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A Thymine:Thymine Mismatch Enhances the Pluramycin Alkylation Site Downstream of the TBP−TATA Box Complex

Seung-Joo Lee and Laurence H. Hurley*

Contribution from the Drug Dynamics Institute, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712

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Abstract: The DNA groove interactions of the pluramycins determine the base-pair specificity to the 5′-side of the covalently modified guanine. The DNA reactivity of these drugs at defined sites can be further increased by structural and dynamic DNA distortion induced by TATA binding protein (TBP) binding to the TATA box. This enhanced drug reactivity has led to the proposal that protein-induced DNA conformational dynamics might be responsible for the more selective biological consequences of the pluramycins. To identify the structural and/or dynamic determinants that account for the enhanced drug reactivity, DNA heteroduplexes that contain base mismatches were examined for enhanced alkylation by altromycin B. The results demonstrate that base mismatches located at the 5′-side of the target guanine enhance drug reactivity. An analysis of the structural and dynamic properties of the base mismatches demonstrates that the pluramycin reactivities are not only determined by dynamic conformation of the base mismatch, which improves the accessibility of the drug to the DNA helix, but also by the specific groove interactions between the DNA and the drug, which results in stabilization of the precovalent drug−DNA complex. Having established that the highest pluramycin reactivity with heteroduplex DNA is produced by a thymine:thymine (T:T) mismatch, we next addressed how the same mismatch affects pluramycin reactivity in the flanking region to the TBP−TATA box complex. When this mismatch is introduced into the downstream flanking sequence of the TATA box, TBP binding cooperatively enhances the drug alkylation on a downstream guanine adjacent to the inserted mismatch. While an obvious structural similarity between the T:T mismatch and the TBP-induced effects at a downstream site of the TATA box does not exist, we propose that the base pair destabilizing effects of the T:T mismatch may resemble the dynamically accessible intercalation site on the downstream side of the TATA box induced by binding of TBP to the TATA box.

Introduction

The pluramycins are a family of DNA intercalating antitumor agents that thread through the helix and covalently modify N7 of guanine (Figure 1).[1] The reaction of these drugs with DNA exhibits a base specificity to the 5′-side of the alkylated guanine.[2] The sugar substituents at various positions of the pluramycins are primarily responsible for the sequence-dependent DNA alkylation reactivity of the drug.[2] These carbohydrate moieties steer the reactive epoxide of the drug into the proximity of the nucleophilic alkylation site on DNA.[2] The precovalent binding complex is stabilized by hydrogen bonding between the amino sugar and the pyrimidine base in the minor groove.[3]

Despite the apparent two-base-pair sequence specificity of DNA alkylation by the pluramycins, this is insufficient to

* To whom correspondence should be addressed. Telephone: (512) 471-4841. Fax: (512) 471-2746. E-mail: dg-dna@mail.utexas.edu.

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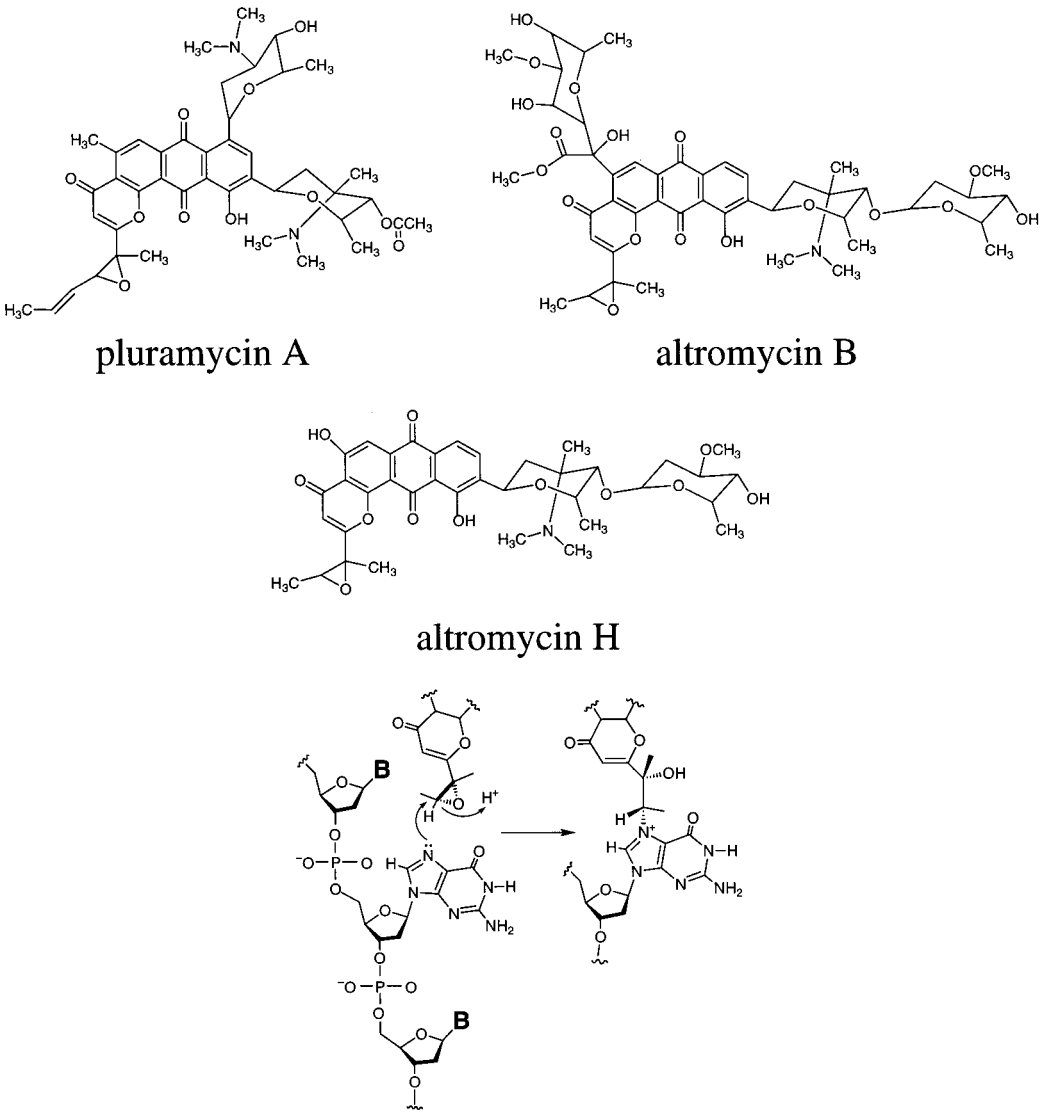

Figure 1. (A) Structures of examples of the pluramycin group of antitumor antibiotics. (B) Mechanism of covalent modification of DNA by altromycin B. N7 of guanine performs nucleophilic attack on the epoxide of the drug, forming a cationic lesion on the DNA. Subsequent thermal depurination results in DNA strand breakage.[3a] Boldface B = base.

rationalize the selective biological activity of these drugs. In a recent study, however, we have demonstrated that a preferred receptor DNA site exists in the initial protein−DNA complex of transcription, which is detected in the form of enhanced pluramycin A modification upon TBP binding to the TATA box.[4] This has suggested that dynamic cellular events might provide "hot spots" for the pluramycins, which could lead to relevant biological consequences.[5]

A candidate structure that might induce high pluramycin reactivity is a DNA base mismatch, which arises during recombination or error-prone replication. Although a base mismatch does not cause global distortion of the DNA helix,[6] it destabilizes the duplex both thermodynamically and kinetically, resulting in a dynamic perturbation of several base pairs around the mismatch site.[7] Because mismatch repair systems that correct these DNA aberrations are essential to avoid tumor development,[8] the selective and effective recognition of these abnormal structures has been of great interest in mismatch repair studies.[9]

In the present study, we have investigated the effect of a spectrum of single base mismatches on the alkylation reactivity of altromycin B. A comparison of the drug reactivity with the mismatch duplex DNA and a normal base-pair duplex reveals that the base mismatch interactions can modulate the drug alkylation reactivity. Base mismatches that facilitate both the intercalation of the drug into the duplex DNA and specific drug interaction with DNA result in significant enhancement of drug reactivity. The introduction of a T:T mismatch into the flanking sequence of a TATA box cooperatively enhances the reactivity of drug modification specifically on the downstream site upon binding of TBP. While an obvious structural similarity between

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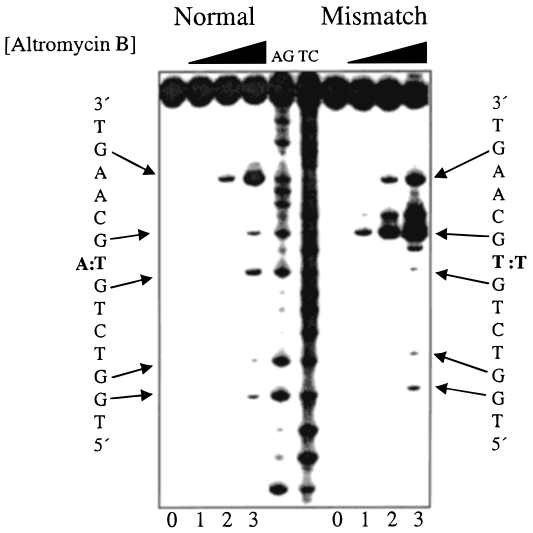

Figure 2. The effect of a T:T mismatch on DNA alkylation reactivity by altromycin B. Normal base pair (left) and mismatch containing (right) 28-mer DNA duplexes have the same DNA sequence except for the base pairs indicated in bold. Control DNA without drug treatment is shown in lane 0. Concentrations of altromycin B used in lanes 1−3 were 10, 50, and 250 nM, respectively. AG and TC are Maxam−Gilbert sequencing marker lanes. DNA sequences are shown to the left and right of the gels. Arrows indicate sites of altromycin B modification.

the T:T mismatch and the TBP-induced distortion at the downstream site of the TATA box does not exist, we propose that the base pair destabilizing effects of the T:T mismatch may resemble the dynamically accessible intercalation site on the downstream side of the TATA box induced by binding of TBP to the TATA box.

Results

A. DNA Base Mismatches Modulate the DNA Alkylation Reactivity of Altromycin B. The sequence-specific DNA alkylation by the pluramycins is predominately determined by the base pair to the 5′-side of the alkylated guanine;[2] however, subtle local helical parameters, such as minor groove geometry and the dynamics of the neighboring DNA sequence, also influence DNA alkylation reactivity of the threading intercalators.[2,10] Another example of DNA dynamics that modulates the sequence-dependent drug alkylation has been demonstrated with the TBP-bound promoter.[4] To gain insight into the molecular basis for the altered pluramycin reactivity induced by local DNA dynamics, DNA base mismatches were incorporated into the duplex DNA, and their effects on the DNA alkylation reactivity of altromycin B were determined.

By annealing a radiolabeled same-sequence DNA strand with different complementary strands, both normal Watson−Crick base pair and mismatch duplexes were prepared. After covalent modification of N7 of guanine in DNA with altromycin B, the level of alkylation was determined by a DNA strand breakage assay.[1]

A. 1. Thymine:Thymine Mismatch on the 5′-side of the Target Guanine Significantly Increases the Altromycin B Reactivity. A comparison of the reactivity of altromycin B with a normal duplex and the same duplex containing a T:T mismatch is shown in Figure 2. The effect of the T:T mismatch on altromycin B reactivity with its target guanine is limited to the immediately adjacent 5′- and 3′-sides of the mismatch. For

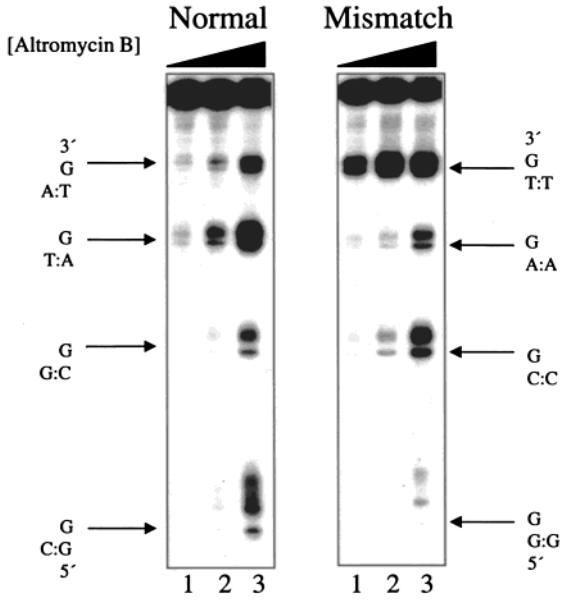

Figure 3. The effect of various base-pair mismatches on altromycin B alkylation reactivity. The 66-mer DNA duplexes contain either normal base pairs (left) or base-pair mismatches (right) on the 5′-sides of the targeted guanines. Concentrations of altromycin B used in lanes 1−3 were 0.05, 0.5, and 5 μM, respectively. Base pairs on the 5′-side of the alkylated guanines are shown to the left and right of the gels. Arrows indicate sites of altromycin B modification. The multiple bands corresponding to altromycin B alkylation are due to incomplete strand breakage reaction.

altromycin B modification of a guanine that has the T:T mismatch on the 5′-side, i.e., 5′-TG<u>T</u>G*-3′ (underlined thymine indicates mismatched base and asterisked guanine denotes alkylated base), the alkylation reactivity produced by the lowest concentration of the drug (lane 1) in the mismatch duplex is roughly equal to that achieved by the highest concentration (lane 3) in the normal duplex. This result indicates that the relative altromycin B reactivity is enhanced at least 25-fold upon substituting a T:T for a T:A at the 5′-side base pair to the target guanine. In contrast, positioning of the same mismatch on the 3′-side of the target guanine (5′-TG*<u>T</u>G-3′) slightly reduces the alkylation reactivity. This suggests that a base-pair destabilization on the 5′-side of the modified guanine, i.e., the drug chromophore intercalation site, significantly affects the alkylation reactivity of altromycin B.

A. 2. The More Destabilizing Mismatches Generate the Higher Alkylation Reactivity Sites for Altromycin B. To extend the investigation of the effect of base mismatch on drug reactivity, 12 possible mismatches were placed on the 5′-side of the target guanine and embedded in the DNA sequence 5′-ATCXGAT-3′/3′-TAGYCTA-5′ (X, Y = A, C, G, or T) and examined for altromycin B alkylation reactivity. A subset of the results is shown in Figure 3 and the complete results are summarized in Table 1. As shown in the left panel of Figure 3, the 5′-side base-pair preference (A:T > T:A > G:C, C:G) of altromycin B reactivity for the normal duplex is in good agreement with our previous report.[2] For the base mismatches shown in the right panel of Figure 3, pyrimidine:pyrimidine (py:py) mismatches generate more reactive sites for altromycin B alkylation than purine:purine (pu:pu) mismatches (T:T > C:C > A:A > G:G). Because py:py mismatches are more destabilizing and expandable than pu:pu mismatches,[11] this suggests that

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Table 1. Effect of Mismatched Base Substitutions on Altromycin B Alkylation of 3′ Guanine in the 66-Mer

base substitution[a]			reactivity change[b]
A:T	→	A:A	−
	→	A:G	−
	→	A:C	−
T:A	→	T:G	0
	→	T:C	0
	→	T:T	++
G:C	→	G:A	0
	→	G:G	−
	→	G:T	0
C:G	→	C:A	0
	→	C:C	+
	→	C:T	+

[a] Base pair on the 5′-side of the alkylated guanine. [b] (−) decreased alkylation, (0) no change, (+) increased alkylation, (+) and (++) represent enhancement of drug reactivity by 10- and 100-fold, respectively.

a more destabilizing mismatch provides a better opportunity for the drug to intercalate into the destabilized DNA site. Furthermore, a $\pi-\pi$ aromatic base stacking interaction, which occurs primarily in pu:pu mismatches,[12] might interfere with the intercalation by reducing the space for the bulky altromycin B molecule.

A. 3. Mismatches That Facilitate Specific Groove Interactions Enhance the DNA Alkylation Reactivity of Altromycin B. To determine if there is any DNA base parameter (i.e., hydrogen bonding potential) that is beneficial for drug reactivity, the effects of base substitution to the 5′-side of the target guanine on altromycin B alkylation reactivity are compared in Table 1. This comparison reveals that a pyrimidine mismatch at the 5′-side of the target guanine is necessary for increased altromycin B reactivity (i.e., T:T; C:C; C:T). The replacement of the 5′-side thymine in the normal A:T base pair with any mismatched base pair decreases the altromycin B alkylation reactivity by 10-fold. These results are in accord with our previous NMR-based modeling study, which suggested that a crucial hydrogen bonding interaction between the O2 carbonyl of a pyrimidine and the dimethylamino sugar substituent of altromycin B in the minor groove is important for stabilizing the precovalent complex.[3a] Therefore, mismatches that provide the 5′-side thymine or cytosine enhance the drug reactivity by most probably facilitating groove interactions (hydrogen bonding) that stabilize the precovalent drug−DNA complex or by creating a more favorable site for the transition state in the covalent reaction. When mispaired with a purine, pyrimidines do not give enhanced reactivity.

The T:T mismatch is of particular interest because it induces the greatest enhancement of altromycin B reactivity with DNA. The same enhancement of the alkylation reactivity by the T:T mismatch was observed with another pluramycin, altromycin H (Figure 1), which has the same substituent located in the minor groove of DNA (data not shown). This reinforces the idea that the altrose sugar, a common feature of altromycins B and H, is involved in the specific interaction with the T:T mismatch. It seems that the unpaired pyrimidines in the mismatch enhance the chances of stabilizing hydrogen bonding with the protonated amino sugar, and this results in the greatest enhancement of reactivity for the altromycins.

A. 4. Pyrimidine:Pyrimidine Mismatches Provide Better Alkylation Reactivity Sites for Pluramycin than Normal A:T Base Pairs, While the Purine:Purine Mismatch Sites Are the Least Reactive to Pluramycin Alkylation. For optimum pluramycin reactivity, destabilized base pairing *and* the opportunity for specific groove interactions (hydrogen bonding) appear to be necessary. Both of these features are found in py:py mismatches, while they are absent in pu:pu mismatches. The normal A:T base pairing provides the hydrogen bonding potential but lacks the destabilizing effect of the py:py mismatch. Thus, the hierarchy of pluramycin alkylation sites py:py mismatch > A:T base pair > pu:pu mismatch is rationalized.

B. A Downstream T:T Mismatch Promotes the Specific Binding Mode of the TBP−TATA Box Complex That Enhances Altromycin B Alkylation upon TBP Binding. The similar enhanced pluramycin reactivity found in both the TBP-bound promoter[4] and the mismatch duplex DNA (in this study) implies that the TBP−TATA box interaction might induce a dynamic and/or structural DNA distortion that is in some way comparable to the base-pair mismatch. To test this structural and/or dynamic commonality, a T:T mismatch (see before) was introduced into either the upstream or downstream side of the human myoglobin TATA box. The mismatch mutant promoter or the wild-type (WT) promoter was modified with altromycin B in the absence or presence of TBP. The strand breakage products of drug-modified DNA samples were separated on a sequencing gel with sequencing markers (Figure 4A). The altromycin B alkylation reactivity was analyzed after normalization, and the results are summarized in Figure 4B.

B. 1. Similar to Pluramycin A, Altromycin B Preferentially Alkylates the Downstream Flanking Sequence of the TBP−TATA Box Complex. The altromycin B alkylation pattern of the WT promoter (the middle panel in Figure 4B) is generally similar to the previous results with pluramycin A in that the binding of TBP to the TATA box creates an enhanced alkylation reactivity site.[4] Although both pluramycin analogues show enhanced alkylation reactivity on guanines at the same base-pair step, the different sugar substituents of the two drugs determine the preferential drug reaction sites. Altromycin B modification shows enhanced reactivities on guanines in both the top and bottom strands of the same downstream base step (5′-AAAACG*-3′ and 3′-TTTTG*C-5′). Furthermore, in contrast to pluramycin A, the enhancement of altromycin B reactivity is greater on the bottom strand than on the top strand, showing that the TBP-induced DNA conformational dynamics are more favorable for altromycin B alkylation on the bottom strand site than the top strand site.

B. 2. The DNA Sequence Context Adjacent to the Mismatch also Influences Altromycin B Reactivity. As anticipated, even in the absence of TBP, the insertion of a T:T mismatch into either the upstream or downstream flanking sequence of the TATA box creates an extremely reactive altromycin B alkylation site in comparison to the normal base pair (white bars of upstream mutant (Um) and downstream mutant (Dm) in Figure 4B). However, the mismatches generate different drug reactivities depending on the neighboring sequence context. For example, although both downstream guanines in the top and bottom strands of the downstream mutant promoter (white bars of the right panel in Figure 4B) have the same base pairs to the 5′-side of the alkylated guanine (5′-CTG*), the bottom strand guanine shows about a 3-fold greater reactivity than the top strand. The different drug reactivities induced by the same mismatch suggest that a helical feature of the adjacent A-tract, which narrows the minor groove,[13] might also influence the alkylation reactivity in the mismatch region.[2,10]

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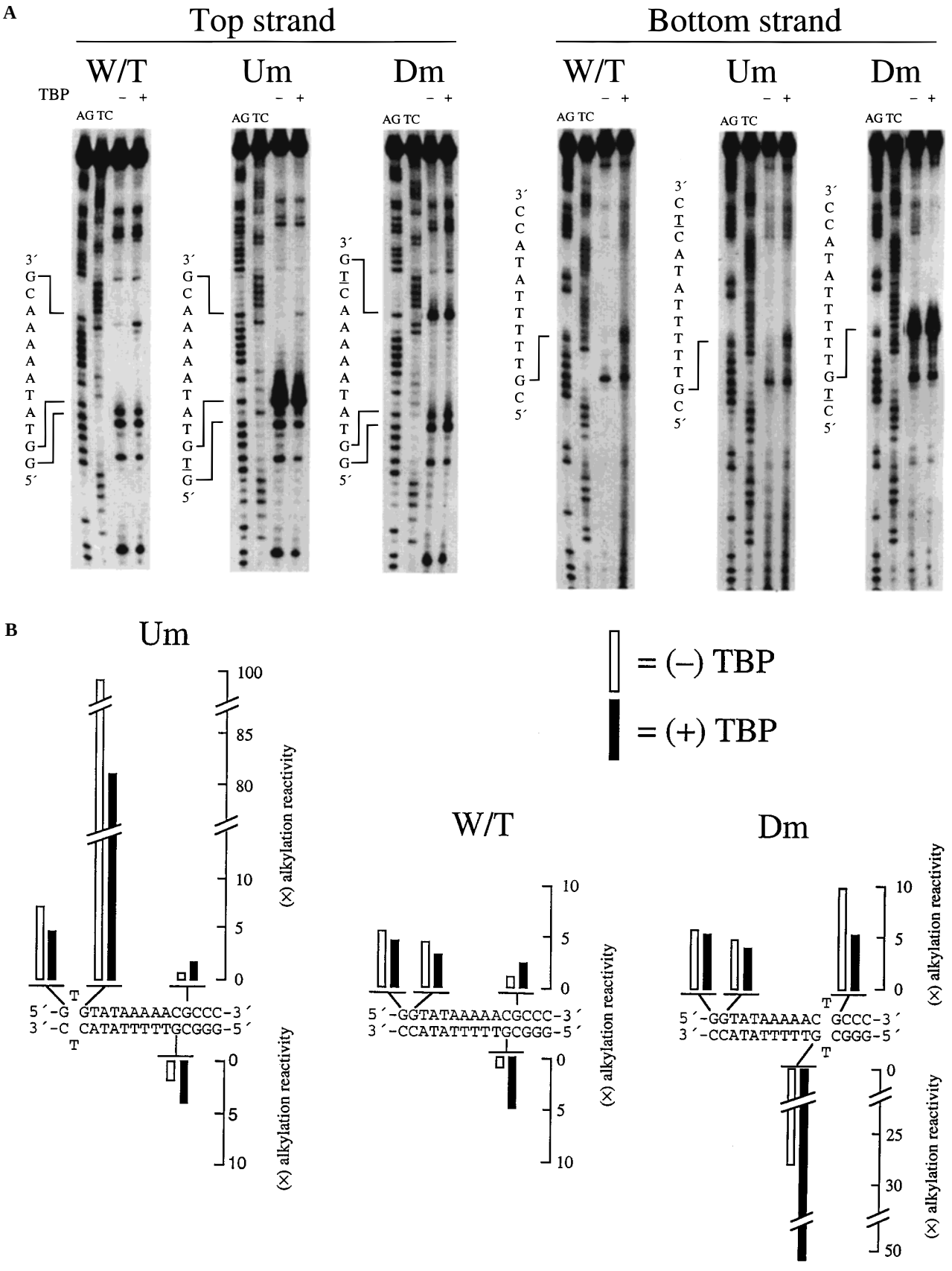

Figure 4. DNA alkylation by altromycin B of various promoter DNA sequences in the absence (−) or presence (+) of TBP. (A) The PAGE of the various promoters modified by altromycin B in either the absence (−) or presence (+) of TBP. W/T represents the wild-type promoter, and Um and Dm denote mutant promoters that contain a T:T mismatch in the upstream and downstream flanking regions of the TATA box, respectively. Underlined thymines in DNA sequences indicate the mismatched bases. Compared sites for guanine alkylation by altromycin B are marked with a solid line. (B) Histogram summary of relative alkylation by altromycin B of the flanking region of the promoters. Alkylation intensity is proportional to the height of bars on the modified guanine. White and black bars indicate alkylation in the absence and presence of TBP, respectively.

B. 3. Only the T:T Mismatch on the Downstream Side of the TBP−TATA Box Complex Cooperatively Enhances Altromycin B Reactivity in the Presence of TBP. The binding of TBP to the mutant promoters induces differential effects on the altromycin B alkylation of guanines, whose reactivities are already increased by the introduced T:T mismatch. After binding of TBP, the upstream and downstream guanines in the top strand of the mutant promoters both show less enhancement of drug alkylation than in the unbound promoters (black bars of Um and Dm in Figure 4B). However, quite uniquely, drug modification on the bottom strand guanine in the downstream mutant promoter is further enhanced by TBP binding. One possible explanation for this cooperative increase in drug reactivity is that the TBP-induced DNA distortion on the mutant promoter readjusts the dynamic mismatch DNA region to a more favorable DNA conformation for altromycin B reaction, i.e., creates a more favored site for the transition state in the covalent reaction. Alternatively, a second possibility that we favor is that the dynamic conformation of the introduced mismatch might participate in the further stabilization of the "specific binding mode" of the TBP−TATA box complex by facilitating the TBP-induced DNA distortion, which results in an additive enhancement of drug reactivity.[4]

Discussion

Determination of the effect of DNA mismatches on altromycin B DNA alkylation reactivity reveals that the destabilizing base pairing of py:py mismatches to the 5′-side of the alkylation site produces enhanced alkylation on the adjacent 3′ guanine. The greatest enhanced drug reactivity is produced by a T:T mismatch positioned on the 5′-side of the target guanine. We propose that this is due to the additive effects of (1) the destabilized unpaired base pairs, which facilitate the intercalation of the drug, and (2) the availability of specific hydrogen bonding from O2 of thymine with the amino sugar of the drug located in the minor groove, which may further stabilize the precovalent binding form or favor the transition state for the covalent reaction. The enhanced pluramycin modification site found downstream of the TATA box in the presence of TBP may have some similarities to the local destabilizing effect on base pairing produced by a T:T mismatch duplex. This commonality in molecular recognition of pluramycin reactivity is strengthened by the observation that only the T:T mismatch located on the downstream side of the TATA box cooperatively enhances the downstream-specific altromycin B reactivity upon TBP binding.

Perturbations in DNA structure, either transient melting of several base pairs or less destructive unstacking and buckling without base pair disruption, are necessary for intercalator binding.[14] DNA structures that readily undergo such a deformation are susceptible to intercalating ligand binding. Many examples of DNA bulges or base mismatches affecting ligand binding have been reported with planar intercalating compounds such as ethidium bromide, neocarzinostatin, bleomycin, methidiumpropyl-EDTA, 9-aminoacridine, dynemicin, nogalamycin, and $[Rh(bpy)_2(chrysi)]^{3+}$ (chrysi = 5,6-chrysenequinone diimine).[15] Similarly, altromycin B undergoes preferential recognition of base mismatches, which destabilize DNA structure so that the drug has improved access to the intercalation site at smaller energy cost for helix expansion. However, a simply expanded DNA structure produced by the mismatch is not the complete explanation for the elevated altromycin B reactivity. Because the pluramycins both intercalate into and then subsequently alkylate duplex DNA, a DNA structure that complements complicated interactions with the drug is required for enhanced alkylation of guanine. For productive DNA alkylation of N7 of guanine by the pluramycins, the reactive epoxide of the drug needs to be aligned and brought into proximity to N7 of guanine for alkylation to occur.[2] The interactions of the sugar substituents of the drug with the DNA base are proposed to play a steering role in positioning the alkylation moiety and consequently to determine the DNA sequence specificity of the pluramycins.[2] Mismatched bases in dynamic motion contain less well defined conformations and allow more varied rotation of the sugar substituents to search for beneficial groove interactions that will stabilize the precovalent binding complex or favor the transition state for covalent reaction. In support of the results of our previous NMR-based modeling study,[3a] it is the 5′-side base substitution that affects altromycin B reactivity (Table 1). For high altromycin B reactivity, it is necessary to have a py:py mismatch perhaps so that the O2 can form a hydrogen bond with the dimethylamino group of the sugar substituent of the drug in the minor groove. We propose that satisfying both the requirements of accessibility and the specific hydrogen bonding interactions, the flexible T:T mismatch duplex DNA affords the optimum conditions for maximum increase of alkylation reactivity of altromycin B.

Because the binding of proteins to duplex DNA often produces severe constraints that destabilize DNA conformations,[16] it is possible that these circumstances might mimic the effects of base mismatches and result in enhanced DNA alkylation. The enhanced pluramycin A reactivity on the downstream flanking sequence of the TBP-bound TATA box is a clear example of increased drug reactivity induced by protein binding to DNA.[4] This particular protein-induced DNA deformation appears to have some similarities in its molecular basis for high pluramycin reactivity to the T:T mismatch duplex. While the X-ray structures of the TBP−TATA box complexes[17] show no obvious structural distortion on the 5′-side of the downstream pluramycin alkylation site, we have proposed a "specific binding mode" that results from the large TBP-imposed distortion of the TATA box, which is asymmetrically distributed by the TATA sequence to the downstream side.[4] Such a distortion produces transient destabilization of the local DNA structure located specifically at the pluramycin intercalation-alkylation site. Since py:py mismatches and, in particular, the T:T mismatch also result in local helix destabilization, it is not too surprising that TBP and the T:T mismatch can result in the cooperative effect demonstrated in this article. NMR studies on the T:T mismatch have reported that the mismatched bases pose interchangeable wobble pairing without causing global

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distortion on duplex DNA.[18] The dynamic and structural parallels between the two deformed and destabilized DNAs (i.e., the TBP−TATA box complex and the T:T mismatch) are reinforced by the altromycin B modification on mutant promoters in which a T:T mismatch is introduced into both flanking sequences of the TATA box. Although the base-pair destabilizing effects of both the upstream and downstream T:T mismatches produce a high altromycin B reactivity site in the absence of TBP, *only the downstream mismatch* cooperatively enhances the drug reactivity on the TBP-bound promoter. This additive effect of the T:T base mismatch on the downstream-specific enhancement of drug reactivity suggests that the downstream mismatch mimics and reinforces the "specific binding mode" of the binary complex between TBP and the TATA sequence, which results in a *cooperative* enhancement of the drug modification.

Enhanced Selectivity of DNA-Reactive Drugs by Targeting Protein−DNA Complexes. One of us has argued previously that the specificity and selectivity of small molecular weight ligands that react with DNA is really achieved as a result of targeting protein−DNA complexes.[5] We have recently documented a clear case of a topoisomerase II-induced site-directed alkylation of DNA by psorospermin,[19,20] and it is likely that therapeutically useful drugs such as adriamycin, camptothecin, *m*-AMSA, the antibacterial quinolones, and antineoplastic quinobenzoxazines also achieve their selectivity by targeting topoisomerase I− or II−DNA complexes.[5b] However, in all of these previously documented cases, the drug-binding or -reactive site is within the confines of the protein−DNA complex. In the case described here, the protein-induced reactivity site is *outside* the range of the direct contacts between the protein (TBP) and DNA (TATA box). Here we argue for the role of a protein-induced transient unwound site with dynamic features that mimic a T:T mismatch site. Altromycin recognizes both of these sites with enhanced reactivity and superimposition of the two results in a cooperative effect. While the TBP−TATA box may be too ubiquitous to be a clinically relevant example, other DNA binding proteins that are overexpressed in cancer cells that induce dynamic alterations in DNA (i.e., topoisomerases) may be useful targets.

Materials and Methods

Chemicals and proteins. Altromycin B was kindly provided by Abbott Laboratories, Chicago, IL. Electrophoretic reagents (acrylamide, bisacrylamide, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine, Tris, and boric acid) were purchased from J. T. Baker, TN. T_4 polynucleotide kinase, [γ-^{32}P] ATP, and X-ray film were purchased from Amersham. TBP (isoform 2) from *Arabidopsis thaliana* was a generous gift from Stephen K. Burley, Rockefeller University, NY. The oligonucleotide synthesis, purification, radioactive labeling, and duplex formation were carried out according to the standard methodology.

A series of 66-mer duplexes was prepared by the combinational annealing of two top strands (T1−T2) with three bottom strands (B1−B3). The DNA sequence of the top strand is 5′-TTATTATGTTATTATC**O**GATTATTATC**P**GATTATTATC**Q**GATTATTAT-C**R**GATTATTACATTATTC-3′ (for T1, **O** = G, **P** = C, **Q** = A, and **R** = T; for T2, **O** = A, **P** = T, **Q** = C, and **R** = G), and the sequence of the bottom strand is 3′-AATAATACA/ATAATAG**W**CTAATAATAG**X**-CTAATAATAG**Y**CTAATAATAG**Z**CTAATAATGTAATAAG-5′ (for B1, **W** = C, **X** = G, **Y** = T, and **Z** = A; for B2, **W** = T, **X** = A, **Y** = G, and **Z** = C; for B3, **W** = G, **X** = C, **Y** = A, and **Z** = T).

Altromycin B Modification on Duplex DNA and Determination of the DNA Alkylation. Between 1 and 10 ng of radioactive DNA samples were modified with the indicated amount of drug in a total volume of 10 μL for 4 h at room temperature. For the determination of DNA alkylation on the specific sites of DNA, a strand breakage assay was performed at 95 °C for 20 min with or without 1 M piperidine.[1] Strand breakage products were separated on a 10% or 16% denaturing sequencing gel with Maxam−Gilbert sequencing marker lanes.

Altromycin B Modification on TBP−TATA Box Complexes. A 5′-end radiolabeled 64-mer duplex DNA containing a single TATA box was used for the binding reaction.[4] The TATA oligonucleotide (6 ng) was incubated with 32 ng of TBP in 10 μL binding buffer (25 mM Tris-HCl, 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.01% NP-40, and 10% glycerol, pH 8.0) at 37 °C for 30 min. After the addition of 1 μL of 100 μM drug solution, reactions were incubated for 5 min, mixed with 3 ng of unlabeled competitor DNA, and loaded onto a 5% native gel to separate the TBP-bound DNA from unbound DNA. The gel was exposed to X-ray film, and DNA samples showing individual bands were eluted with an elution buffer (0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$, 1 mM EDTA, 0.1% SDS, pH 8.0). Samples were further purified by organic solvent extraction and ethanol precipitation and finally subjected to a strand breakage assay in 0.2 M piperidine. Dried samples were separated on a 10% sequencing gel. The intensity of DNA cleavage was quantitated on a PhosphorImager (Molecular Dynamics) and normalized with an internal standard site whose drug alkylation is not affected by the protein binding.[21]

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