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# TAPP (tetra-*p*-amidinophenoxyneopentane) inhibits the binding of nuclear factors to target DNA sequences

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## Summary

The aromatic polyamidine tetra-*p*-amidinophenoxyneopentane (TAPP), a compound exhibiting strong anti tumor activity both 'in vivo' and 'in vitro', was tested for the ability to bind DNA and to interfere with protein-DNA interactions. In this paper we show that TAPP binds DNA and inhibits the binding of the nuclear protein factors GTATA/IFN-γ, OTF-1, NFE-1 and Sp1 with specific target DNA sequences present in the HLA-DRα gene, in the γ-globin gene and in the LTR of HIV-1. These inhibitory effects of TAPP are comparable to those of distamycin, a DNA-binding drug able to suppress DNA-protein interactions. The gel retardation approach described in the present paper could represent a powerful tool to identify potential antitumor and antiviral drugs.

## Introduction

The aromatic polyamidine tetra-*p*-amidinophenoxyneopentane (TAPP) is a strong inhibitor of both in vitro tumor cell growth (Nastruzzi et al., 1988a,b, 1989a) and in vivo tumorigenicity of neoplastic cells xenografted into nude mice (Bartolazzi et al., 1989). A number of papers suggest that one of the mechanisms of action of these and structurally related drugs could be the inhibition of tumor-associated proteinases (Nastruzzi et al., 1989a,b, 1989b; Gambari et al., 1990a). In line with this hypothesis is the finding that aromatic polyamidines inhibit in vitro invasiveness of tumorigenic cells (Gambari et al., 1990a), a process which appears to be largely dependent on the secretion of proteinases by tumor cells (Liotta, 1986). In addition, TAPP is a strong inhibitor of a variety of proteinases, including kallikrein, factor Xa, urokinase, chymotrypsin and trypsin (Menegatti et al., 1982, 1987; Ferroni et al., 1984). Due to their in vitro effects on serine proteinases, aromatic polyamidines have been proposed for the control of a large spectrum of disease processes, such as inflammation, thrombosis and complement-dependent immune reactions (Geratz et al., 1973). On the other hand, a recent report from our laboratory suggests that TAPP may exert its antiproliferative effects on tumor as well as on normal cells through alternative mechanism(s) of action

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(Gambari et al., 1990a). The observation that the chemical structure of TAPP is similar to that of some DNA-binding drugs such as berenil (Neidle et al., 1987), hydroxystilbamidine (Festy and Daune, 1973) and 4′,6-diamidino-2-phenylindole (DAPI) (Portugal and Waring, 1988) prompted us to investigate (a) possible interactions between TAPP and DNA and (b) possible interference by TAPP of the binding of nuclear transcriptional factors to cis-elements of promoter sequences.

With this aim we performed gel retardation experiments using nuclear extracts from a variety of cells and synthetic double-stranded oligonucleotides mimicking the promoter regions of eukaryotic and viral genes (HLA-DR$\alpha$, $\gamma$-globin, HIV-1) (Das et al., 1981; Jones et al., 1986; Martin et al., 1989) containing the target sequences of transcriptional factors (GTATA/IFN-$\gamma$-B3, NFE-1, OTF-1, Sp1) (Briggs et al., 1986; Martin et al., 1987; Barbieri et al., 1990).

## Materials and Methods

*Cell lines and culture conditions*

The human leukemic K562 (Lozzio and Lozzio, 1972), the melanoma Colo 38 (Gambari et al., 1987) and the B-lymphoid WI-L2 (Nastruzzi et al., 1988a) cell lines used in this study were grown in $\alpha$-medium (Gibco) supplemented with 10% FCS in 5% $CO_2$ at 37°C.

*Synthetic oligonucleotides*

The sequences of the synthetic oligonucleotides used in this study are shown in Fig. 3. The $-156/-201$ oligonucleotide of the $\gamma$-globin promoter ($\gamma$-globin mer) was a gift from Professor Sergio Ottolenghi, Ist. Genetica Medica e dei Microorganismi, Milano, Italy. GTATA/IFN-$\gamma$ and Sp1 mers were synthesized using a Pharmacia Gene Assembler Plus DNA synthesizer and the phosphoramidite method. Equimolar amounts of each strand were 5′-labelled with [$\gamma$-$^{32}$P]ATP by the enzyme T4 polynucleotide kinase (Genenco), combined, heated for 5 min at 80°C in 0.5 M NaCl and annealed for at least 30 min at room temperature. The complete annealing of the two molecular species was assessed by comparing the migration path of the DNA duplex with that of single strand oligonucleotides after electrophoresis in a 16% low ionic strength polyacrylamide gel.

*Electrophoretic mobility shift assay*

The electrophoretic mobility shift assay (for brevity: gel retardation) was performed as originally described (Fried and Crothers, 1981) with minor modifications (Barbieri et al., 1990). Nuclear extracts were prepared according to Dignam et al. (1983) at a protein concentration (BCA assay, Pierce Rockford, IL, U.S.A.) of 1–5 mg/ml. Binding reactions were, unless otherwise specified, set up in binding buffer (20 mM Tris-HCl, pH 7.6, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 0.01% Triton X-100, 5% glycerol, 0.5 mM spermidine), in the presence of increasing amounts of poly(dI : dC) · poly(dI : dC) (Pharmacia, Uppsala, Sweden), 1 $\mu$g of nuclear extract proteins and 0.25 ng of end-labelled double-stranded oligonucleotides (approx. 50 000 Cerenkov counted cpm), in a total volume of 25 $\mu$l. After 30 min at room temperature, samples were electrophoresed at constant voltage (300 V for 2 h) through a low ionic strength (0.35 × TBE buffer) (1 × TBE = 0.089 M Tris-borate, 0.089 M boric acid, 0.008 M EDTA) on 10% polyacrylamide gels until the tracking dye (bromophenol blue) reached the end of a 16 cm slab. Gels were dried and exposed to X-Omat Kodak films at $-80$°C with intensifying screens. The order of addition of the reagents was the following: (a) poly(dI : dC) · poly(dI : dC); (b) labelled oligonucleotides; (c) binding buffer; (d) nuclear extracts (Barbieri et al., 1990).

## Results

*Binding of TAPP to DNA*

Fig. 1 shows the chemical structure of TAPP and those of other polyamidines reported elsewhere to display DNA-binding activity, such as berenil (Neidle et al., 1987), hydroxystilbamidine (Festy and Daune, 1973) and 4′,6-diamidino-2-phenylindole (DAPI) (Portugal and Waring, 1988). Fig. 2 shows a chromatographic run through a Sephadex G-200 column of (A) sonicated salmon sperm DNA (ssDNA), (B) free TAPP and (C) an

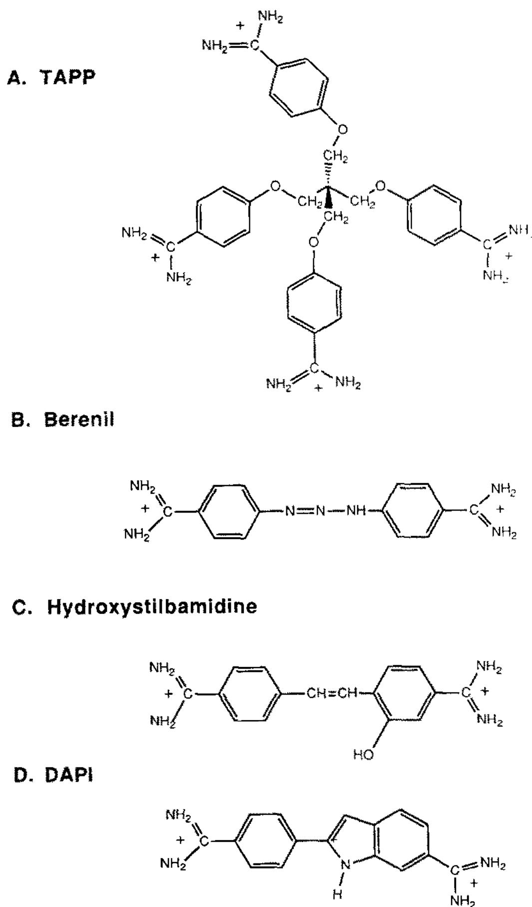


Fig. 1. Structures of (A) tetra-*p*-amidinophenoxyneopentane (TAPP), (B) berenil, (C) hydroxystilbamidine and (D) DAPI (4′,6-diamidino-2-phenylindole).

ssDNA/TAPP mixture. As clearly evident in this latter case, TAPP co-elutes with ssDNA, therefore suggesting that TAPP-DNA interactions occur under these experimental conditions. In agreement with this interpretation, TAPP was also found to retard the electrophoretic mobility of double-stranded oligonucleotides on polyacrylamide gels (data not shown).

*Binding of nuclear factors to synthetic oligonucleotides mimicking cis-elements of eukaryotic and viral genes*

Fig. 3(A,B) shows the sequences of the upstream regions of the human HLA-DR$\alpha$ and $\gamma$-globin genes. In addition, Fig. 3C shows the HIV-1 regulatory LTR region containing the Sp1 and NF-kB consensus elements (Jones et al., 1986). As reported elsewhere (Briggs et al., 1986; Martin et al., 1987; Barbieri et al., 1990), these regions are specifically recognized by nuclear factors. The synthetic oligonucleotides used in the present investigation are also reported in Fig. 3(A–C). When gel retardation experiments are performed using nuclear extracts and the $^{32}$P-labelled double-stranded GTATA/ IFN-$\gamma$, $\gamma$-globin and Sp1 oligonucleotides, typical gel retardation patterns are obtained as shown in panels D–F of Fig. 3.

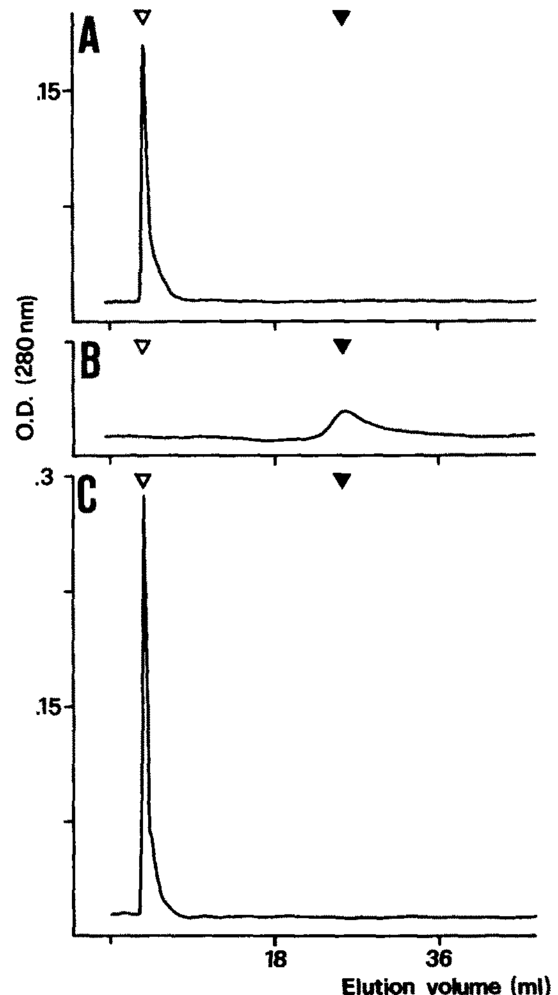


Fig. 2. Binding of TAPP to DNA. Sephadex G-200 chromatography of (A) 80 $\mu$g of sonicated salmon sperm DNA, (B) 25 $\mu$l of a 2 mM solution of TAPP and (C) TAPP pre-incubated for 10 min with ssDNA. (▽) ssDNA; (▼) free TAPP.

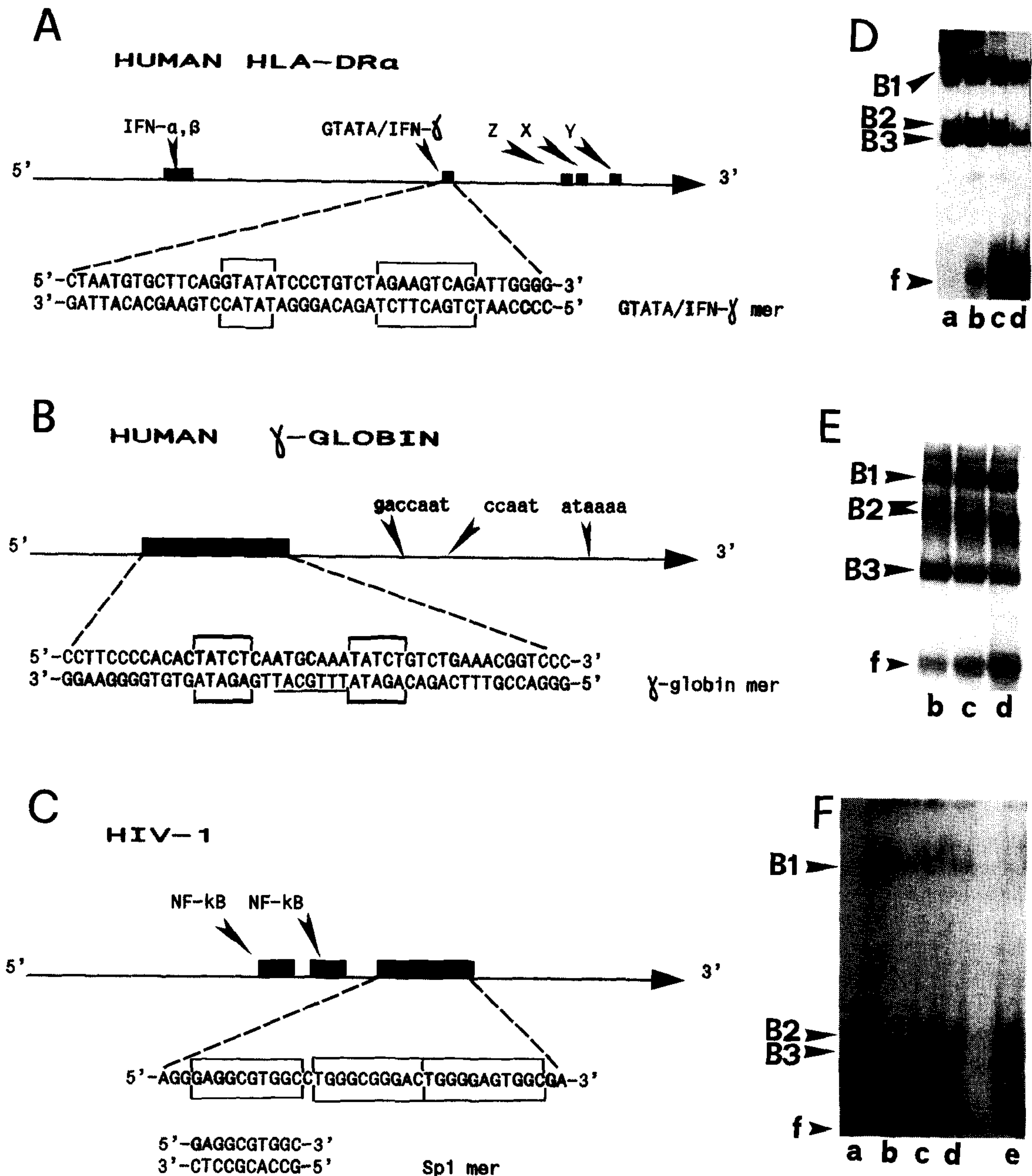


Fig. 3. Location of the DNA elements of (A) human HLA-DRα gene, (B) human γ-globin gene and (C) HIV-1, recognized by the GTATA/IFN-γ (DRα), NFE-1 and OTF-1 (γ-globin) and Sp1 (HIV-1) nuclear factors. Starts of transcription are indicated by arrows. DNA sequences involved in the binding of GTATA/IFN-γ, NFE-1 and Sp1 factors are boxed, the octamer motif recognized by OTF-1 (γ-globin gene) is underlined. The sequences of the synthetic oligonucleotides (GTATA/IFN-γ mer, γ-globin mer and Sp1 mer) employed in the gel retardation experiments are reported. In panels D–F typical gel retardation experiments are shown in which the $^{32}$P-labelled mers have been incubated in 25 μl reaction in the presence of nuclear extracts and poly(dI : dC) · poly(dI : dC) (a, 50 ng; b, 100 ng; c, 200 ng; d, 400 ng; e, 800 ng). After 15 min the reaction mixtures are electrophoresed through a 10% polyacrylamide gel. The retarded bands identifying the nuclear factors are indicated. f, free oligonucleotides. In these experiments nuclear extracts from the B-lymphoid WI-L2 (D), erythromyeloid K562 (E) and melanoma Colo 38 (F) cell lines have been used. GTATA/IFN-γ factor is identified in panel D by the B3 retarded band (Barbieri et al., 1990); OTF-1 and NFE-1 factors are identified in panel E by the B1 and B2/B3 bands, respectively (Martin et al., 1989); the Sp1 monomer and dimer are identified in panel F by the retarded B2 and B3 bands (Briggs et al., 1986).

Specificity was assessed, as described elsewhere (Barbieri et al., 1990), by the effects on the gel retardation pattern of increasing concentrations of poly(dI : dC) · poly(dI : dC). For instance, the B1 and the B2 factors which strongly bind to the GTATA/IFN-γ mer at low poly(dI : dC) · poly (dI : dC) concentrations (50–100 ng/25 μl of reaction) tend to disappear when greater amounts (400 ng/25 μl of reaction) of poly(dI : dC) · poly(dI : dC) are used (Fig. 3D).

Conversely, the specific B3 retarded band, but not B1 and B2 disappear when 50–100 ng of cold GTATA/IFN-γ mer is included in the reaction (data not shown). Similar results were obtained with the γ-globin mer and with the Sp1 mer.

In accordance with data previously published by our and other groups, in the case of the human HLA-DRα gene the B3 retarded band (Fig. 3D) identifies the GTATA/IFN-γ factor (Barbieri et al., 1990); in the case of the γ-globin gene, the B1 identifies OTF-1, while the B2/B3 retarded bands identify the NFE-1 factor (Fig. 3E) (Broggini et al., 1989; Martin et al., 1989); in the case of HIV-1 the retarded bands B2/B3 (Fig. 3F) identify binding of Sp1 to the Sp1 10-mer (Briggs et al., 1986).

*Effects of TAPP on the binding of nuclear factors to target DNA sequences*

Fig. 4(A–C) shows that TAPP inhibits the binding of GTATA/IFN-γ-B3, OTF-1, NFE-1 and Sp1 nuclear factors to target sequences present in the synthesized oligonucleotides. In these experiments the TAPP concentration needed to obtain a 50% inhibition of nuclear protein binding to the $^{32}$P-labelled mers is about 25–50 μM for

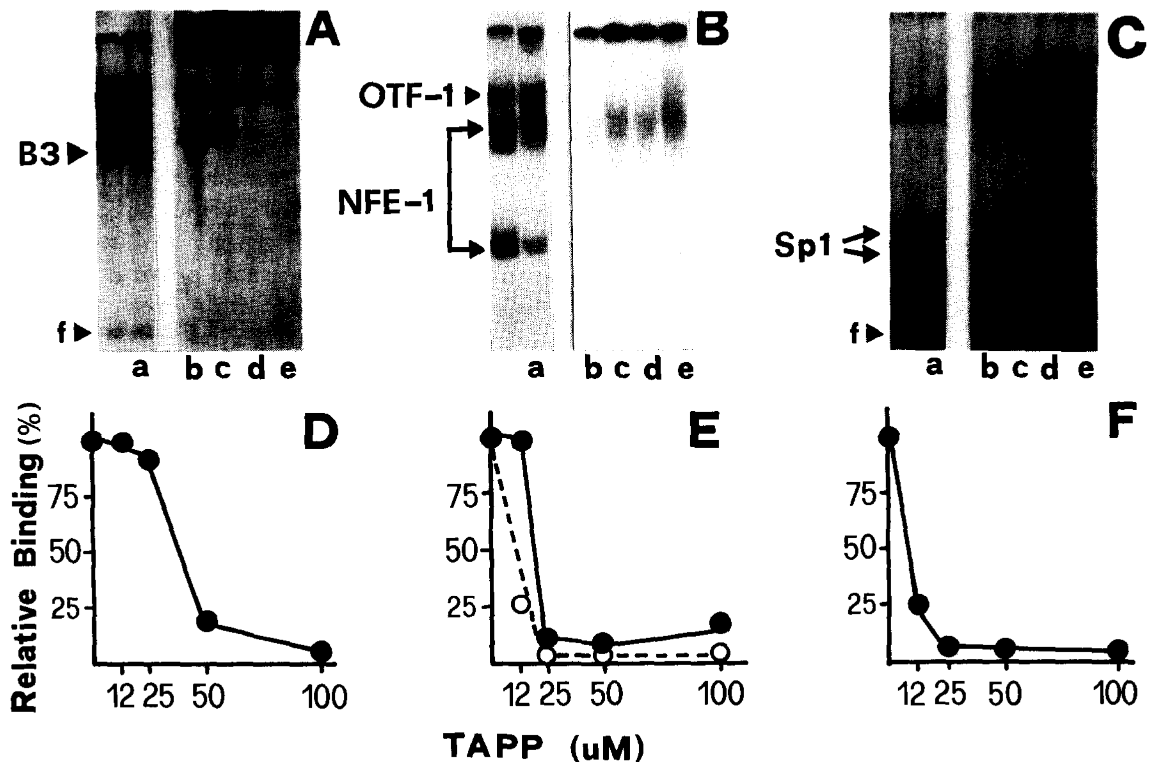


Fig. 4. Effects of TAPP on the binding of nuclear factors to the GTATA/IFN-γ (A,D), γ-globin (B,E) and Sp1 (C,F) mers. Binding reactions were performed in the presence of 400 ng of poly(dI : dC) · poly(dI : dC) and the 12 (a), 25 (b), 50 (c), 100 (d), 200 (e) μM TAPP. Incubations of $^{32}$P-labelled mers with nuclear extracts were carried on for 15 min and then the reaction mixtures were electrophoresed through a 10% polyacrylamide gel. Retarded bands identifying the nuclear factors are indicated. f, free oligonucleotides.

GTATA/IFN-$\gamma$, 6–12 $\mu$M for the $\gamma$-globin mer and 6 $\mu$M for Spl (Fig. 4D–F).

The effects of TAPP on the $\gamma$-globin mer deserve further comment, since two factors, OTF-1 and NFE-1, are able to bind to this oligonucleotide (Martin et al., 1987). Densitometric analysis (Fig. 4E) suggests that the binding of the erythroid specific NFE-1 transcriptional factor is slightly more sensitive to TAPP addition than the binding of OTF-1.

The effects described in Fig. 4 are comparable to those observed when we add to the binding reaction the well-known DNA-binding drug distamycin (Gambari et al., 1990b), that binds preferentially AT-rich sequences (Arcamone et al., 1989) and it has been recently reported to be able to inhibit a variety of molecular events related to DNA-protein interactions (Broggini et al., 1989; Gambari et al., 1990b).

## Discussion

The aromatic polyamidine tetra-*p*-amidinophenoxyneopentane (TAPP) is a strong inhibitor of proteinases, including trypsin, kallikrein, chymotrypsin and factor X. Therefore, this and other synthetic proteinase inhibitors have been suggested for the experimental treatment of a number of human pathologies, notably in pulmonary emphysema (Trainor, 1987) and cancer (Nastruzzi et al., 1989a; Gambari et al., 1990a).

The main conclusion gathered from the results presented in this paper is that TAPP does interfere with the binding of nuclear transfactors with specific target sequences. This effect is comparable to that observed with distamycin, a DNA-binding drug which does selectively bind to AT-containing DNA elements (Arcamone et al., 1989) and is able to suppress DNA-protein binding (Broggini et al., 1989; Gambari et al., 1990b). The gel retardation approach described in the present paper could represent a powerful tool to screen for potential antitumor and antiviral drugs. The inhibition of protein-DNA interactions described in this paper (Fig. 4) is likely to be due to an interaction between TAPP and DNA, since as suggested by Fig. 2, TAPP is able to bind DNA. In addition, the chemical structure of TAPP is homologous to that of berenil, DAPI and hydroxystilbamidine, all of which exhibit DNA-binding capability (Festy and Daune, 1973; Neidle et al., 1987, Portugal and Waring, 1988). Despite the fact that our data do not demonstrate that TAPP binds DNA in intact cells, they do suggest that this is one of the mechanisms accounting for the strong antiproliferative effects of this compound on tumor as well as normal cells (Nastruzzi et al., 1989a). This hypothesis is in agreement with previously published data suggesting that TAPP is more active than other known proteinase inhibitors (leupeptin, antipain, Bowman-Birk, PMSF, benzamidine) in inhibiting the growth of neoplastic cells (Gambari et al., 1990a).

We hypothesize that TAPP could inhibit gene transcription by altering the activity of transacting factors. With respect to this point we like to emphasize that the binding of transfactors to cis-elements of retroviral genomes is an essential prerequisite to activate transcription (Wingender, 1988). For instance, during the activation of latent or persistent forms of HIV-1 in AIDS (acquired immune deficiency syndrome), the interaction between both host cell factors (NF-kB, Spl) and viral proteins (tat) to specific target DNA sequences is a critical step for (a) transcriptional activation of fully integrated provirus and (b) stability of unintegrated viral reverse transcribed DNA (Jones et al., 1986; Greene et al., 1990). On the other hand, DNA binding drugs usually exhibit antibacterial activity (Arcamone et al., 1989). Accordingly, the results presented in this paper appear to be of some interest, as they suggest that TAPP and related drugs could be proposed as anti-bacterial and/or antiviral agents. On the other hand, we would like to point out that, as for other DNA-binding drugs (such as distamycin and daunomycin) (Arcamone et al., 1989), TAPP treatment of experimental animals could lead to long-term side effects (such as neoplastic transformation). It is indeed well known that activation of cellular oncogenes could be due to pharmacologically mediated inhibition of the binding of negative transcriptional factors to specific DNA sequences (Varmus, 1984).

Therefore, our data indicate that strict precau-

tions are required in using TAPP and related compounds in the experimental therapy of disease processes, such as those occurring during inflammation, thrombosis and complement dependent immune reactions (Geratz et al., 1973), due to its DNA-binding properties.

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