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Bisanthracycline WP631 inhibits basal and Sp1-activated transcription initiation *in vitro*

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ABSTRACT

An *in vitro* transcription assay was used to compare the capacity of the bisintercalating anthracycline WP631 (which displays a remarkably high DNA-binding affinity) and the monointercalating anthracycline daunomycin to inhibit transcription initiation of the adenovirus major late promoter linked to a G-less transcribed DNA template. Both drugs inhibit basal RNA synthesis in a concentration-dependent way, and the drug concentrations required to inhibit transcription initiation are similar. However, in this study WP631 was around 15 times more efficient at inhibiting transcription initiation when used with an adenovirus promoter containing an upstream Sp1-protein binding site under experimental conditions in which the Sp1 protein acted as a transactivator *in vitro*. The differences in the ability of each drug to inhibit transcription initiation were related to the competition between Sp1 and the drugs for the same binding site. Concentrations of WP631 as low as 60 nM could inhibit the Sp1-activated transcription initiation *in vitro*. In contrast, the concentration of daunomycin required to inhibit Sp1-activated transcription by 50% was almost the same as the concentration required to inhibit basal transcription. The efficiency of WP631 at displacing Sp1 from its putative binding site was confirmed using gel retardation and footprinting assays. These results are the first unequivocal example of a direct effect of an intercalator on activated transcription initiation.

INTRODUCTION

Regulating the rate of transcription initiation by RNA polymerase II is an important mechanism by which cells establish the suitable expression of their genes (1–3). It is known that the regulation of gene transcription demands sequence-specific recognition of a gene promoter by protein factors and several interactions between these proteins (3–6). While several protein factors are common to all class II promoters, the expression of some genes requires additional factors known as transactivators. Sp1 is a zinc-finger transcription factor that activates transcription in many cellular promoters (3,6–8). It recognizes the sequence 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3' (9). Transcriptional activation by the Sp1 protein involves composite effects including conformational changes in DNA structure and the interaction with other protein factors as well as specific protein–protein contacts (5,6).

Many antitumor drugs presently in clinical use are considered to perform their activity by binding to DNA (10–12). These small molecules, which can bind to specific DNA sequences, might control gene expression by interfering with DNA–protein interactions (10,13–18). Nevertheless, there are grounds to believe that, because affinity is a dominant factor in all these interactions, most of the drugs analyzed to date might have a relatively lower affinity than protein factors (15,17,19,20). Thus, the concentrations of drug required to interfere with the transcription of eukaryotic genes in most cases might be quite high (in the μM range). It would therefore be desirable to obtain drugs that exhibit a binding constant of the same order of magnitude as the protein factors.

The recently designed and synthesized bisanthracycline WP631 (Fig. 1), bisintercalates into DNA with a binding affinity close to that of some proteins (21–23) and, interestingly, circumvents multidrug resistance in some cell lines (21). Crystallographic and NMR studies on WP631 binding to a short oligonucleotide have revealed how the two aglycones of WP631 intercalate between the CpG steps in a way that is reminiscent of the binding of two daunomycin molecules (23,24). The long axes of the chromophores of WP631 and daunomycin are oriented almost perpendicular to those of the flanking bases. In this respect, we foresaw that WP631 could inhibit the interaction between some nuclear protein factors and their binding sites with extraordinary efficiency, and thus might regulate gene expression by altering the binding of regulatory proteins.

In the present study, we sought to determine the influence of WP631 on an Sp1-transactivated promoter *in vitro*, by comparing its effects with an Sp1-lacking promoter and the monointercalator daunomycin, which has a lower DNA binding constant. Since the Sp1 site contains G/C-rich tracts, including CpG steps (5,9), we expected both anthracyclines to bind to the same regions but with different binding affinities. We found that both drugs inhibited the basal RNA synthesis of an adenovirus promoter in a concentration-dependent way. However, WP631 was more efficient at inhibiting transcription initiation when we used a plasmid which contained an Sp1-binding site under experimental conditions in which Sp1 acts as a gene activator (6). The two plasmids used in our experiments contained

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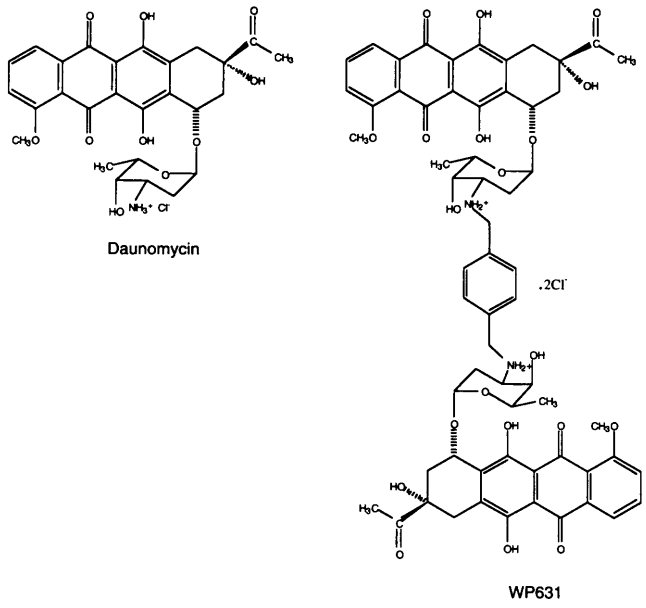


Figure 1. Chemical structures of daunomycin and WP631.

a G-less template (4,25) (Fig. 2). From a practical standpoint, this means that we have been able to distinguish unambiguously between effects that are due to the decrease in transcription initiation and those due to elongation.

MATERIALS AND METHODS

Drugs, DNA templates and proteins

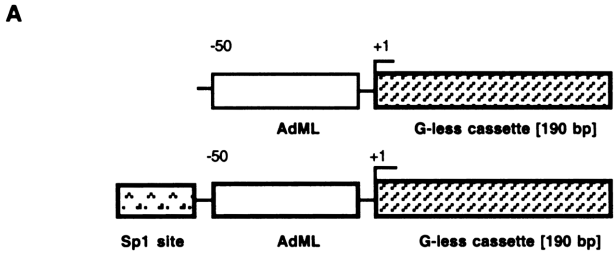
The bisintercalating anthracycline WP631 was synthesized as described previously (21). Daunomycin was purchased from Sigma (St Louis, MO). Both drugs were dissolved in 10 mM Tris–HCl (pH 7.4) containing 20 mM KCl. Any undissolved drug was removed by low-speed centrifugation. The drug concentrations were determined using ε_{480} (daunomycin) = 11 500 $M^{-1}cm^{-1}$ and ε_{480} (WP631) = 13 400 $M^{-1}cm^{-1}$.

For RNA polymerase II transcription studies (see below), the templates were a plasmid containing the AdML50[180] (AdML) promoter (25), and AdSP01, which we obtained by the insertion of the oligonucleotide 5'-GAATTCGGGGCG-GGGCGAATTC-3', which contains a consensus Sp1 site, within the unique *Eco*RI in the AdML promoter. The sequence of the relevant part of both plasmids (i.e., the promoter plus the G-less template) was confirmed (Fig. 2) using an Abi Prism 377 automatic DNA sequencer (Perkin-Elmer, Langen, Germany), at the Serveis Cientifico-Tecnics of the University of Barcelona.

HeLa cell nuclear extracts were prepared as a source of general transcription factors as described in (26). Pure human Sp1 protein was purchased from Promega (Madison, WI).

Transcription *in vitro*

The effects of daunomycin and WP631 on the transcription initiation of AdML and AdSP01 promoters were measured using 200 ng of supercoiled templates and 50 µg of HeLa nuclear extract, in a final volume of 25 µl containing 30 mM HEPES–KOH (pH 7.9), 7 mM $MgCl_2$, 5 µM $ZnSO_4$, 1 mM dithiothreitol (DTT), 0.2 mM EDTA, 2% PEG 8000 (Sigma), 400 µM each of ATP and CTP, 1 µM UTP and 10 µCi of [α-^{32}P]UTP (800 Ci/mmol; Amersham, Madrid, Spain). The reaction also contained 1 mM 3'-*O*-methyl-GTP (Pharmacia Biotech, Uppsala, Sweden) and 15 U RNase-T1 (Calbiochem, San Diego, CA) (4) in the presence of the amounts of either drug indicated in the legends to the figures. Transcription was allowed to proceed for 60 min at 30°C, after which the samples were phenol extracted and ethanol precipitated before gel loading. In all the



B

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-80   |   -70   |   -60   |   -50   |   -40   |   -30   |   -20   |   -10   |
-83                                                                    GAA   -81
-80  TTGAATTCGG GGCGGGGCGA ATTCAATTCG GGGTGTTCCT GAAGGGGGGC TATAAAAGGG GGTGGGGGCG CGTTCGTCCT   -1
  1  CTTCCATACC TTCCTCCATC TATACCACCC TACTCTCCTT TCCTCATTAT TCCTCCTATT ATCTTCTCCT CTTCTCTCCT   80
 81  TCTTCTATAT TTCCCAAATC TATCATCATT CACTCTCACC CCCTCTTCCT TCACTCCCAT TCTATTCTAC TCCTTTCCCT  160
161  TTCCATATCC CCTCCACCCC CCCCCCCG                                                          188
       |   10    |   20    |   30    |   40    |   50    |   60    |   70    |   80
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Figure 2. (**A**) Scheme of the G-less templates used in the analysis of the effects of daunomycin and WP631 in the transcription experiments *in vitro*. They are the AdML50[180] (AdML) and the AdSP01 promoters (see the main text for details). (**B**) Nucleotide sequence of the upper strand of the AdSP01 promoter and the G-less cassette template. The Sp1-binding site, the TATA box and the INR box are indicated. The initiation site is nucleotide +1.

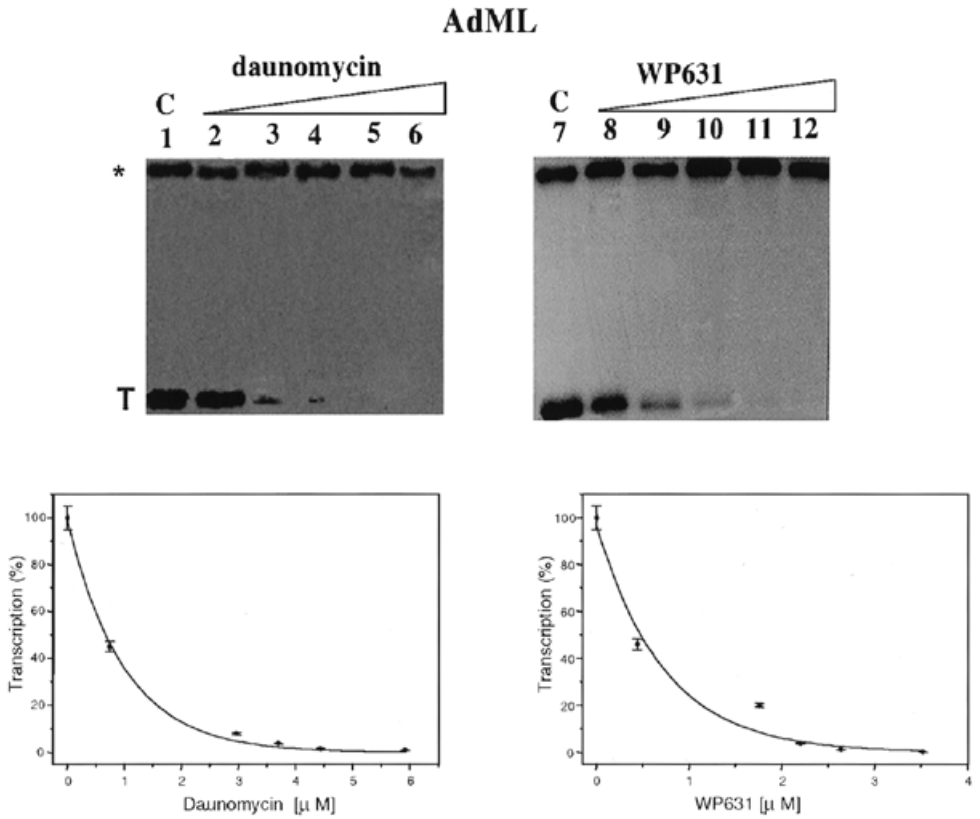

Figure 3. Influence of daunomycin and WP631 on transcription from the AdML promoter. The top panels show a representative transcription assay *in vitro*. In all cases, an unrelated RNA was added as a recovery and loading control (indicated by an asterisk). Lanes 1 and 7, transcripts obtained using a HeLa cell extract; lanes 2–6, effect of increasing amounts of daunomycin (0.74, 2.96, 3.70, 4.44 and 5.92 μM respectively); lanes 8–12, effect of increasing amounts of WP631 (0.44, 1.76, 2.20, 2.64 and 3.52 μM respectively). The bottom panels represent a quantitative analysis of the effects of daunomycin and WP631 on transcription *in vitro* from the AdML promoter. The densitometric profiles of the experimental results shown in the two top panels were normalized with respect to the total amount of loaded radioactivity using the loading control marker. The C_{50} values (i.e., the drug concentrations required to reduce transcription by 50%) were derived by fitting an exponential decay curve to plots of transcription (%) versus drug concentration. The data represent the mean of at least three independent experiments (mean values ± SD).

transcription experiments, an internal control for recovery and gel loading (an unrelated T7-transcript) was used.

Transcripts were analyzed by high-voltage electrophoresis in 90 mM Tris–borate, 2 mM EDTA (pH 8.3) using 8% polyacrylamide gels containing 7 M urea. After running, the gels were soaked in distilled water, dried under vacuum and subjected to autoradiography. Quantitative analyses of transcripts were carried out using a Molecular Dynamics computing densitometer and ImageQuant 3.3 software, with the relative amounts of transcripts observed being normalized to the total amount of radioactivity loaded. The C_{50} values (i.e., the drug concentrations that reduced electrophoretic band intensity by 50%) were derived by fitting an exponential decay curve to plots of percentage of transcription versus drug concentration.

Gel retardation assays

Gel retardation (band-shift) assays were performed in a buffer of 10 mM Tris–HCl (pH 7.4) containing 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 30 μg/ml BSA and 5% glycerol. A typical reaction contained ~20 ng of pure Sp1 protein (Promega) and 1500–3000 c.p.m. (~2 nmol in base pairs) of the end-labeled double-stranded oligonucleotide: 5'-GAATTCG-GGGCGGGGCGAATTC-3', in the presence of 1 μg of poly[d(I–C)] (Boehringer Mannheim, Mannheim, Germany). In reactions containing daunomycin or WP631, either the labeled oligonucleotide was preincubated for 15 min at 30°C with 20–25 ng Sp1 before the addition of different concentrations of the drugs (Fig. 6), or the protein and either drug were added at the same time to mimic the conditions of the transcription *in vitro*. After a further 20 min incubation, the samples were analyzed on 4.5% non-denaturing polyacrylamide gels containing 45 mM Tris–borate, 1 mM EDTA (pH 8.3). After running at low voltage (12 V/cm), the gels were dried under vacuum and subjected to autoradiography. Quantitative analysis of the complex formation was performed with a Molecular Dynamics computing densitometer.

DNase I footprinting

For DNase I footprinting assays, the plasmid containing AdSP01 was first digested with *Pvu*II. The *Pvu*II–*Pvu*II frag-

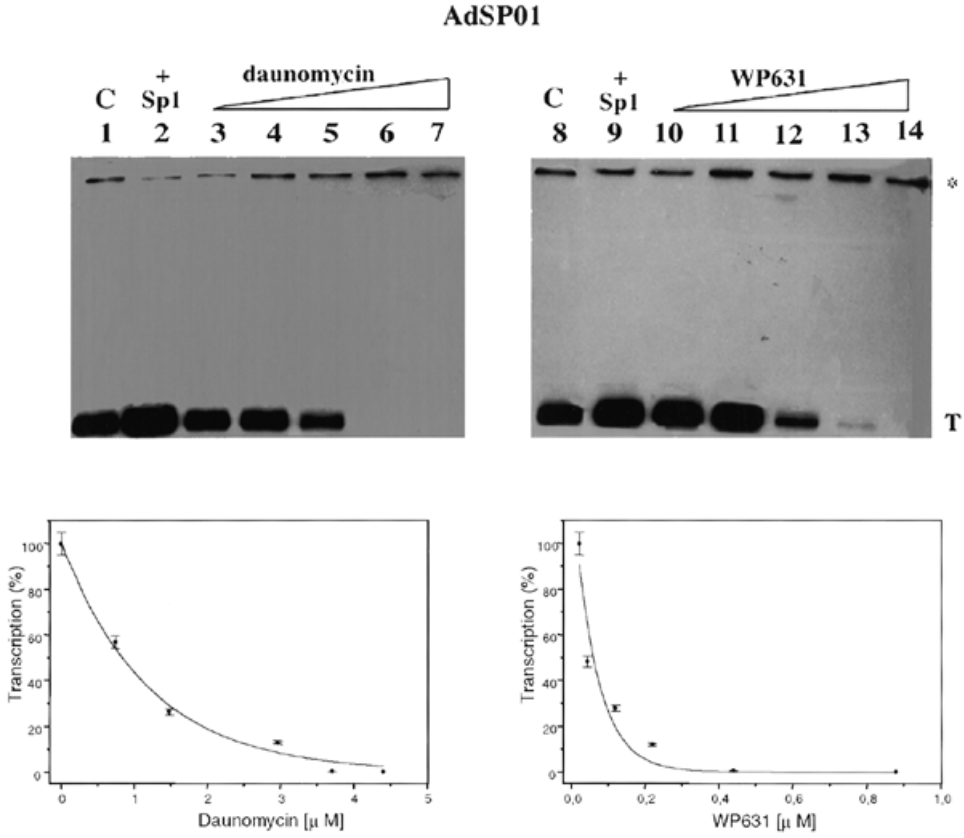

Figure 4. Influence of daunomycin and WP631 on Sp1-activated transcription from the AdSP01 promoter. The top panels show a representative transcription assay *in vitro*. In all cases, an unrelated RNA was added as a recovery and loading control (indicated by an asterisk). Lanes 1 and 8, transcripts obtained using a HeLa cell extract; lanes 2 and 9, Sp1-activated transcription obtained using whole HeLa extract plus added pure Sp1; lanes 3–7, effect of increasing amounts of daunomycin on the Sp1-activated transcription (0.74, 1.48, 2.96, 3.70 and 4.40 μM respectively); lanes 10–14, effect of increasing amounts of WP631 (0.022, 0.044, 0.22, 0.44 and 0.88 μM respectively). The bottom panels represent a quantitative analysis of the effects of daunomycin and WP631 on Sp1-activated transcription of the AdSP01 promoter. Details as described in the legend to Figure 3.

ment was dephosphorylated using Alkaline phosphatase and isolated on an agarose gel. Digestion of this fragment with *Sma*I yielded a 352 bp fragment that was labeled at the 5'-end of the upper strand (Fig. 2), using [γ-^{32}P]ATP and T4 polynucleotide kinase. Next, samples containing 3000 c.p.m. of this DNA fragment (~10 pmol in base pairs), and different concentrations of WP631 and/or of pure Sp1 protein, were digested, in a final volume of 20 μl, with DNase I (Boehringer Mannheim) at a final concentration of 0.01 U/ml for 2 min at 30°C, in a buffer consisting of 10 mM Tris–HCl (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 0.5 mM DTT and 5% glycerol. The reaction mixtures were phenol extracted and, after precipitation with ethanol, dissolved in 85% formamide, 10 mM EDTA, 0.02% bromophenol blue. Samples were heated at 95°C for 2 min prior to electrophoresis. The footprints were resolved by high-voltage electrophoresis in 90 mM Tris–borate, 2 mM EDTA (pH 8.3) using 6% polyacrylamide gels containing 8 M urea, together with a formic acid–piperidine marker specific for adenines plus guanines. After running, the gels were soaked in distilled water, dried under vacuum and subjected to autoradiography.

RESULTS

Daunomycin and WP631 inhibit basal transcription *in vitro*

Figure 3 shows representative results of the transcription initiation by a HeLa cell extract of the AdML promoter in the presence or absence of daunomycin or the bisanthracycline WP631. The plasmid used contained an AdML promoter linked to a synthetic 190 bp DNA template that lacked cytidine residues on the transcribed strand (a G-less cassette) (4,25). Under our experimental conditions (see Materials and Methods), the unique transcript that accumulated was a 190-nt RNA resulting from the accurate initiation at these promoters. Therefore, the inhibition of transcription by increasing amounts of each drug was apparently the result of the drug's effect on the transcription initiation by RNA polymerase II. About 4.5 μM daunomycin fully inhibited transcription from the strong AdML promoter (Fig. 3), while lower concentrations of WP631 (~2.5 μM) were required to produce the same effect. These results suggested that both drugs might have blocked the formation of the transcription initiation complex. Besides, since there were no GpC nor CpG steps in the template region (Fig. 2) neither drug had preferred

intercalating sites in the transcribed region (21,23,27,28) and thereby no detectable effect during the elongation step (see also the footprinting experiments described below). WP631 appeared to be more efficient at preventing transcription factors in the HeLa cell extract from binding to the promoter. At first glance, these results might be related to the tighter WP631 binding to DNA that is due, at least in part, to the bisintercalating nature of WP631 (22,23).

Figure 3 and Table 1 display the results of quantitative analyses of the influence of daunomycin and WP631 on transcription initiation of the AdML promoter *in vitro*. Concentrations of 0.68 μM daunomycin and 0.48 μM WP631 decreased transcription by 50% (C_{50}). The ratio of these inhibiting concentrations (~1.5) seemed to be consistent with differences in DNA binding of the monointercalator daunomycin and the bisintercalator WP631. Nevertheless, this ratio was not high enough to support the claim that WP631 was more effective at inhibiting basal transcription *in vitro*. In any case, the two drugs appeared to compete with transcription factors for DNA-binding sites.

Table 1. Effects of daunomycin and bisanthracycline WP631 on basal and Sp1-activated transcription initiation *in vitro*

Promoter	C_{50} [μM][a]		
	Daunomycin	WP631	r_{50}[b]
AdML	0.68	0.48	1.42
AdSP01	0.84	0.060	14.00

[a]C_{50} indicates the drug concentration that decreases the transcription initiation by 50%.
[b]r_{50} is the ratio of the drug concentrations required to inhibit the *in vitro* transcription by 50% from each promoter.

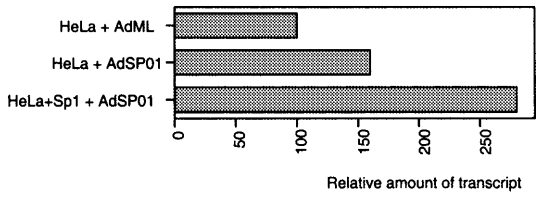

Figure 5. A quantitative comparison of Sp1-activated transcription of the AdSP01 promoter and the basal transcription of the AdML promoter without any added drug. Sp1 protein present in HeLa cell extracts enhanced *in vitro* transcription from the AdSP01 promoter about 1.5 times above that from AdML. The addition of pure Sp1 led to an ~3-fold enhancement of transcription. These results are representative of two different experiments as those displayed in Figures 3 and 4. See the main text for further details.

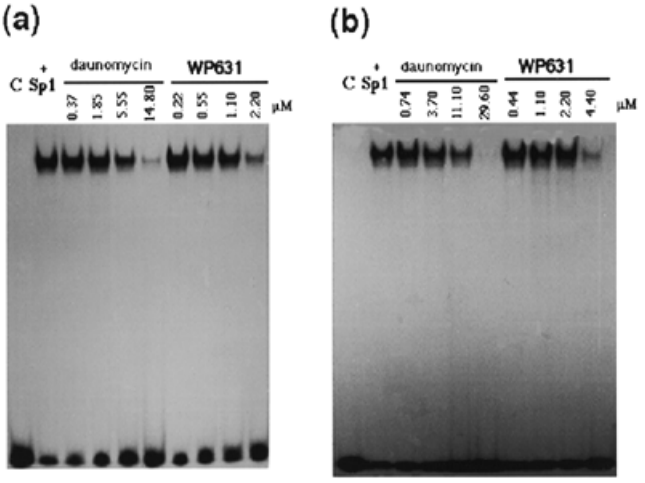

Figure 6. Gel retardation (band-shift) analysis of the effect of daunomycin and WP631 on Sp1 binding to the end-labeled oligonucleotide, which contains its consensus binding sequence. (**a**) Each drug and the protein were added together to the labeled oligonucleotide; (**b**) pure Sp1 protein and the oligonucleotide were pre-incubated before the drugs were added. The concentrations of daunomycin or WP631 added to the binding reactions are indicated in the figure. Tracks labeled C and +Sp1 contained the oligonucleotide alone or in the presence of 20 ng pure Sp1 respectively.

WP631 is very potent at inhibiting Sp1-activated transcription *in vitro*

The plasmid AdSP01, which contains a consensus Sp1-binding site upstream of the adenovirus late promoter (Fig. 2), was used to analyze whether daunomycin and WP631 were able to inhibit transcription *in vitro* through a direct effect on protein–DNA interactions. Pure Sp1 protein, a GC-rich binding one (9), was added in order to activate transcription (3). Figure 4 shows the inhibition by daunomycin or WP631 of the Sp1-activated transcription of the AdSP01 promoter *in vitro*. Since both of the anthracyclines bind to CG-rich regions in DNA (22,27,28), differences in the transcription of the Sp1-activated promoter were considered to be closely related to the different affinities displayed by the two drugs for the putative DNA sequence. Furthermore, the experimental results displayed in Figure 4 could be compared with the basal promoter data (Fig. 3), thus providing a way to discriminate between the undefined effects of daunomycin or WP631 on other protein–DNA complexes and their defined effects on Sp1–DNA complexes. The percentage of transcription of AdSP01, in the presence or absence of any drug (Fig. 4, bottom panels), was normalized in each experiment, considering the transcription of the basal AdML promoter and the activated AdSP01 as 100%. In fact, Sp1 protein present in the HeLa cell extract enhanced the transcription of AdSP01 about 1.5 times (Fig. 5) above that of the AdML promoter (compare lanes 1 or 7 in Fig. 3 with lanes 1 and 2 or 8 and 9 in Fig. 4), and when pure Sp1 was added, the transcription increased another 1.75 times. Consequently, the accumulated activation of the AdSP01 promoter was ~3-fold that of basal transcription (Figs 3–5), which would be expected for Sp1-activated transcription *in vitro* (6,16) since AdSP01 contains a unique Sp1-binding site. Yet, the transactivation was less than that observed in several promoters *in vivo* (6,8). The ability of WP631 to inhibit Sp1-activated transcription was outstanding and observable in the low nanomolar range. WP631 also inhibited the activated transcription more efficaciously than it did basal transcription. The C_{50} of daunomycin was 0.84 μM, while the

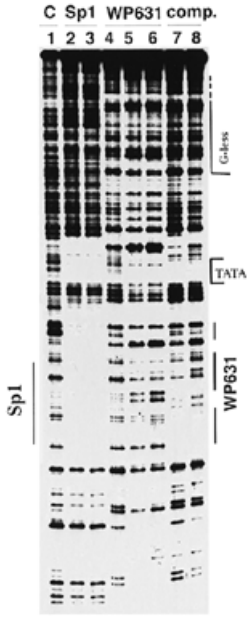

Figure 7. DNase I footprinting of a 352 bp DNA fragment containing the AdSP01 promoter and a G-less cassette. The DNA was labeled at the 5' end of the strand whose sequence is displayed in Figure 2. The top of the gel corresponds, therefore, to the G-less region. Lane 1, labeled DNA treated with DNase I (control); lanes 2 and 3, DNase I cleavage in the presence of 10 and 20 ng of pure Sp1 protein respectively; lanes 4–6, DNase I cleavage in the presence of 1, 3 and 5 μM WP631 respectively; lane 7, DNase I cleavage in the presence of 20 ng pure Sp1 and 3 μM WP631; lane 8, DNase I cleavage in the presence of 20 ng pure Sp1 and 5 μM WP631. The region corresponding to the Sp1 footprint is indicated at the left side of the figure, while the footprints of WP631 on the Sp1-binding site are indicated at the right. The locations of the TATA-box and G-less cassette are also shown.

C_{50} of WP631 was only 0.060 μM (see the quantitative analysis in Fig. 4 and Table 1).

The smaller C_{50} for WP631 (60 nM) as well as the higher ratio between the C_{50} of both drugs (Table 1) would be useful information for those involved in the design of new intercalating agents since the C_{50} is in the nanomolar scale and WP631 competed efficiently with transcription factors. Clearly, WP631 inhibited the Sp1-activated transcription *in vitro* even more efficiently than it did basal transcription.

It is worth mentioning that although transcription was allowed to proceed for 60 min, a transcript of unique size was formed, meaning that once transcription was initiated it did not halt during elongation. This experimental observation strengthens the hypothesis that the drug effects were due to the disruption of DNA–protein interactions in the promoter. Consequently, the amount of drug required to inhibit the initiation appeared to depend on the competition between daunomycin, or WP631, and protein-factors for the CpG sites in the promoter. While previous reports have noted a direct effect of some GC-binding drugs on DNA–Sp1 binding (10,14,16,17), the present results are, to our knowledge, the first unambiguous example of a direct effect of an intercalating agent on the transactivated initiation of transcription *in vitro*.

Sp1 and WP631 might bind to the same DNA sequence

More direct evidence of the inhibitory effects of the two drugs on Sp1 binding was obtained by gel retardation (band-shift) and DNase I footprinting. Gel retardation experiments were carried out using a labeled Sp1 oligonucleotide and pure Sp1 protein. Two different experimental approaches are displayed in Figure 6. Figure 6a shows the drug-mediated inhibition of the interaction between Sp1 and the oligonucleotide without preincubation of the protein–DNA complexes, while Figure 6b shows the effect of the two anthracyclines on a preformed Sp1–DNA complex. The results in Figure 6a were obtained under experimental conditions that mimic better the transcription experiments described above. As expected, the amount of any drug required to disrupt the complex was higher when the DNA–protein complex was preincubated (see legend to Fig. 6). Nonetheless, in the presence of decreasing concentrations of either drug, the formation and stability of the Sp1–DNA complexes was more apparent, indicating that the DNA–protein complex was sensitive to both anthracyclines in a concentration-dependent way. These results attest that WP631 blocked the binding of Sp1 to its putative binding site with higher efficiency than did daunomycin.

The different susceptibility of the Sp1–DNA complex to the two drugs was not at variance with the results observed in the *in vitro* transcription assays described above. In both experiments, WP631 appeared to work more efficiently as an inhibitor. Nevertheless, the concentrations required to inhibit the transcription *in vitro* and to disrupt the Sp1–DNA complex were clearly different (compare the C_{50} values in Table 1 and Fig. 6). The higher drug concentrations needed in the band-shift studies were probably due to the different experimental conditions, related to the buffer composition and the presence of protein factors, other than Sp1, in the transcription assay. The intrinsic flexibility of the longer DNA used in the transcription assays (a supercoiled plasmid) was also peculiar if compared with that of the short linear 22mer oligonucleotide. Since there are experimental grounds for believing that flanking sequences might somewhat stabilize DNA–drug or DNA–protein interactions (1,16), it is possible that this flexibility could facilitate the DNA-bending ability of Sp1 (29), and thus affect the WP631 bisintercalation in the promoter region (see below). Moreover, the nascent RNA transcript could delay transcription initiation differently depending on the dissociation rate of the drugs (30,31).

A DNase I footprinting analysis of the binding of WP631 and Sp1 to the AdSP01 promoter is shown in Figure 7. In the presence of different concentrations of pure Sp1 protein a prominent footprint was located at the putative Sp1 site (Fig. 7, lanes 2 and 3). Sp1 binding also produced a smaller footprint downstream of the Sp1 site, possibly due to a change in the conformation of DNA after binding. This protection from cleavage is not a peculiarity of the AdSP01 since it has been observed on other promoters (16 and references therein). Lanes 4–6 in Figure 7 analyze the interaction of WP631 with the same DNA. Footprints were found at CG-rich regions of the promoter including the Sp1-binding site. More detailed footprinting experiments have suggested that WP631 binds best to regions of mixed sequence, possibly recognizing multiple CG base pairs within an AT environment (I.Fokt, W.Priebe and K.R.Fox, unpublished observations). Although the Sp1-binding

site in the AdSP01 promoter did not contain adenines or thymines (Fig. 2), such bases were present near the 5' site of the sequence, where a WP631 footprint was clearly observed (bottom part of the gel in Fig. 7). When both ligands were added into reaction together, the protein and the drug seemed to compete for the Sp1-binding site partially. It is worth noting that a composite footprinting was observed (lanes 7 and 8), in which the protein appeared to bind better at the 5' edge of the target sequence while WP631 would bind more tightly to the other part of this DNA region. A few enhanced bands that were not observed in lanes containing either ligand alone, did become evident when both ligands were incubated together (compare lanes 2–6 with lanes 7 and 8 in Fig. 7). The presence of such bands could correspond to a ternary complex formed between DNA and both ligands on the same sequence. This interpretation would be consistent with the results obtained in the gel retardation assays (Fig. 6a), in which 3 μM WP631 competed partially with the protein–DNA complex. Concentrations of WP631 higher than those displayed in Figure 7 were required to displace the protein completely (data not shown).

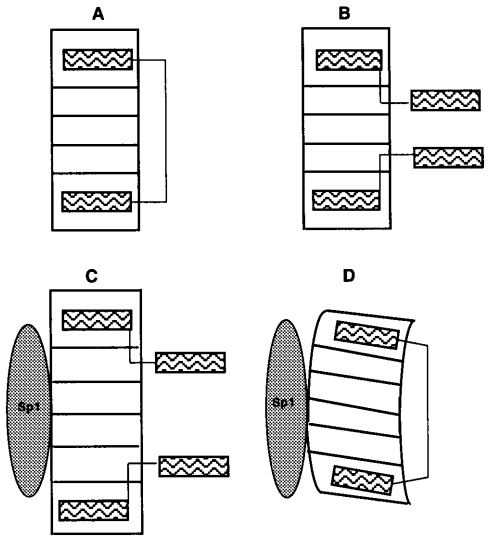

Figure 8. A schematic model of the possible binding modes for the interaction of WP631 with DNA. (**A**) WP631 bisintercalates into DNA and occupies 6 bp. (**B**) Two WP631 molecules monointercalate into DNA and occupy 3 bp. (C) and (D) Tentative models to explain the binding of WP631 to the Sp1-binding site. (**C**) The DNA-binding domain of Sp1 and WP631 cover 7 bp of a putative Sp1-binding site, in which the drug is monointercalated. (**D**) Sp1 binding bends its putative binding site and allows some bisintercalation of WP631 because of a change in the geometry of DNA that makes the bisintercalation cover 7 bp. (A) and (B) were adapted from (22).

DISCUSSION

Studying drug-induced inhibition of transcription may be a very helpful tool in understanding the mechanism of action of DNA-binding drugs (31). Transcription can be inhibited if a DNA-binding drug alters the interactions between a promoter region and some transcription factors, or if it is able to stop the elongation of an initiated transcript. Previous studies using nuclear run-off and run-on transcription assays have suggested that most of the antitumor drugs studied to date would produce inhibition of initiation rather than elongation (15,17). The two plasmids used in our experiments contained a G-less cassette template (4,25) (Fig. 2). From a practical point of view, this means that we have been able to distinguish unambiguously between any drug effect due to the decrease in transcription initiation rather than elongation.

WP631 is a bisintercalating ligand that occupies 6 bp of DNA, while daunomycin is a monointercalating ligand that occupies only 3 bp (22). WP631's larger binding site should make WP631 more selective since the number of potential drug binding sites in DNA should be reduced (20). Indeed, a striking feature of the binding of WP631 to the AdSP01 promoter in our present study was that the C_{50} of WP631 (60 nM) was much lower than that required to inhibit the basal *in vitro* initiation of the AdML promoter (480 nM). Though a WP631 binding site should ideally contain two CpG steps separated by another 2 bp, the consensus Sp1-binding site was slightly longer (Fig. 2), which meant WP631 had to bind to the 5'-CGC-CCCGCCCCG-3' sequence (5'-CGGGGCGGGGCG-3' in the complementary strand). Hence, it is likely that the binding of WP631 to the AdSP01 promoter was facilitated by an alteration of the DNA structure after protein binding, which would have somewhat reduced the distance between the CpG steps. Three CpG steps are available for intercalation, though the occupancy of two CpG steps by one molecule would exclude a second molecule from the binding region. DNase I footprinting analysis (Fig. 7) confirmed that WP631 and Sp1 can bind to the Sp1-binding site in AdSP01 at drug concentrations that only partially disrupt the DNA–protein complex, as observed by band-shift analysis (Fig. 6a). It also denoted that spacious overlapping of the Sp1 and drug binding sites might be required to inhibit the Sp1-activated transcription *in vitro*, a result that would be consistent with the footprinting patterns generated by other drugs binding to an Sp1-binding site (10,14,16).

Figure 8 shows a schematic model of WP631 binding to DNA based on previous structural analyses (23,24). For the sake of comparison, Figure 8A and B corresponds to two possible binding modes of WP631 to a 6 bp-long DNA (22). Figure 8C and D offers tentative explanations for our striking results. Figure 8C shows two molecules that are monointercalated. However, this simple model cannot explain the strong effect of the bisanthracycline WP631 on the Sp1–DNA complex. As Figure 8D shows, we therefore assumed that Sp1 bends the DNA. The bending of the DNA target has been described before [(29) and references therein]. Not only could this bending be essential for the Sp1-activating function, but it could also favor more efficient binding of WP631, thus allowing inhibition of an activated promoter such as AdSP01 at concentrations lower than those required in basal transcription (Table 1).

Sp1 induces a distortion in DNA structure (29,32) and can act synergistically with other protein factors (3,6,7). Our model (Fig. 8D) would explain our results convincingly, while remaining consistent with the emerging hypothesis that DNA plays the role of an allosteric ligand whose binding might alter the binding affinity of other ligands (33). In such a model, the Sp1 protein could act as an allosteric effector, facilitating the binding of WP631 to DNA. If so, then this ability of Sp1 to facilitate WP631 binding might explain why the concentration

of WP631 versus daunomycin required to inhibit transcription from the AdML promoter was only ~1.5 times lower during basal transcription but about 15 times lower in Sp1-activated transcription.

Another likely explanation for the enhanced effect of WP631, but one still compatible with this model, would be that the observed inhibition of the Sp1-activated promoter was due to a larger residence time of WP631 versus daunomycin in this particular DNA sequence. We note that WP631 binds with high affinity ($K_b > 2.7 \times 10^{11}$ M^{-1}) to herring sperm DNA (21,22), and that this binding constant is close to the values of the constant calculated for Sp1 binding to a consensus recognition site (34). In addition, we would also expect the WP631 binding constant to be larger for the preferred binding sites like CGTACG or CGCGCG (24). On the other hand, the binding of the Sp1 transcription factor to DNA appears to be a multistep process (5). If, for example, more than one intermediate is formed due to the different contribution of the three zinc fingers in the protein or to DNA bending, then it is feasible that the DNA–protein complex might first facilitate WP631 binding and thereafter become disrupted by the intercalated drug. In this respect, it is noteworthy that the strong effect of WP631 on AdSP01 was rather distinct and, therefore, presumably specific, while the daunomycin concentrations needed to inhibit the transcription of either AdSP01 or AdML promoters were similar.

The results presented here have both practical and theoretical implications for the analysis and design of new DNA-binding drugs. Together with previous results (10,15–17), they favor a general model in which drugs and transcription factors compete for the same sequences. It is worth noting that daunomycin and WP631 were capable of challenging the *in vitro* transcription of either a basal or a transactivated strong promoter. This experimental situation was not limited to the first round of transcription, but continued over 60 min (Figs 3 and 4).

Although WP631 appears to prefer binding to CGTACG versus CGCCCG sequences (24), we have shown here that WP631 can strongly inhibit Sp1-activated transcription initiation *in vitro* by preventing the binding of Sp1 to the sequence CGGGGCGGGGCG, a sequence clearly distinct from a CGTACG binding site (23). Thus, it should be possible to improve the DNA affinity of bisintercalators like WP631 and therefore their ability to challenge *in vitro* transcription from strong promoters. In the case of WP631, its *p*-xylyl tether could be replaced by another kind of chemical bridge. Hence, we could try to avoid clashes between the linker and the C2-amino groups of guanines, which are, for example, very abundant in the Sp1-binding site. Likewise, we could more selectively discriminate between DNA sequences by enlarging the drug binding site to make it compete with protein factors for binding to the same DNA fragment. Ultimately, the selective targeting of a transcription factor might then provide a way to interfere with transcription regulatory processes *in vivo*.

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