Articles

Effects of Minor Groove Binding Drugs on the Interaction of TATA Box Binding Protein and TFIIA with DNA†

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ABSTRACT: TBP (TATA box binding protein), a general transcription factor required for proper initiation of gene expression by RNA polymerase II, and minor groove binding drugs (MGBs) both interact with DNA within the minor groove at AT sites. This study has evaluated MGBs as inhibitors of DNA/TBP complex formation by gel mobility shift assays. Our results demonstrate that reversible MGBs (DAPI, distamycin A, Hoechst 33258, and netropsin) are effective inhibitors of the formation of DNA/TBP complex and that distamycin A is the most potent (0.16 μM inhibits TBP complex formation by 50%). CC-1065, a drug that covalently binds to DNA in the minor groove, is even more active than distamycin A (0.00085 μM inhibits TBP complex formation by 50%). Significantly more CC-1065 (0.009 μM) is required to break up preformed DNA/TBP complex compared to the drug concentration needed to prevent complex formation. In comparison, the order of drug addition has little influence on the ability of reversible MGBs to disrupt DNA/TBP complex. In the presence of TFIIA, a factor that enhances TBP association with DNA, greater drug concentrations (distamycin A and CC-1065, respectively) are needed to disrupt a preformed complex of DNA/TBP/TFIIA. In comparison to MGBs, drugs capable of binding to DNA by intercalation are generally weaker at blocking TBP complex formation except for hedamycin, which can intercalate and irreversibly bind to DNA and is as effective as reversible MGBs.

Drugs that interact with DNA in the minor groove have been shown to interfere with DNA processing enzymes such as DNA polymerase, RNA polymerase, and topoisomerases I and II (Bruzik et al., 1987; Straney & Crothers, 1987; Woynarowski et al., 1988; McHugh et al., 1989; Mortensen et al., 1990; Beerman et al., 1991; Strol et al., 1993a). In some cases, interference is associated with the drug and enzyme sharing a common sequence preference for binding to DNA. Recently, minor groove binding drugs (MGBs) have been studied to determine whether they influence the regulation of gene expression as a result of affecting the binding of regulatory proteins to their consensus binding sites. For example, distamycin A affected the binding of ubiquitous octamer binding protein (OTF-1) and the erythroid-specific GATAAG protein (NFE-1) to their unique DNA elements (Broggini et al., 1989). It has also been reported that distamycin A was able to inhibit the *Antennapedia* homeodomain protein (*Antp* HD) that binds to DNA at a 5′-ATTA-3′ motif (Affolter et al., 1990; Dorn et al., 1992). Studies of mithramycin indicated that the drug obstructed the binding activity of the regulatory protein Sp1 to GC box sequences on the SV40 early promoter and prevented transcriptional initiation from the c-myc P1 and P2 promoters (Ray et al., 1989; Snyder et al., 1991). In these cases with major groove regulatory proteins, it is suggested that the interference caused by minor groove binding drugs with DNA/protein interaction results in a DNA conformational change rather than a direct impediment due to the presence of drugs at the binding sites.

The general transcription factor TATA box binding protein (TBP) has been well documented to interact with DNA in the minor groove, preferentially at AT regions (Lee et al., 1991; Starr & Hawley, 1991). The binding of TBP to a promoter located upstream from the transcription start site is essential for the accurate and efficient initiation of transcription from many class II promoter sequences [reviewed in Drapkin et al. (1993)]. TBP and associated factors (TAFs) bind to the TATA box followed by other factors (e.g., TFIIA, IIB, IIE/IIF) assembling in an ordered manner to form a preinitiation complex for transcription by RNA polymerase II (Saltzman & Weinmann, 1989; Maldonado et al., 1990; Zhou et al., 1993). A crystallographic study shows that TBP forms a unique saddlelike shape that is distinct from the binding motif of other regulatory proteins (e.g., homeodomain, basic leucine-zipper, zinc-finger, etc.) (Mitchell & Tjian, 1989; Nikolov et al., 1992; Peterson & Baichwal, 1993).

The DNA features recognized by TBP indicate that MGBs are likely to be effective inhibitors of TBP binding. Distamycin A and netropsin are two prototypical MGBs that have been extensively characterized (Waring, 1981; Van Dyke & Dervan, 1983; Kopka et al., 1985; Portugal & Waring, 1988). With a crescent-shaped structure composed of planar aromatic groups joined by amide bonds, both distamycin A and netropsin bind deeply within the minor groove, specifically at AT-rich sequences. Hydrogen bonds, van der Waals interactions, and electrostatic interactions all contribute to DNA/drug complex

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stability without intercalating between DNA base pairs. Because of such a specific, sequence-dependent binding activity, these drugs interfere with a wide range of biological effectors (Bruzik et al., 1987; Straney & Crothers, 1987; Kas et al., 1993; Strol et al., 1993b).

CC-1065 represents a class of minor groove binding drugs (cyclopropylpyrroloindole, CPI) which form covalent adducts with DNA bases (Hurley et al., 1984, 1988; Reynolds et al., 1986). Originally isolated from *Streptomyces zelensis*, CC-1065 contains two identical benzodipyrrole units and an indolequinone system bearing a reactive cyclopropane ring (Hanka et al., 1978; Reynolds et al., 1986). Studies of the site and mechanism of binding of CC-1065 to DNA have shown that this drug also binds to AT-rich sites throughout the DNA minor groove (Li et al., 1982; Swenson et al., 1982), but unlike distamycin A and netropsin, CC-1065 is able to form a covalent adduct on N3 of adenine forming an essentially irreversible DNA/drug complex (Warpehoski et al., 1992). Cytotoxicity tests and antitumor studies *in vitro* have indicated that CC-1065 is one of most potent antitumor drugs ever found (Martin et al., 1978; Bhuyan et al., 1982; Li et al., 1982). Adozelesin (U-73 975), a synthetic analog of CC-1065, also shows strong antitumor activity and is under evaluation as a new type of clinical antitumor drug (Weiland & Dooley, 1991).

The sequence preference shared between TBP and MGBs as well as the similarity in the mode of interaction with DNA suggests that these drugs may strongly affect the biological interaction of TBP to DNA by obstructing complex formation. In the present paper, different types of MGBs are examined for their abilities to interfere with the binding of TBP to DNA. The effect of intercalating agents on TBP complex formation is also evaluated.

MATERIALS AND METHODS

Drugs. Distamycin A and DAPI (4′,6-diamidino-2-phenylindole) were purchased from Sigma Chemical Co. (St. Louis, MO). Hoechst 33258 (bisbenzimide) was obtained from Aldrich Chemical Co. (Milwaukee, WI), and netropsin was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). These four drugs were prepared in distilled water and stored at −20 °C. CPI compounds (CC-1065 and adozelesin), kindly provided by Upjohn Co. (Kalamazoo, MI), were diluted in dimethyl sulfoxide (DMSO) as stock solutions and stored at −20 °C. mAMSA (Warner Lambert Pharmaceutical Co., Morris Plains, NJ) was made in DMSO. Ethidium bromide (Sigma Chemical Co.) was reconstituted in sterile distilled water in an amber bottle. Hedamycin was obtained from the National Cancer Institute and was solubilized in 0.1 N HCl.

Oligonucleotides. Three oligonucleotides and their complementary strands were prepared by phosphoamidite synthesis. The 24mer 5′-GAAGGGGGGCTATAAAAGGGGGTG-3′, positioned at −18 to −41 of the adenovirus major late promoter (AdML promoter; wt TATA fragment), was used as a DNA target throughout the study (RPCI, Buffalo, NY). A DNA sequence modified within the TATA region (Horikoshi et al., 1989), 5′-TAGAGAA-3′, was used as a TATA mutant which should not bind TBP (mt TATA fragment; National Bioscience Inc., Plymouth, MN). A 25mer, 5′-TCGACGCCCTCGCCCCCGCGCCGGG-3′, served as a nonspecific DNA control (RPCI).

Preparation of Double-Stranded DNA Fragment. For preparing radioisotope-labeled, double-stranded DNA fragments (ds-DNA), chemically synthesized single-stranded DNA oligonucleotides were first purified by separation over a Poly-pak column (Glen Research, Sterling, VA). Oligonucleotides were annealed as described (Lee et al., 1991). Briefly, equal molar amounts of purified single-stranded oligonucleotides were mixed with their complementary strand in TE buffer (10 mM Tris-HCl with 1 mM EDTA, pH 8.0). Subsequently, the mixtures were heated at 90 °C for 3 min followed by slow-cooling to room temperature. End-labeling with [γ-^{32}P]ATP using T_4 polynucleotide kinase was performed according to the manufacturer's instructions (New England BioLabs, Beverly, MA), followed by PCI extraction. Labeled double-stranded oligonucleotides were further purified by passage through a Sephadex G-25 column (Boehringer-Mannheim Co.) and dilution in TE buffer.

Purified Transcription Factors. Human TBP was produced and purified from *Escherichia coli* using nickel-chelate affinity chromatography (Hochuli et al., 1987) under conditions recommended by the manufacturer (Quiagen Inc., Chatsworth, CA). The plasmid pDS56-hTBP was a gift from T. Kerpolla and T. Curran (Roche Institute of Molecular Biology) and expressed human TBP fused to six histidine residues at the NH_2-terminus of the protein. Following nickel-chelate affinity chromatography in the presence of 6 M guanidine hydrochloride, the purified, denatured TBP was slowly refolded via stepwise dialysis. The eluate from the nickel column (~2.5 mL) was first dialyzed in 4-L volumes of a 2× buffer consisting 25 mM MES, pH 6.5, 5% glycerol, 1 mM DTT, and either 1 or 0.1 M guanidine hydrochloride. The final dialysis consisted of 4 L of the above buffer lacking guanidine. Soluble protein yields averaged 400–500 μg/L of bacterial culture. Purity was assessed by SDS–PAGE/Coomassie blue staining and was generally >98% (Figure 1A). The yTFIIA (produced and purified from *E. coli*) was a kind gift of M. Schmidt (University of Pittsburgh Medical School), and the purity was >90% as judged by Coomassie blue staining (unpublished data, F.J.R.).

Gel Mobility Shift Assay. A modification of the gel mobility shift assay was performed as described (Peterson et al., 1990). In general, 5 ng of TBP was incubated with 2 nM labeled DNA fragment at 30 °C for 30 min in a reaction buffer consisting of 20 mM Hepes–KOH, pH 7.9, 25 mM KCl, 10% glycerol, 0.025% NP-40, 100 μg/mL bovine serum albumin (BSA), 0.5 mM dithiothreitol (DTT), 0.8 mM spermidine, 0.1 mM EDTA, and 2 mM $MgCl_2$ in a final volume of 10 μL. The mixtures were separated by electrophoresis in a 4% native polyacrylamide gel in 0.5× TBE buffer (45 mM Tris-HCl, 44 mM boric acid, and 10 mM EDTA) run at 240 V for 30 min, at room temperature. The gel was fixed in 10% acetic acid for 5 min and then dried and exposed to Kodak XRP-5 film. The intensities of radioisotope-labeled DNA/TBP complex or free DNA were measured using a Molecular Dynamics Densitometer (Molecular Dynamics, Sunnyvale, CA). Fifty percent inhibition of DNA/TBP complex formation (IC_{50}) was measured by comparing drug-treated samples with the corresponding control.

Drug Assays. Two types of drug assays were utilized. For a standard assay (drug treatment of the oligonucleotide precedes addition of the protein), four minor groove binding drugs (DAPI, distamycin A, Hoechst 33258, and netropsin) and CPI compounds (CC-1065 and adozelesin) as well as intercalating agents (mAMSA, ethidium bromide, and hedamycin) were used. A 2 nM labeled ds-DNA fragment was treated with the indicated drug concentration in a reaction buffer at 30 °C for 30 min. Then, 5 ng of TBP was added to the reaction for an additional 30-min incubation. In the

reverse assay, drug treatment of the oligonucleotide follows addition of the protein. A 2 nM labeled DNA fragment was incubated with 5 ng of TBP at 30 °C for 30 min followed by drug treatment (distamycin A or CC-1065). In the case of TFIIA supplement, mixtures of 5 ng of TBP and 0.4 ng of TFIIA were incubated with 2 nM labeled DNA fragment prior to or following drug addition. All samples were analyzed on a 4% native polyacrylamide gel, and the final results were analyzed as described for the mobility shift assay.

Detection of DNA Adduct Formation by CC-1065. CC-1065 was diluted in distilled water to 0.001, 0.02, 0.5, and 10 μM working stocks. The drugs were incubated with 2 nM labeled DNA fragment in a reaction buffer at 30 °C for 30 min. The mixtures were heated at 90 °C for 30 min following the incubation. Heated samples mixed with an equal volume of 2× formamide loading buffer (90% deionized formamide/0.5% bromophenol blue in TBE buffer) were analyzed by electrophoresis on a 17% denaturing polyacrylamide gel in 1× TBE buffer (90 mM Tris-HCl, 88 mM boric acid, 20 mM EDTA, and 6 M urea). After running at 280 V for 4 h, the gel was exposed to Kodak XRP-5 film. The intensity of radioisotope-labeled, denatured, single-stranded DNA fragment was quantitated by a densitometer.

RESULTS

Gel Mobility Shift Assay. Gel mobility shift assays are a facile way to determine the specificity of protein binding to DNA, and to provide a means of quantitating DNA/protein complex formation. The gel mobility shift assay was used to study in detail how MGBs interfere with the binding of TBP to a selected DNA fragment derived from the AdML promoter containing a TATA element. TBP and MGBs share a similar recognition pattern for DNA; since both bind preferentially in the minor groove at AT sites, it is likely that MGBs would effectively compete with TBP for DNA binding, resulting in a weak or disrupted complex. To evaluate the efficacy of drugs as inhibitors of complex formation, we first set up conditions of a gel shift assay to maximize complex formation using a fixed amount of labeled DNA fragment (2 nM) and fresh aliquots of highly purified, recombinant hTBP (Figure 1A). Our purpose was to determine the lowest amount of TBP that would provide an optimal level of complex. Figure 1B indicates that 5 ng of TBP was able to form the greatest amount of complex; below 5 ng, complex formation was reduced. The temperature for the incubation and electrophoresis conditions were optimized to enhance the stability and activity of DNA/protein binding. Carrier DNA was tested as a means to minimize well retention of the oligonucleotides in the presence of proteins. The specificity of the DNA fragment to compete for TBP binding was also examined. About 10-fold unlabeled DNA fragment (wt TATA fragment) is needed to compete for the binding of TBP while the same amounts (10-fold) of TATA mutant fragment and a nonspecific control DNA fragment do not compete (data not shown).

Inhibition of TBP Binding to DNA by Reversible Minor Groove Binding Drugs. A number of reversible MGBs that occupy variously sized sites on DNA were studied to determine whether their presence on DNA would prevent TBP complex formation. The structure of distamycin A, a typical minor groove binding drug, is shown in Figure 2. The effect of DAPI, distamycin A, Hoechst 33258, and netropsin on DNA binding of TBP was analyzed by gel mobility shift assay. In a standard assay, DNA was treated with the drug followed by TBP addition. A representative gel mobility shift assay in the

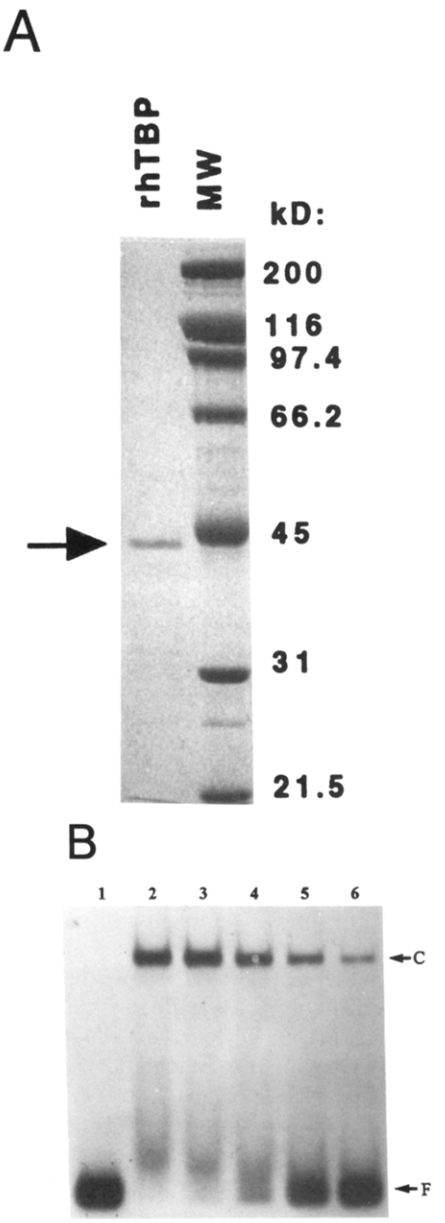
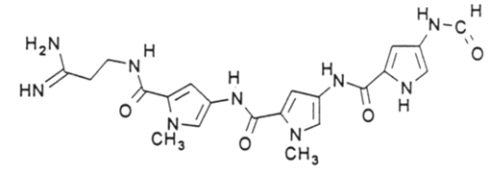


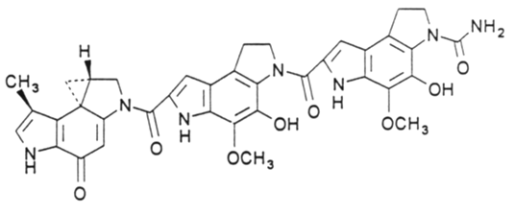
FIGURE 1: (A) SDS–PAGE analysis of purified recombinant human TBP. A ~0.5-μg aliquot of purified TBP was resolved on a 10% SDS–polyacrylamide gel followed by Coomassie blue staining. The molecular mass markers are Broad Range Marker (Bio-Rad Laboratories). The arrow indicates the ~43-kDa rhTBP polypeptide. (B) Determination of the optimal binding activity of TBP on DNA by a gel mobility assay. Two nanomolar ^{32}P-labeled DNA fragment was incubated with varying levels of TBP followed by electrophoresis through a 4% native polyacrylamide gel, and the result was visualized by autoradiography. Lane 1 contains labeled DNA fragment without TBP addition as a DNA control. Lanes 2–6 are labeled DNA fragment incubated with TBP at concentrations of 7.5, 5.0, 2.5, 1.2, and 0.6 ng separately. The letter C indicates DNA/TBP complex (upper bands), and the letter F indicates free DNA (bottom bands).

presence of distamycin A (Figure 3A) demonstrates that DNA/TBP complex formation is completely blocked at 1.2 μM distamycin A, while the first indication of the inhibition of complex formation is seen at only 0.08 μM drug. The amount of distamycin A required to prevent 50% DNA/TBP complex formation, compared to nontreated controls (IC_{50}), is 0.16 μM. The rank order of IC_{50} values for the four drugs tested is distamycin A (0.16 μM), netropsin (0.24 μM), DAPI (0.68 μM), and then Hoechst 33258 (1.2 μM) (Figure 3B). For all four drugs, the profile of the inhibition curve is similar.

Effect of Distamycin A on DNA Pretreated with Proteins. Distamycin A, the most potent inhibitor of TBP complex



Distamycin A



CC-1065

FIGURE 2: Structures of distamycin A and CC-1065.

formation, was investigated further to determine whether minor groove binding drugs can disrupt the DNA/protein complex. In such a reverse assay, DNA and TBP were incubated prior to distamycin A addition. In Figure 4A, about 0.27 μM distamycin A is able to displace the protein by 50%, which is close to the IC_{50} concentration in the standard assay (0.16 μM). It is possible that the relatively weak interaction of TBP and DNA may explain the displacement of TBP by distamycin A in the reverse assay. In the presence of TFIIA, DNA/TBP complex stability can be significantly enhanced (Meisterernst & Roeder, 1991). Under our assay conditions, TFIIA alone does not bind to the DNA, but does enhance the ability of TBP to form a complex with increased mobility relative to the DNA/TBP complex (Figure 4B). We determined whether a preformed complex of TBP and TFIIA would be more difficult to dissociate by distamycin A. When TBP and TFIIA are added together, about 2.5 times more drug (0.7 μM) is required to reduce the complex formation by 50% than TBP alone (Figure 4A).

Inhibition of DNA/TBP Complex Formation by CC-1065. CC-1065 belongs to the family of covalent minor groove binding drugs which bond to the N3 of adenine (Hurley et al., 1984) (Figure 2). Specifically, bond formation requires a minimum of a three-base AT binding site with an adenine at the 3′ end (Hurley et al., 1990). The AdML promoter contains such a site with the 4 base pair (AAAA) region. In a standard assay, DNA was preincubated with CC-1065 for 30 min to allow the drug to bind to the DNA minor groove and also allow bond formation to occur. For CC-1065, 0.85 nM drug was sufficient to reduce TBP complex by 50%, which makes this drug 200-fold more effective than distamycin A (Figure 5). CC-1065's potent inhibitory effect on complex formation suggests that covalent bonding to DNA contributes greatly to the activity of CC-1065. In contrast, 0.8 μM adozelesin, another drug of the CPI family, is required to reach the same effectiveness as CC-1065, making adozelesin nearly 1000-fold less potent (data not shown).

Influence of the Order of Addition of CC-1065 on Complex Formation. The previous data show that CC-1065 obstructs the TBP complex very efficiently under conditions that allow the drug to covalently bind to the DNA. Would the presence of TBP on DNA before drug treatment prevent bond formation and thus reduce the drug effectiveness to the level of the noncovalently binding MGBs? To answer this question, DNA and TBP were incubated before CC-1065 addition. Under these conditions, 9 nM CC-1065 is needed to reduce the complex by 50%. This is 10-fold more drug than is needed

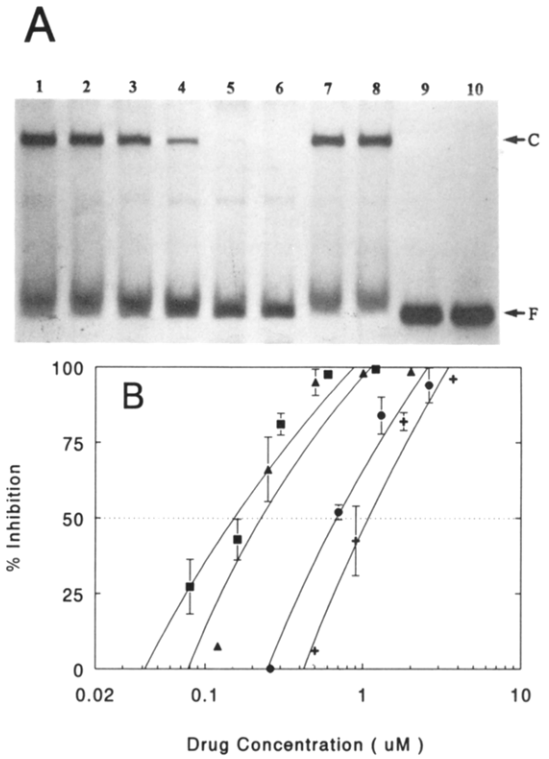


FIGURE 3: (A) Representative gel mobility shift assay determining the ability of distamycin A to block TBP complex formation. Incubation of labeled DNA fragment with the indicated drug concentration at 30 °C for 30 min preceded the addition of TBP. Complexes were separated from free DNA by electrophoresis on a 4% native polyacrylamide gel followed by autoradiography. Lanes 1–6 denote labeled DNA fragments incubated with distamycin A at concentrations of 0.04, 0.08, 0.16, 0.3, 0.6, and 1.2 μM, respectively. Lanes 7 and 8 represent a DNA/TBP complex with no drug treatment. Lanes 9 and 10 are duplicates of DNA controls. The letter C indicates DNA/TBP complex (upper bands), and the letter F indicates free DNA (bottom bands). (B) Inhibition curves representing blockage of TBP complex formation by various reversible MGBs. The effect of reversible MGBs on the binding of TBP to DNA was evaluated as described above with varying concentrations of distamycin A (■), netropsin (▲), DAPI (●), and Hoechst 33258 (+) respectively. The intensity of each respective band after autoradiography was quantitated with a densitometer, and the effect of each drug on the inhibition of DNA/protein complex was represented as a percentage of inhibition by comparing the intensity of drug-treated sample to the control. The (···) symbol denotes the level of drug required to inhibit complex formation by 50% (IC_{50}). The data represent the mean of at least three experiments (mean values ± SD).

in a standard assay (Figure 6). We have demonstrated that the presence of TFIIA diminishes the ability of distamycin A to block the interaction of DNA and TBP (Figure 4A). Whether CC-1065 would be similarly affected has also been studied. The reaction of CC-1065-treated DNA with TBP, in the presence or absence of TFIIA, shows that a very similar drug concentration (1.0 and 0.85 nM, respectively) is required for CC-1065 to impede complex formation by 50%. When TBP and TFIIA are added to DNA prior to CC-1065, a much greater increase in drug concentration (even at 200 nM drug, only 25% decrease in complex was observed) is needed to disrupt the complex. Our results demonstrate that access to the DNA is more critical for covalent linkage of a minor groove binding agent than for a drug (e.g., distamycin A) which simply binds electrostatically by hydrogen bonds within the minor groove.

Detection of Adduct Formation on DNA by CC-1065. It has been reported that the DNA sequence 5′-AAAAA is the preferential target for CC-1065 bond formation (Hurley et al., 1984; Reynolds et al., 1985). We wished to verify that

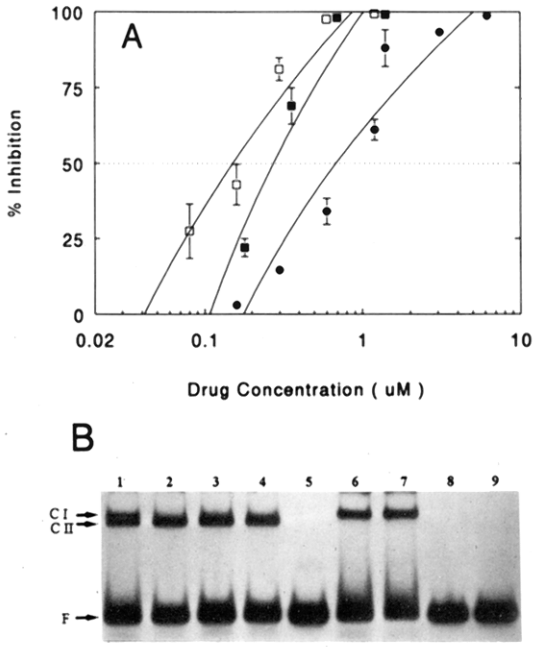


FIGURE 4: (A) Detection of the effect of distamycin A on DNA pretreated with general transcription factors. Labeled DNA fragment and TBP (■) or mixtures of TBP and TFIIA (●) were incubated prior to drug addition. The percentage of inhibition of complex formation was measured by following the electrophoresis as described in Figure 3. The (□) symbol is the result of a standard assay where labeled DNA was treated with distamycin A in advance of TBP, and the (···) symbol refers to the IC_{50}. Results are the mean of at least two experiments (mean values ● SD). (B) Demonstration of the binding activity of TBP to DNA in the presence of TFIIA. TBP and TFIIA in a series of dilution were incubated with labeled DNA fragment, and reaction patterns were determined by a gel mobility shift assay. Lanes 1–4 refer to labeled DNA fragment with mixtures of 5 ng of TBP and TFIIA at concentrations of 0.1, 0.2, 0.4, and 0.6 ng, respectively. Lane 5 contains the mixture of labeled DNA fragment and 0.4 ng of TFIIA. Lanes 6 and 7 are duplicates of the complex with TBP alone. Lanes 8 and 9 indicate DNA control. C I (top bands) and C II (lower bands) indicate DNA/TBP and DNA/TBP/TFIIA complexes, respectively, and the letter F indicates free DNA (bottom bands).

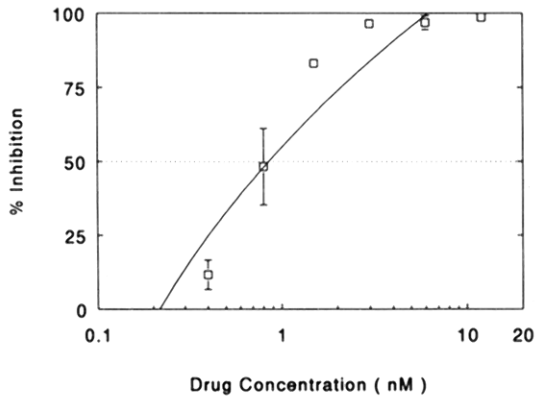


FIGURE 5: Inhibition of TBP complex formation by CC-1065. The effect of CC-1065 on TBP binding to DNA was evaluated by interaction of labeled DNA fragment with the indicated drug concentration of CC-1065 (□) prior to TBP addition. Electrophoresis and measurement of the percentage of inhibition of complex formation were performed as described in Figure 3. The (···) symbol refers to IC_{50}. The data represent the mean of at least three replicates (mean values ± SD).

CC-1065 was able to covalently bind to the AdML DNA

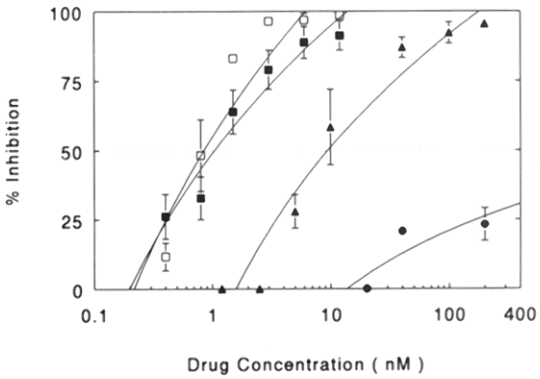


FIGURE 6: Effect of CC-1065 on the binding of mixtures of TBP and TFIIA to DNA. The ability of CC-1065 to prevent TBP complex formation in the presence of TFIIA was tested. CC-1065 at the indicated drug concentration and labeled DNA fragment were incubated before addition of TBP (□) or of a mixture of TBP and TFIIA (■). Reversibly, TBP (▲) or a mixture of TBP and TFIIA (●) was incubated with labeled DNA fragment for 30 min at 30 °C followed by the addition of CC-1065. Gel mobility shift assays were carried out, and inhibition of complex formation by CC-1065 was determined as described in Figure 3. The (···) symbol refers to IC_{50}. The data are the mean of at least two experiments (mean values ± SD).

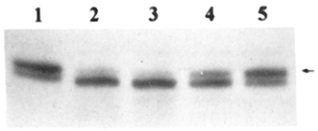


FIGURE 7: Detection of adduct formation on DNA by CC-1065. A direct examination of alkylating bond formation on DNA with CC-1065 carried out. Labeled DNA fragment was incubated with each drug at the indicated concentration followed by heating at 90 °C for 30 min to induce strand breaks at drug bonding sites. Samples were analyzed on a 17% denatured urea–polyacrylamide gel. The separated single-stranded DNA fragments (upper and lower bands) were visualized after autoradiography. Lane 1 refer to non-drug-treated DNA. Lanes 2–5 contain labeled DNA fragment treated with CC-1065 at concentrations of 1000, 50, 2, and 0.1 nM, respectively. The upper band is indicated by the arrow.

DNA since such adducts are heat-labile, and thus result in DNA breakage upon heat treatment (Hurley et al., 1984). CC-1065-mediated adduct formation was examined. In Figure 7, two separate bands correspond to the two complementary DNA strands (upper and lower bands in the control). The intensity of the upper band decreased when the DNA fragment was pretreated with CC-1065 at concentrations of 1 μM and 50 nM, respectively (lanes 2 and 3, which completely block TBP complex formation). At 2 nM CC-1065, which partially blocks complex formation in a gel mobility shift assay, there is a comparable loss in the intensity of the upper band (lane 4). At a drug concentration (0.1 nM) that does not affect complex formation, there is no loss in the intensity of the upper band, suggesting that the drug is not covalently attached to the AdML sequence (lane 5).

Effect of Intercalating Agents on TBP Complex Formation. The drugs studied to this point, MGBs and CC-1065, bind to DNA through the minor groove. Another type of DNA binding agent that may disrupt complex formation would be drugs capable of binding DNA by intercalation. With this type of agent, interference with complex formation would likely be caused by a general decomformation of the helix due to intercalation rather than a direct competition for TBP

Table 1

	[drug] (μM)[a]
minor groove binding drug	
distamycin A	0.16
netropsin	0.24
DAPI	0.68
Hoechst 33258	1.20
CC-1065	0.00085
intercalating agent	
hedamycin	0.25
ethidium bromide	6.8
mAMSA	>50

[a] Concentration required for each drug to inhibit 50% of the DNA/TBP complex is the mean of three replicates of standard assays.

addition to intercalating, hedamycin may covalently bond to N7 of guanine (Sun et al., 1993). The results (Table 1) show that 0.25 μM hedamycin reduced complex formation by 50%. This is comparable to a number of the reversible MGBs. In contrast, ethidium bromide inhibits the complex much less effectively than hedamycin and is also less effective than MGBs which react with DNA reversibly. mAMSA (>50 μM for IC_{50}) is the weakest drug shown in Table 1 for inhibition of TBP complex formation.

DISCUSSION

The purpose of this study was to evaluate how treatment by DNA-interactive drugs affects the binding of the general transcription factor TBP to its consensus sequence derived from the AdML promoter. Three different categories of drugs, reversible MGBs, CPI drugs, and intercalating agents, were tested. These drugs differed in their sequence preferences, their mode of interaction with DNA, and their ability to bond to DNA. Experiments were performed by adding drugs to reactions in advance, or by following DNA/protein complex formation (standard and reverse assays). The role of TFIIA in the binding activity of TBP to DNA, in the presence of drug, was also examined.

Reversible MGBs interact with DNA by forming hydrogen bonds with the O2 of thymine as well as the N3 of adenine in the minor groove (Kopka et al., 1985; Pjura et al., 1987; Larsen et al., 1989). Still, there is some variation in how they bind DNA. Distamycin A and netropsin, which recognize five base pairs of DNA, are the stronger inhibitors in the reduction of DNA/TBP complex formation, followed by DAPI and then Hoechst 33258 which react only with four and three base pairs, respectively (Figure 3B) (Portugal & Waring, 1988). A similar drug inhibition pattern was found in a previous study for these reversible MGBs showing that the catalytic activity of topoisomerases I and II was diminished, distamycin A being the most potent drug (Woynarowski et al., 1988; McHugh et al., 1989). Inhibition is suggested to be a consequence of drug competition with TBP for a common site within the DNA minor groove.

Previous studies have demonstrated that distamycin A interfered with the interaction of certain regulatory proteins that bind to the DNA major groove, although higher levels of distamycin A were required compared to TBP. For example, preincubation of DNA with ~2 μM distamycin A prevented binding of purified *Antp* HD homeodomain protein binding to AT-rich sequences (Dorn et al., 1992). Another study has demonstrated that even greater drug concentrations (25–200 μM) of distamycin A were required to inhibit completely complex formation by OTF-1 and NFE-1 (in this study, cellular extracts were used rather than purified proteins) to their respective DNA elements (Broggini et al., 1989).

When the DNA fragment was exposed to TBP prior to treatment with distamycin A, the drug concentration needed to inhibit complex formation by 50% is close to the level needed in the standard assay (Figure 4A). In contrast, reverse assays performed with the Antp HD homeodomain protein required higher distamycin A concentrations (3–12 μM) to reduce complex formation (Dorn et al., 1992). Broggini et al. (1989) have reported that pretreatment of DNA with distamycin A was an absolute requirement for blocking protein interaction. The fact that we find little difference in the drug requirement to prevent TBP binding in both standard and reverse assays suggests that inhibition of a minor groove binding protein by a minor groove binding drug can occur in a direct manner, and thus may account for stronger drug action compared to major groove binders.

In contrast to reversible MGBs, the CPI agent CC-1065 not only binds within the minor groove but also binds to DNA covalently. The sequence 5′-AAAAA has been defined as a preferential CC-1065 bonding site (Hurley et al., 1984; Reynolds et al., 1985). The selected DNA fragment contains a TATA element (5′-TATAAAA) which should present several potential binding sites for CC-1065. The fact that 0.00085 μM CC-1065 is sufficient to diminish complex formation by 50% (200-fold more effective than distamycin A) in a standard assay suggests that covalent binding within the TATA region effectively blocks TBP complex formation. However, a dramatic change in drug requirement (about 10-fold) is seen if TBP is present prior to treatment with CC-1065 (Figure 6). Apparently, access to DNA is important for CC-1065 binding. While data from our laboratory suggest that CC-1065 can covalently bind to DNA and chromatin with nearly equal efficiency (McHugh et al., personal communication), it appears that protein binding within the minor groove can serve as a significant barrier to adduct formation. In contrast, a minor groove binding drug like distamycin A is not influenced by the order of protein addition. Preincubation of DNA with TFIIA and TBP, followed by drug addition of either CC-1065 or distamycin A, requires a greater drug concentration to prevent complex formation: 10- and 2.5-fold, respectively. In this respect, the inhibition profile of a reversible MGB drug and CC-1065 is similar.

Adozelesin (U-73 975), a structural analog of CC-1065, was also tested for its ability to block TBP complex formation. An optimal consensus sequence, 5′-(T/A)-T/A-T-A*-(C/G)-(G)-3′, has been identified for adozelesin, suggesting that, like CC-1065, the 5′-TATAAAA-3′ sequence would be a binding site (Weiland & Dooley, 1991). An unexpected 3 orders of magnitude more adozelesin (0.8 μM) was needed for preventing 50% of the complex formation compared to CC-1065 (data not shown). A contributing factor to this difference could be sequence specificity since a previous study demonstrated that adenine is required for bond interaction of adozelesin and 5′-T/A and 3′-G/C nucleotides are necessary when the drug forms adducts (Weiland & Dooley, 1991). While a longer incubation time (4 h) did improve adozelesin activity, other factors in addition to site preference must be playing a role to account for the nearly 1000-fold difference in activity between the two CPI drugs.

To relate CPI drug activities to their potential to bond the TATA region of the AdML promoter, a direct examination of covalent bond sites was performed. A heat-labile lesion is generated when CPI drugs bond N3 of adenine to form an adduct and can be detected by heating drug-treated DNA to induce strand breakage (Hurley et al., 1984). The band corresponding to the single-stranded DNA fragment contain-

ing the 5′-TATAAAA-3′ (upper band) sequence disappears after heating at 90 °C with doses of CC-1065 which block TBP complex formation. In comparison, little or no change in the DNA fragment is detected after similar treatment with adozelesin (data not shown). These results suggest that the activity of CPI drugs in inhibition of DNA/TBP complex formation parallels their ability to form covalent linkages on the DNA.

The ability of reversible MGBs and CPI drugs to inhibit TBP complex formation appears to depend upon interaction between the drug and the DNA. Whether drugs that induce conformational change in DNA would be effective inhibitors of TBP complex formation was addressed by utilizing a group of intercalating agents (mAMSA, ethidium bromide, and hedamycin) which bind DNA between adjacent base pairs and unwind DNA (Waring, 1976, 1981; Reinhardt & Krugh, 1978; Wilson et al., 1981; Bennett, 1982). Our results from standard gel mobility shift assays reveal that hedamycin is the most effective of the three drugs in inhibition of complex formation (0.25 μM for IC_{50}) and also is the only intercalating drug to act in the range of the MGBs (Table 1). In a recent study of altromycin B, a hedamycin analog, the drug was observed to covalently bind to DNA at N7 of guanine (Sun et al., 1993). By analogy, hedamycin bonding to DNA may be important to its ability to inhibit TBP complex formation. These results indicate that alteration of DNA structure induced by hedamycin is stronger than classic intercalating agents (e.g., ethidium bromide and mAMSA required 6.8 and >50 μM, respectively for IC_{50}). The relatively weak inhibition by the latter compared to MGBs suggests that DNA conformational change is less important than direct occupation of recognition sites.

In summary, the results demonstrate that both reversible MGBs and CC-1065 interfere with TBP complexes more strongly than classic intercalating agents. Sequence preference, mode of reaction, and bond formation have been identified as three important factors in determining drug inhibition activity on TBP complex formation. This study can be viewed as a model for further drug development in which drugs will be designed to interfere with a specific DNA element that contains binding sites for general or specific regulatory proteins. The next step will be to determine the ability of DNA binding drugs to disrupt transcription factor induction of expression under cell-free and cellular conditions. Ultimately, selective targeting of a transcription factor may provide a means to interfere with the regulatory processes mediated by the factor.

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