Nucleic Acids Research, 1995, Vol. 23, No. 10 **1657–1663**

Distamycin A and tallimustine inhibit TBP binding and basal *in vitro* transcription

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Received March 2, 1995; Revised and Accepted April 5, 1995

ABSTRACT

The antibiotic distamycin A is a DNA minor groove binding drug (MGB) that recognizes a stretch of a least four ATs. The alkylating benzoyl mustard derivative tallimustine (FCE 24517) has powerfull anti-tumor activity. Using the electrophoretic mobility shift assay (EMSA) we determined that both compounds can prevent binding of TBP and, with 10-fold higher concentration, TBP–TFIIA (DA) and TBP–TFIIA–TFIIB (DAB) to a TATA box. Once formed, the DA and DAB complexes are more resistant to MGB challenge. Both drugs can inhibit basal *in vitro* transcription of a minimal TATA-containing promoter and similar concentrations are necessary for binding and transcriptional inhibition. Tallimustine shows strong selectivity by decreasing only correctly initiated transcripts. Even at high doses (20 μM), however, they cannot disturb a competent pre-initiation complex or Pol II progression. This functional *in vitro* model will provide a way to investigate the activity of sequence-specific DNA binding drugs with potential anti-viral and anti-tumour activity and to develop novel more selective compounds.

INTRODUCTION

Distamycin A is a sequence-specific DNA binding compound which recognizes AT-rich stretches by interacting non-covalently with the minor groove of B-DNA and replacing the spine of hydration (1,2).

Co-crystals with selected oligonucleotides identified the details of these interactions (3,4). Sequence selectivity arises from steric hindrance of the exocyclic C2 amino group of the guanine and by van der Walls interactions in the narrower minor groove associated with AT pairs (4). It also distorts DNA by inducing conformational changes in the flanking sequences (3).

Unlike distamycin A, alkylating derivatives have shown powerful anti-tumor activity (6–8). One such minor groove binder (MGB) is tallimustine, a benzoyl nitrogen mustard that has additional sequence-specific requirements for DNA alkylation (8,9). Distamycin A has been shown to interfere with the activity of topoisomerase I (10), topoisomerase II (11) and DNA polymerases (12), as well as the binding of structural proteins such as histone H1 with AT-rich SAR sequences (13) and HMG-1 to satellite DNA (14). The exact mechanisms by which these compounds exert their action *in vivo* are still elusive; in particular it is not clear why tallimustine, but not distamycin A, has anti-tumor activity.

One of the possibilities is that they might regulate gene expression by altering the binding of important regulatory proteins to their natural target sequences. Indeed, recent studies indicate that MGBs are able to inhibit binding of several transcription factors that recognize specific AT-rich sequences, including Gata1, Octa (15), Antennapedia, Ftz (16) and TBP (17). The latter observation is of particular importance, given the pivotal role that TBP has in the mechanisms of transcriptional activation. However, the actual influence of these compounds on the transcription processes has yet to be tested.

The AT-rich stretch known as the TATA box is the most common element in eukaryotic Pol II promoters (18) and plays a key role in regulating the overall level of transcription and in positioning the initiation start site (19,20).

It is usually located between –25 and –30 bp upstream of the initiation start site and appears to bind a single DNA binding protein, TBP, whose gene has been cloned from different species; protein sequence alignments clearly identified the C-terminal 180 amino acids as an extremely conserved domain, encoding the DNA binding part of the protein.

Crystallographic studies of yeast and *Arabidopsis thaliana* TBP, alone or in combination with TATA sequences, have shown a saddle-like structure that binds by making contacts through a concave surface with the minor groove of the DNA (21–24). In addition, TBP is able to significantly bend the double helix towards the major groove by an angle of 80°, resulting in a remarkable widening and flattening of the minor groove (23–24). Biochemical and *in vitro* transcription studies indicate that TBP is associated with other polypeptides as part of the multi-subunit

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TFIID complex (25), which nucleates the initial events in the ordered assembly of the pre-initiation complex, involving other general transcription factors: TFIIA, -IIB, -IIE, -IIF, -IIG, -IIH and Pol II (26,27).

A number of reasons thus lead us to postulate that MGBs could interfere with transcription by inhibiting TBP binding. To test this hypothesis we used the electrophoretic mobility shift assay (EMSA) with recombinant human and yeast TBP, purified TFIIA and recombinant TFIIB for *in vitro* binding studies and a sensitive TBP-dependent *in vitro* transcription system for functional experiments.

MATERIALS AND METHODS

Drugs and protein purifications

Distamycin A was dissolved in DMSO at a 10 mM stock concentration; tallimustine was dissolved in DMF at 5 mM. Stock solutions were kept at –20°C and freshly diluted in H_2O shortly before use.

Recombinant yeast and human TBP (yTBP and hTBP) were produced in *Escherichia coli* from the T7 expression vector and purified as previously described (28); production and purification of recombinant human TFIIB was as previously described (29). TFIIA was purified through heparin Ultrogel, DEAE, Phenyl5PW and HAP.t31 columns (30).

For the experiments shown in Figure 3, hTBP was produced from inclusion bodies, according to the described method (31): inclusion bodies were resuspended in 6 M GnCl and slowly dialyzed at 4°C against NDB buffer (20% glycerol, 20 mM HEPES, pH 7.9, 100 mM KCl, 0.5 mM EDTA, 1 mM dTT) over a period of 4 h. The resulting material was centrifuged in a Eppendorf centrifuge for 10 min and the supernatant stored in liquid nitrogen. Under these conditions TBP was at least 80% pure.

EMSA

Binding reactions were performed with a 37mer containing the AdML TATA box (GAAGGGGGTATAAAAGGGGGTGGGGGCGTTCGTCCT); 0.1 ng end-labeled oligonucleotide was incubated with 1–5 ng (2–10 nM) yTBP or 0.2–0.5 µg hTBP in 8% glycerol, 2% PEG 6000, 10 mM HEPES, pH 7.9, 50 mM KCl, 5 mM ammonium sulfate, 5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM dTT in a final volume of 15 µl. After 30 min at 30°C, samples were loaded on a 5% 0.5× TBE gel containing 2 mM $MgCl_2$, 5% glycerol, 1 mM dTT and run in a 0.5 TBE/2 mM $MgCl_2$ buffer for 3 h. The gels were dried and exposed to Kodak XAR films. When indicated, 0.5 µl partially purified TFIIA and 20 ng recombinant TFIIB were used. EMSA for the DAB complex were performed in acrylamide gels without $MgCl_2$ and dTT.

EMSA experiments in which the SP1 binding oligonucleotide was used (GATCCCCCGCCCC) were performed with 5 µg K562 nuclear extracts in 5% glycerol, 50 mM KCl, 20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 1 mM dTT.

In vitro transcription

The DNA template used for *in vitro* transcriptions was pAL5 (32). Briefly, it contains the rabbit β-globin first exon and promoter sequences up to position –41 (AGGACTTGGGCATAAAAGGCAGAGCAGGGCAGCTGCTGCT +1).

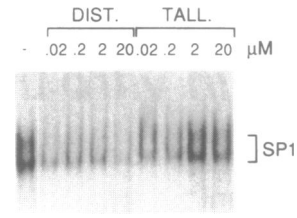

Figure 1. Effect of MGBs on SP1 binding. Increasing concentrations of MGBs were pre-incubated with an SP1 binding oligonucleotide, before addition of K562 nuclear extracts and EMSA analysis. Bands corresponding to specific SP1 complexes are indicated.

The preparation of nuclear extracts from exponentially growing K562 cells, the *in vitro* transcription reactions and RNA purification procedures were performed exactly as previously detailed (33). In all reactions 4 µl extract and 50 ng template DNA were used. S1 mapping analysis was done as described (34), using a single-stranded DNA probe that, upon hybridization with pAL5-transcribed RNA, generates a 60 bp fragment corresponding to the correct +1 β-globin signal and a 70 bp fragment corresponding to read-through (RT) transcripts (see 30–32).

RESULTS

MGBs inhibit TBP–TATA interactions

As a first step to understand a possible role of MGBs in regulating transcription, we set up experiments to verify the specificity of MGB compounds. First we used the electrophoretic mobility shift assay (EMSA) to test whether binding of these drugs was able to inhibit DNA binding proteins that do not recognize AT-rich sequences. For this purpose SP1 was a logical candidate, since it is known to recognize GC-rich sequences (35). Figure 1 shows that the binding of SP1 to DNA is completely unaffected by pretreatment of the target oligonucleotide with dystamicin A or tallimustine, even at high concentrations.

A second control experiment was performed to see whether MGBs would stably bind to recombinant TBP, thus possibly altering normal protein–protein interactions of TBP with natural partners such as TFIIA or with the target TATA sequence. TFIIA is a heteromeric positive regulator of transcription which has no intrinsic capacity to bind DNA (36–38): the TBP–TFIIA–TATA (DA) complex can be visualized in EMSA and was shown to be specific and to contain TBP, both by competition analysis with unlabeled TATA-containing oligonucleotides and by anti-TBP antibody challange (not shown; see 28–30).

We incubated different concentrations of hTBP with distamycin or tallimustine at a high (20 µM) concentration and subsequently, after dialysis to remove the two drugs, with TFIIA and an oligonucleotide containing a high affinity binding site for TBP (39–41): if stable interactions or modifications of TBP occurred, one would expect a severe decrease in formation of the DA complex resulting from the association of TBP with TFIIA and the TATA box. Results shown in Figure 2 show no significant variation in the ability of distamicin A- or tallimustine-treated hTBP to interact with TFIIA and DNA, compared with untreated controls, over three TBP concentrations. Thus there is no direct adverse effect of MGBs on TBP stability, conformation or affinity for DNA.

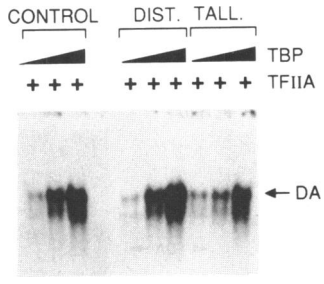

Figure 2. Effect of MGBs on TBP and TFIIA proteins. MGBs were pre-incubated at a 20 μM concentration with increasing amounts of hTBP for 15 min at 30°C and then removed by dialysis at 4°C for 30 min. TBPs were then added to binding reactions containing TFIIA and an AdML TATA oligonucleotide and run on EMSA. The DA complex is indicated.

We therefore started to analyze possible inhibitory effects of the two drugs on binding of *Saccharomyces cerevisiae* and human recombinant TBP to the TATA oligonucleotide employed above: the oligonucleotide is exclusively GC-rich and hence not a target for these drugs, aside from the central TATAAAA (39–41). When incubated with 1–5 ng (2–5 nM) yTBP alone, the labeled TATA oligonucleotide formed a retarded complex (Fig. 3A, lane 1); this band is specific, since it can be competed by an excess of wild-type cold oligonucleotide, but not by an oligonucleotide mutated in the TATA box (data not shown; see 28). It should be noted that under these conditions the free oligonucleotide is always in excess with respect to the recombinant protein, no more than 20% being complexed and shifted by TBP.

When we pre-incubated the labeled oligonucleotide with increasing concentrations (20 nM–20 μM) of distamycin A and the alkylating derivative tallimustine before addition of recombinant yTBP we observed a clear decrease in TBP–TATA complex formation at 2 μM (Fig. 3A, lanes 2–9). In parallel experiments we changed the order of addition, by pre-incubating the recombinant protein with the DNA before addition of the drugs. Results of the latter experiments were very similar (Fig. 3B, lanes 1–9) to the one reported in Figure 3A. Tallimustine appears to be slightly less efficient (2-fold) than distamycin A (compare lanes 4 and 8 in Fig. 3A and B).

We next tested recombinant hTBP in the EMSA under the same experimental conditions. Compared with yTBP, a higher hTBP concentration (100- to 500-fold) was necessary to detect a retarded band (28–30). Inhibition was already evident at 200 nM and was complete at 2 μM (Fig. 3C, lanes 1–10). Essentially identical results were obtained inverting the order of addition, namely pre-incubating the target oligonucleotide with hTBP first (not shown). With respect to yTBP, hTBP was thus more susceptible to MGB interference. Taken together, these experiments indicate that MGB drugs can either inhibit or displace TBP binding from the target sequence, by competing with a common site on the DNA.

MGBs inhibit DA and DAB complex formation

We then performed dose–response inhibition experiments on the DA complex, similar to the ones described before for the TBP–TATA complex. At low concentrations (10 ng) hTBP does not bind DNA in a detectable way (Fig. 4A, lane 1), neither does partially purified TFIIA from HeLa cells (lane 2), however, when hTBP and TFIIA were mixed together the DA complex was

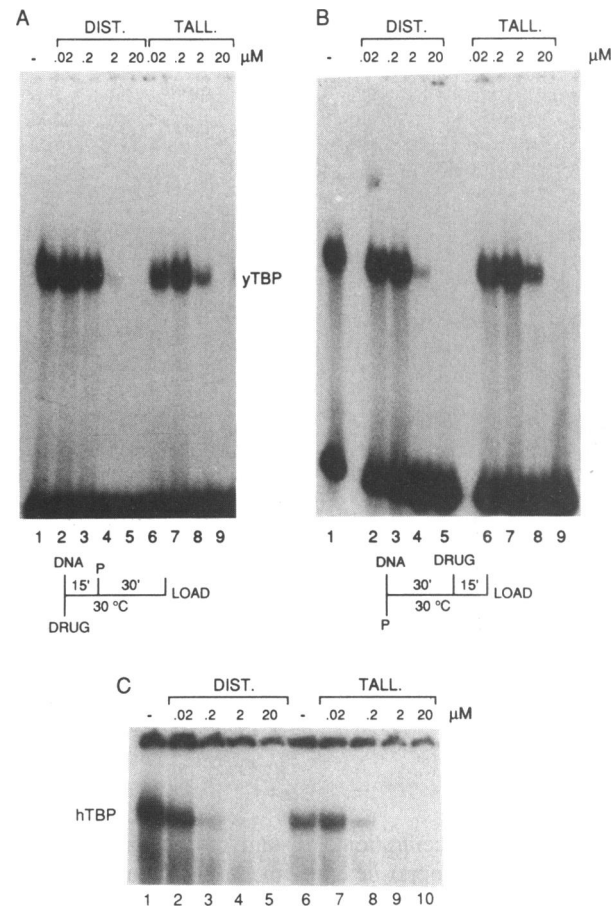

Figure 3. Inhibition of TBP–TATA interaction by distamycin A and tallimustine. (**A**) The AdML oligonucleotide was first incubated with increasing concentrations of the indicated drug and then 2 ng yTBP (labeled P in the scheme) was added and further incubated for 30 min. (**B**) yTBP and the AdML oligonucleotide were incubated before addition of the indicated amount of each drug. (**C**) As (B) except that 500 ng hTBP was used.

visible (lane 3). Another faster migrating complex was apparent on these gels and is due to non-specific interactions of a contaminant present in the TFIIA fraction (labeled NS; see 28–30). Results of inhibition experiments are shown in Figure 4A (lanes 4–11): a 10-fold higher dose of MGB drugs was necessary to inhibit binding with respect to the hTBP–TATA complex, best evidenced from the lack of inhibition at 200 nM. A slightly different picture was revealed when we changed the order of addition; formation of the DA complex before addition of either distamycin A or tallimustine yielded a reduction (80%) only at the highest (20 μM) dose (Fig. 4B, lanes 4–11). It should be noted that in the experiments in Figure 4, the non-specific complex (NS) is unaffected even at 20 μM, confirming that the two MGB drugs only act on sequence-specific proteins.

We next added to the system recombinant TFIIB, another essential component of the pre-initiation complex known to stabilize the binding of TBP to DNA (26,27). Figure 5 shows that addition of TFIIB to the DA complex results in the formation of a new complex (DAB) of slower electrophoretic mobility (Fig. 5A and B, lanes 1 and 2). When the DAB complex was first formed and then challenged with MGBs, inhibition was observed

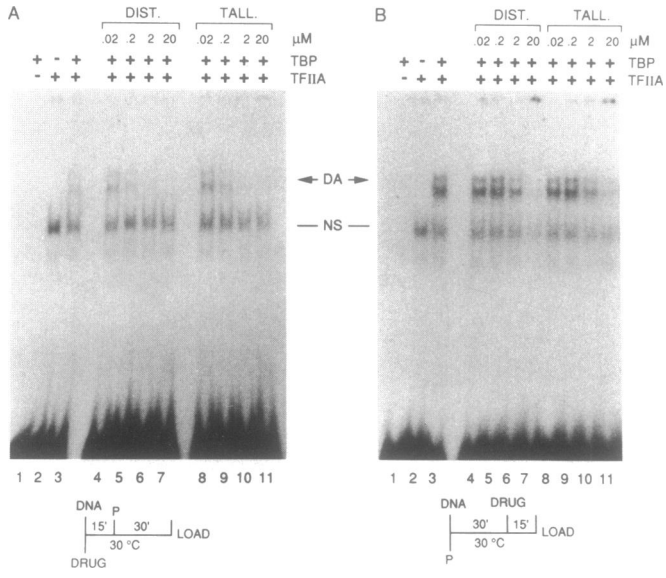

Figure 4. Inhibition of the DA complex by distamycin A and tallimustine. (**A**) EMSA of AdML TATA oligonucleotide with 10 ng hTBP (lane 1) or 0.5 μl partially purified TFIIA (lane 2). The same amount of hTBP and TFIIA together with (lanes 4–11) or without (lane 3) increasing amounts of the indicated drug. (**B**) As (A) except that the MGBs were added after DA complex formation (see scheme).

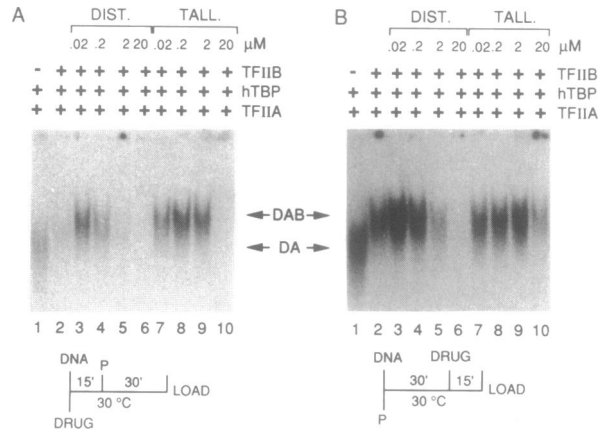

Figure 5. Inhibition of the DAB complex by MGBs. (**A**) EMSA of AdML TATA box oligonucleotide with 10 ng hTBP and 0.5 μl TFIIA (lane 1) or 20 ng recombinant TFIIB without MGBs (lane 2) or with increasing concentrations of both drugs (lanes 3–10). DNA was first incubated with MGBs. (**B**) As (A) except that MGBs were added after DAB complex formation.

only at a 20 μM concentration of distamycin and hardly at all with tallimustine. In the opposite experiment, the picture was similar to that observed with the DA complex, with the exception of a lack of inhibition at 2 μM tallimustine (Fig. 5A, lane 9). Interestingly, low concentrations of both drugs reproducibly increased the binding of the DAB complex to DNA (compare lanes 2 and 3, 4, 7 and 8 in Fig. 5A and B).

We can conclude that TFIIA and TFIIB, by stabilization of the TBP–TATA interaction, can efficiently prevent displacement by the MGBs, whereas both distamycin A and tallimustine are still efficient at inhibiting binding of the DA and DAB complexes.

MGBs inhibit *in vitro* transcription

It has been well established that TBP is absolutely required for promoter recognition by all three RNA polymerases (25). Genetic and biochemical analysis of several TATA-containing promoters indicate that TBP recognition of the TATA box is the primary event in the formation of the pre-initiation transcription complex (25–27). Since this interaction can be inhibited by distamycin A and tallimustine, we wished to verify whether they could be detrimental to the process of transcriptional activation. It is not known, in fact, whether an initiation complex, which binds tightly and stably to a promoter, can be perturbed by such compounds nor whether they can interfere with Pol II elongation.

For this purpose we used the *in vitro* transcription system that we have recently developed (31,33), adapted to a minimal TATA-containing promoter. It consists of human erythroid K562 nuclear extracts transcribing a β-globin reporter gene under the control of a minimal β-globin promoter, pAL5, containing up to nucleotide –41, thus being devoid of any upstream activating sequences and absolutely dependent both on the TATA box and on the presence of TBP (30,32). Only basal transcription can be scored with this construct (42). Finally, this promoter contains no other DNA stretch that might be susceptible to MGB binding (see sequence in Materials and Methods). Upon incubation of the template DNA with the nuclear extract and addition of nucleotides, transcription ensues and continues for multiple rounds over a 40 min period (31,43–45). The resulting RNA is purified and analyzed by S1 mapping, using a single-stranded, end-labeled DNA probe (34). Two signals are expected from this basal promoter: the correct β-globin +1 and a RT signal coming from spurious weak start sites along the vector. The latter is expected to be strong, since the system is devoid of any upstream activator that will focus the transcriptional machinery on the TATA box.

We first wanted to verify whether MGBs might have a general negative effect on transcriptional initiation or elongation. We thus exploited the fact that in a number of systems, including ours, incubation of extracts with the template DNA leads to the rapid formation (over a period of 15 min) of a pre-initiation complex that is fully competent for transcription and is resistant to Sarkosyl, competing oligonucleotides and antibodies (31,43–45). We pre-incubated pAL5 with the K562 extract first, so that a competent pre-initiation complex is formed; then we added the drugs at different concentrations. It should be noted that the amount of plasmid used is comparable in molar terms with the amount of TATA oligonucleotide used in the EMSA. Figure 6 shows that no significant decrease in either spurious RT transcripts or the correct +1 signal was observed, indicating that the transcription complex on the promoter is quite resistant to MGB challenge. Therefore, lack of transcriptional repression rules out direct negative effects that the drugs might have on the proteins of the transcriptional apparatus or on the elongation process.

Similarly, we performed experiments changing the order of addition of the components. We pre-incubated template DNA with increasing concentrations of both MGB drugs before addition of the transcription extract and NTPs, according to the scheme depicted in Figure 7. A clear inhibition of the specific +1 signal was already evident with both drugs at 2 μM.

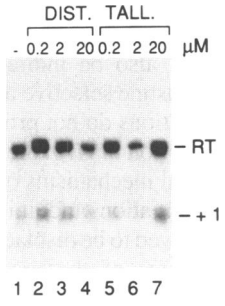

Figure 6. Effect of distamycin A and tallimustine on *in vitro* transcription after pre-initiation complex formation. The pAL5 template DNA containing a minimal β-globin promoter was first incubated with K562 nuclear extract after which drugs were added at different concentrations and incubation continued; nucleotide (NTPs) addition started transcription, which was stopped after 40 min. RNA was purified, hybridized and analyzed by S1 mapping. The sample in lane 1 is the control with no drug. RT is read-through transcription starting from spurious upstream vector sequences, while +1 corresponds to the correctly initiated β-globin signal.

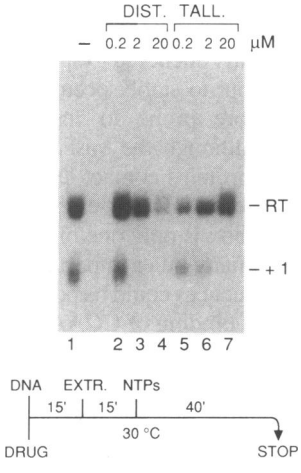

Figure 7. Inhibition of *in vitro* transcription by distamycin A and tallimustine. The transcription read-out of the experiment is as in Figure 6, except that MGBs were incubated with the template pAL5 DNA before addition of the transcription extract and nucleotides (NTPs). Samples were treated as in the experiments in Figure 6.

Surprisingly though, while at 20 μM distamycin A the RT transcripts were decreased, at 20 μM tallimustine the RT signal is at normal levels: the ratio between RT and +1 transcription increases considerably from 200 nM to 20 μM (see Fig. 7, lanes 5–7). We conclude that distamycin A has a specific effect on the +1 signal, best seen at 2 μM, and a more non-specific one at 20 μM (inhibition of RT transcription coming from spurious start sites in the vector), but tallimustine only shows a specific inhibition of the β-globin promoter over the three concentrations. This finding reinforces the notion that there is no general inhibitory activity on the transcriptional apparatus or on Pol II progression.

DISCUSSION

In this study we evaluated the inhibitory effects of the MGBs distamycin A and the alkylating derivative tallimustine on the interactions between the general transcription factors TBP, TFIIA and TFIIB and their target TATA box sequence, in terms of both DNA binding and transcriptional activation.

The oligonucleotide that we used contained no other possible binding site for these two drugs but the sequence TATAAAA, which is recognized by TBP (39–41), and specificity for AT-rich sequences was confirmed by lack of competition on SP1. A possible direct negative effect of the two compounds on recombinant TBP is ruled out by MGB–TBP binding experiments. Moreover, yTBP and hTBP concentrations necessary to shift the TATA oligonucleotide differed considerably, yet inhibitory doses follow apparent DNA binding affinity (yTBP > hTBP) and not protein concentration (hTBP > yTBP). We can conclude that direct competition for a common recognition sequence is thus responsible for the binding data presented.

Several points can be made. (i) Both yTBP and hTBP alone are susceptible to >90% inhibition by the drugs at concentration of 2 μM, a result in good agreement with the study of Chiang *et al.* (17), who found that the concentration of distamycin A necessary to produce 50% inhibition of the hTBP–TATA complex is 160 nM and complete inhibition was observed at 1 μM. Higher concentrations (10-fold) were reported to be necessary to inhibit DNA binding of the Antp homeodomain (16), OTF1 and GATA1 (15). (ii) Both drugs shows very similar dose–response curves when changing the order of addition, indicating that MGBs can interfere not only with TBP–DNA interactions, but can also displace the complex once it is formed. This is somewhat surprising, considering that the yTBP–TATA complex has been shown to have a very slow dissociation rate, with a half-life of >1 h (46). However, the same kinetic studies suggested that binding of yTBP to the TATA box is a multi-step process, which would help explain the data presented here. If one (or more) less stable intermediate(s) is formed, it is conceivable that it might still be attackable by MGBs, shifting the equilibrium toward dissociation of the complex(es). Interestingly, co-crystals of TBP and the TATA box have indicated that the underside concave surface of TBP makes direct hydrophobic interaction with a widened AT-containing minor groove: this will represent the working model to understand MGBs displacment activity. (iii) The effects of MGBs on the DA and DAB complexes are rather different. While inhibition was still evident on pre-incubating the DNA and the drugs first, if the DA and DAB complexes are first formed, displacement can be observed only at much higher MGB concentrations (20- and 100-fold). In the study of Chiang *et al.*, addition of yeast TFIIA before drug challenge also led to a stabilization of the TBP–TATA complex, while the reverse experiment was not tried (17). The higher drug concentrations required in our study are probably due to the different experimental conditions: (a) in our assays free DNA was always in large excess (no more than 5–15% of the oligonucleotide is shifted); (b) we have used hTFIIA (as opposed to yeast); (c) a longer TATA oligonucleotide (37mer versus 24mer). It is known that addition of hTFIIA extends the TBP footprints of TATA elements (36,37,47,48), suggesting that flanking sequences somewhat stabilize the DNA–protein interactions.

These data are in agreement with previous observations on the stabilizing role of TFIIA and TFIIB in promoter recognition by TBP (47,48). The remarkable resistance of pre-formed DA and DAB complexes described here suggests that TFIIA, by binding to the TBP–DNA complex, might somehow shield access to the minor groove of MGBs. CC1065, another sequence-specific

MGB able to alkylate adenine N3, also behaves in a similar way (see 17; data not shown).

In these DNA binding interference studies no striking difference between distamycin A and tallimustine was obvious, except that slightly higher doses (2-fold) of the latter were consistently necessary for inhibition under all conditions tested. This behavior might be related to a lower affinity for DNA in general or to a more pronounced sequence specificity of tallimustine, which indeed has been shown to preferentially alkylate the A in the sequence TTTTGA or the second A in TTTTAA (8,9).

The functional *in vitro* transcription experiments shown here represent the first evidence that MGBs are able to alter the transcription rate of a promoter, by interfering with TBP/TFIID binding.

Several lines of data lead to this conclusion. (i) We used a minimal promoter containing only the β-globin TATA box; this sequence is surrounded by a GC-rich area, which could not be bound by MGBs of the distamycin A family. For the relatively low levels of basal transcription the TATA box is essential (19,42). (ii) The doses of both drugs necessary for DA/DAB–TATA inhibition parallel well the doses that are inhibitory in the transcription system. (iii) It is sufficient to pre-incubate the extract with the template DNA, under conditions that allow pre-initiation complex to form, to essentially abolish the inhibitory effect. The two drugs do not affect elongation of either the correct +1 signal or the spurious RT transcripts, even at high doses, implying that it is not by obstructing RNA Pol II progression or the elongation complex in general that the inhibitory effect is brought about. Thus these compounds are unlikely to represent general non-specific inhibitors of transcription.

It is believed that in living cells (and consequently in nuclear extracts) TBP is tightly complexed with several TAFs (TBP associated factors), forming TFIID complexes (25). Inhibition of basal *in vitro* transcription by MGBs suggests that they also prevent binding of TFIID to DNA, which is not too surprising, since TBP represents the major TATA-contacting moiety of the complex. The affinity of MGBs for DNA is far lower than that of TBP alone (K_d values are 10^{-7} and 10^{-9} respectively) and it should be considered that the other components of the initiation complex, TFIIA, TFIIB and the TAFs in particular, will further increase the difference, by stabilizing TBP on the TATA box and making contacts with neighbouring sequences. In addition, our system allows multiple rounds of re-initiation (31,33): we find it remarkable that these drugs, despite the difference in affinity, are able to resist the challange of the general transcription machinery and that inhibition is not limited to the first round, but remains strong over the transcription reaction, suggesting that MGB–TATA interactions cannot be easily displaced by transcriptional factors.

Perhaps the most surprising result in this study is that, unlike distamycin A, tallimustine only showed inhibition of the correct +1 signal, even at high concentrations. From the binding studies one could in fact anticipate that tallimustine would require higher concentrations to achieve the dystamicin A inhibitory effect. While distamycin A showed inhibition at lower doses for +1 than for RT 'non-specific' transcription, the tallimustine response was slower, but much more specific. These data can be explained by considering the higher affinity of distamycin A for AT sequences, also demonstrated by the binding studies, and the fact that tallimustine contacts two additional nucleotides (9). The requirement for additional nucleotides might confer a higher sequence selectivity to the latter drug, which would have fewer or no other target sequences on the plasmid in addition to the TATA box in the minimal promoter and hence be less effective in inhibition of RT transcription. This might also be indirectly suggested by the already mentioned tallimustine selective alkylation pattern (8,9).

Although these observations do not prove directly that MGBs do interfere with transcription *in vivo*, they could give important indications of the potential mechanisms of action of MGBs, still largely unknown. Pre-initiation and initiation complexes are stable *in vivo* and are believed to be displaced by DNA replication events (49). The resistance of the pre-initiation complex to inhibition by MGBs even at high concentrations is not surprising, in the light of their remarkable stability once bound to promoter sequences. This observation suggests that MGB activity *in vivo* is unlikely to be related to perturbation of ongoing transcription and that they need naked DNA to bind to essential regulatory elements. Thus the activity of MGBs *in vivo* could be limited to a certain phase of the cell cycle, when the DNA is replicated and becomes a possible target for these drugs. We favor a model in which MGBs and transcription factors compete for a specific DNA sequence and it would be a matter of affinity and concentration of the two competitors to determine which first recognizes a given regulatory element. In this scenario it is conceivable that alkylating agents such as tallimustine (or CC1065), being covalently anchored to specific DNA sequences, although no more prompt to stably occupy available sites than distamycin A, are more prone to resist the challenge of transcription factors. Although the vast majority of sequence-specific DNA binding proteins contact DNA through the major groove, the set of experiments presented here is intended to indicate that the TATA box is only one of the possible targets of these drugs. Certainly, many other important regulatory proteins recognizing AT-rich sequences could be potential targets for these molecules: inhibition of binding of CCAAT-box binding protein NF-Y (CP1-CBF) by MGBs appears to be highly dependent on neighbouring sequences (A. Ronchi, in preparation).

We are confident that the functional assay described here will be an important tool in elucidation of the molecular mechanisms underlying the action of these drugs, as we have started to perform here, and in the screening of new highly selective sequence-specific compounds.

ACKNOWLEDGEMENTS

We would like to thank S. Ottolenghi and F. Badaracco for critically reviewing the manuscript. This work was supported by a Pharmacia/FCE grant to RM, Progetto Finalizzato ACRO and AIRC grants to S. Ottolenghi and ACRO and AIRC grants to MD. MB is supported by a Milano Ricerche fellowship.

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