthrapyran system. The alkylating epoxide at R4 is quite variable, from the diepoxide of hedamycin to the olefinic epoxide of rubiflavin and the unconjugated epoxide of the altromycins (Figure 1). ^{1}H-NMR studies show that, while reaction takes place at the least substituted carbon of altromycin B,[4a] in the case of hedamycin, covalent reaction takes place at the most substituted carbon of the terminal epoxide.[4b] Since there is no reason to expect the alkylating epoxides of the altromycins or hedamycin to have different inherent reactivities, we propose that the higher reactivity of hedamycin is due to the longer reach of the epoxide, which reduces the need for guanine to distort from its optimally base-paired position in order for alkylation to occur. Evidence to support this minimized distortion proposal is available from molecular modeling of duplex adduct structures of the hedamycin and altromycin B adducts. While in the case of the altromycin B−DNA adduct, the covalently modified guanine is tilted into the major groove, for the hedamycin−DNA adduct (Figure 9 (top)), the longer reach allows for a planar orientation of the equivalent alkylated guanine (Figure 9 (bottom). The difference in overall reactivity of the altromycins in groups A and B most probably relies on the ability of the C5 substituents in group A to shift the C2 epoxide into proximity of N7.

Conclusions

The pluramycins are a unique group of DNA-reactive drugs that exhibit sequence selectivity determined at the covalent bonding step due to minor and major groove steering interactions, which mediate a proximity effect between the epoxide and N7 of guanine. The overall reactivity of individual classes within this group of antitumor antibiotics also appears to be dependent upon the reach of the epoxide in the major groove.

Materials and Methods

Altromycins B, H, and I, pluramycin, hedamycin, and rubiflavin were obtained from Abbott Laboratories, Chicago, IL. Electrophoretic reagents (acrylamide, TEMED, ammonium persulfate, and bis(acrylamide) were purchased from Biorad, T4 polynucleotide kinase from U.S. Biochemical Corp., [γ-^{32}P]ATP and X-ray film from ICN, and pCAT from Promega.

Preparation of Oligonucleotides. A series of oligonucleotides (Table 1) was synthesized on an automated DNA synthesizer (Applied Biosystems 381A) using the phosphoramidite method.[14] Labeled duplexes were prepared as described previously.[15]

Isolation and End-Labeling of a 189-mer Restriction Fragment from Plasmid DNA. Plasmid DNA pCAT was digested with Ncol followed by dephosphorylation with bacterial alkaline phosphatase treatment. Restriction fragments were purified by phenol/$CHCl_3$ extraction and ethanol precipitation and kinated with [γ-^{32}P]ATP and polynucleotide kinase. 5′ end-labeled restriction fragments were digested with PvuII to generate a unique end-labeled restriction fragment, and the desired fragment was separated on a 6% nondenaturing polyacrylamide gel. The wet gel was exposed onto autoradiographic film, and the desired fragment was located and excised. The DNA was removed from the gel by crushing and vortexing at room temperature overnight in 10 mM Tris-HCl (pH 7.6) and 10 mM NaCl.

Drug-Binding Reactions, DNA Strand Breakage Assay, and DNase I Footprinting. Drug-binding reactions were carried out at room temperature in a solution containing 10 mM Tris-HCl (pH 7.6), 10 mM NaCl, DNA, and the indicated amount of drug molecules. These reactions were stopped by phenol/$CHCl_3$ extraction and ethanol precipitation to remove unbound drug molecules. DNA pellets were dried and redissolved in DDW. For the strand breakage assay to identify the alkylation sites, drug-modified DNA samples were heated to 95 °C with piperidine (1 M) for 15 min. DNA samples were dried, resuspended in DDW, dried again, and dissolved in alkaline dye (80% formamide, 10 mM NaOH). These samples were subjected to denaturing polyacrylamide gel electrophoresis in parallel with DNA sequencing, as described previously.[16] DNase I footprinting was carried out as described in the legend for Figure 6.

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(14) The sequence 5′-TG*- was used rather than 5′-CG- because this sequence was chosen prior to the analysis of the sequence specificity experiments described in this paper. However, while 5′-CG*- is preferred over 5′-TG*-, the interactions we describe here are generally applicable to 5′-PyG*-.

(15) Galt, M. J., Ed. *Oligonucleotide Synthesis—A Practical Approach*; IRL: Oxford, U.K.

(16) Lin, C. H.; Sun, D.; Hurley, L. H. *Chem. Res. Toxicol.* **1991**, *4*, 21−26.

(17) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499−560.

(18) Molecular models were generatd upon the basis of information derived from two-dimensional studies of the bis(hedamycin)−[d(GATG*TACATC)]$_2$ and bis(altromycin B)−[d(GAAG*TACTTC)]$_2$ diadducts. Structures were generated using B-form DNA, which was then adjusted to dock the drug molecules as predicted from NMR results. Models were altered either in MIDAS[19] or with constrained belly dynamics to achieve a structure consistent with inter-drug−DNA NOE connectivities and were then minimized in AMBER 4.0.[20]

(19) Ferrin, T. E.; Huang, C. C.; Jarvis, L. E.; Langridge, R. *J. Mol. Graphics* **1988**, *6*, 13−27.

(20) Pearlman, D. A.; Case, D. A.; Caldwell, J. C.; Seibel, G. L.; Singh, U. C.; Weiner, D.; Kollman, P. A. *Amber 4.0*; University of California: San Francisco, CA, 1991.