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COMMENTARY

DNA-BINDING ACTIVITY AND BIOLOGICAL EFFECTS OF AROMATIC POLYAMIDINES

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The recent advances in molecular biology, especially those related to the study of the regulation of transcription in eukaryotes, prokaryotes and viruses, have introduced a number of technologies that have proven to be very useful in increasing our understanding of the molecular events responsible for the control of gene expression [1–6]. These advances have both theoretical and practical implications, since a large number of molecular events governing gene expression could be possible targets of antitumor and antiviral drugs [7–13].

Among the most important achievements in biochemical pharmacology is the demonstration that many antitumor and antiviral durgs (a) exert their biological activity through extensive interactions with DNA [7–12], and (b) exhibit some degree of sequence selectivity [13, 14]. This is very important, since the interaction between transcriptional factors and specific DNA motifs present in the promoters of eukaryotic genes [1, 4] and in the long terminal repeats (LTRs)‡ of retroviruses [6, 15–18] is one of the most important steps for transcriptional activation of genes in a cell-cycle or differentiation-specific manner.

With these considerations in mind, the design, synthesis and testing of DNA-binding drugs displaying sequence selectivity [13, 14] are of great interest. This would, in principle, direct the biological effects of such compounds to regulatory DNA sequences of a limited number of genes, potentially leading to specific alteration of transcription.

In this commentary, we review some of the biological activities and DNA-binding properties of aromatic polyamidines. Among this rather heterogeneous group of compounds [19–38], the most studied molecules are bis-benzamidines, such as stilbamidine [25], berenil [26–28], 1,5-bis(*p*-amidinophenoxy)pentane (pentamidine) [29–33], 1,3-bis(*p*-amidinophenoxy)propane (propamidine or DAPP) [34, 35] and 4′,6-diamidino-2-phenylindole (DAPI) [36] (see structures depicted in Fig. 1). In addition, our research group recently conducted a number of experiments on the biological activity and mechanism of action of tetra-benzamidine compounds, such as tetra-*p*-amidinophenoxy-neopentane (TAPP) [34, 35, 37–40].

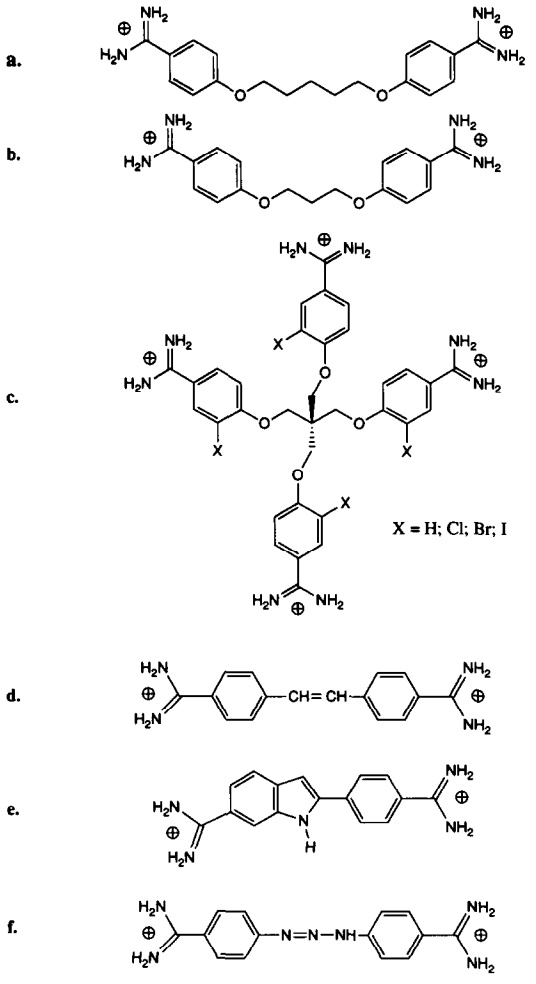

Fig. 1. Chemical structures of six aromatic polyamidines for which biological activities and DNA-binding properties have been described. For chemical synthesis and characterization of the compounds reported in the figure, see Refs. 19–24, 31 and 32. Key: (a) pentamidine; (b) propamidine; (c) TAPP; (d) stilbaminide; (e) DAPI; and (f) berenil.

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‡ Abbreviations: LTRs, long terminal repeats; DAPI, 4′,6-diamidino-2-phenylindole; TAPP, tetra-*p*-amidinophenoxy-neopentane; GTFs, general transcriptional factors; EMSA, electrophoretic mobility shift assay; PCR, polymerase-chain reaction; and NRE, negative regulatory element.

Concerning the nomenclature of the compounds analysed in the present commentary, it should be underlined that the name "pentamidine" refers to the number of carbon atoms constituting the major hydrocarbon chain of the compound and not to the number of benzamidine residues (in contrast to the tetra-benzamidine TAPP).

BIOLOGICAL EFFECTS OF AROMATIC DI- AND POLY-AMIDINES

Many reports are available showing that aromatic polyamidines are antimicrobial agents proven to be useful for the therapy of a number of protozoal infections [for a partial list of recently published papers, see Refs. 30, 31, 41–44]. For instance, the bis-benzamidine pentamidine was demonstrated to be an effective agent for the treatment of *Pneumocystis carinii* pneumonia, an opportunistic infection very common in AIDS patients [45, 46].

A comparative screening of the activities of 33 analogues of pentamidine (used at a concentration of 10 mg/kg/day given intravenously, with the exception of compounds displaying low solubility or high toxicity) against a rat model of *P. carinii* pneumonia demonstrated that most of the compounds tested showed biological activity, leading to an absence of intense areas of focal infections with only scattered cysts present in less than 10% of the lungs [30]. Furthermore, some of the compounds analysed exhibited a much lower toxicity when compared with pentamidine [30].

The biological effects of aromatic polyamidines are not restricted to the infection causecd by *P. carinii*, since recent reports showed that this class of compounds also can be used against *Trypanosoma brucei*, *Trypanosoma rhodesiense* and other agents of infectious diseases [40]. In fact, significant *in vitro* activity of aromatic diamidines against *Giardia lamblia*, *Plasmodium falciparum* and *Leishmania mexicana* has been reported [30–32].

Aromatic di- and tetraamidines, including DAPP and TAPP, also have been described to inhibit the growth of both normal and transformed *in vitro* cultured cell lines [34, 35, 37]. For instance, our research group correlated the *in vitro* effects of aromatic polyamidines on cell proliferation to alterations of cell cycle parameters, analysed by propidium iodide staining of cells and flow microfluorometry [47]. The data obtained suggest that aromatic tetraamidines inhibit the progression of tumor cells through all cell-cycle phases (namely G_1, early S, late S, G_2). The lengthening of the different phases of the cell cycle is not the only biological effect on tumor cell lines. Indeed, aromatic tetraamidines display other interesting *in vitro* activities: despite not inhibiting the attachment of neoplastic cells to *in vitro* reconstituted basement membrane, they strongly inhibit oriented migration of tumor cells and invasion of tumor cells through Matrigel™ [40, 48]. This effect is rather general and was obtained by studying the human breast cancer MCF7 cell line [40] as well as keratinocytes and fibroblasts transformed to a tumor phenotype by transfection with activated Ki-*ras* and Ha-*ras* oncogenes, respectively [48].

These results are of interest, especially when related to data reported elsewhere [23, 24, 49, 50], demonstrating that aromatic polyamidines exhibit antitumor activity when administered *in vivo*. A preliminary report by Parsons [49] described a tris-benzamidine that was able to inhibit, to some extent, the *in vivo* growth of Sarcoma 180 tumors subcutaneously implanted in hybrid mice. In addition, experiments from our group demonstrated that TAPP-Br (the 2′-bromo-derivative of TAPP; see structure depicted in Fig. 1) inhibits the *in vivo* growth of human melanoma M10 cells xenografted into nude mice [50]. Similar *in vivo* antitumor activity was demonstrated when TAPP-Br was injected into nude mice xenografted with the highly tumorigenic FH06T1-1 cells (Chinese hamster fibroblasts transformed to neoplastic phenotype by integration of multiple copies of the activated human Ha-*ras*-1 oncogene under the transcriptional control of SV40 and MoMLV enhancers) [51, 52]. Furthermore, recent data from our laboratory indicate that when TAPP-Br is topically applied to nude mice by using lecithin gels, it can be percutaneously absorbed, leading to a sharp inhibition of the *in vivo* growth of tumors previously implanted by subcutaneous injection of FH06T1-1 cells [52, 53].

Another biological activity of these compounds is related to their potential antiviral properties. In our hand, TAPP-Br was found to inhibit the *de novo* infection of CCRF-CEM cells by HIV-1 (Federico M. and Gambari R. unpublished results). This interesting effect, however, has been obtained with drug concentrations that are very close to those causing antiproliferative activity.

With respect to the antiproliferative activity, it is well established, from both *in vitro* and *in vivo* studies, that aromatic polyamidines can cause toxic effects [30, 35, 43–46, 50], leading, for instance, to a decrease in body weight of the treated animals [50]. Concerning these adverse effects, it should be pointed out that the toxicity of this class of compounds can be overcome by a variety of drug-delivery approaches. In this respect, we have elsewhere reported that TAPP-Br and related compounds can be efficiently entrapped in liposomes [54] and in gelatin [55] or pullulan [56] microspheres. In all cases, we have demonstrated that the biological activity of these compounds towards *in vitro* cultured tumor cell lines is maintained or even increased when they are delivered by liposomes or microspheres. With respect to delivery methods, it should be underlined that pentamidine is usually administered in aerosolized free [41, 42, 57] or liposomal [58] forms to AIDS patients affected by *P. carinii* pneumonia, in order to avoid the high toxic effects of the drug. Despite the fact that the biological effects of aromatic polyamidines have been well described, suggesting interesting therapeutical applications in a variety of human diseases, the molecular mechanism(s) of action of these compounds is far from being fully elucidated.

One of the mechanisms of action of aromatic polyamidines could be related to the antiproteinase activity demonstrated by these compounds [59–62]. Indeed, many authors have reported that TAPP [38, 39, 61] and pentamidine [59, 60] inhibit a large

spectrum of serine proteinases, including trypsin, kallikrein, chymotrypsin, and factor Xa. Structure–activity studies indicate that aromatic polyamidines containing two, three and four benzamidine rings are all active against serine-proteinases [62]. In addition, halo-derivatives of di- and polyamidines retain comparable antiproteinase activity. On the contrary, haloderivatives are much more active than their non-halogenated counterparts in inhibiting *in vitro* tumor cell growth [35]. This discrepancy between antiproteinase activity of *in vitro* inhibition of cell proliferation could be explained in view of recent results (summarized in the next section) demonstrating that these drugs display DNA-binding properties.

DNA-BINDING PROPERTIES OF AROMATIC POLYAMIDINES

No conclusive evidence exists to fully elucidate the molecular basis of *in vitro* and *in vivo* biological effects of aromatic polyamidines. Speculative explanations for the mechanism of action of aromatic polyamidines, however, should take into consideration the large number of reports showing that these compounds display strong DNA-binding activity.

As already pointed out, recent studies in molecular and biochemical pharmacology have demonstrated that a large number of antitumor/antiviral drugs that interact with DNA exhibit some degree of sequence selectivity, this feature possibly being responsible for their biological activity [63–72]. Therefore, a number of laboratories started a systematic analysis of aromatic di- and polyamidines with respect to their ability to bind to nucleic acids (especially DNA). Aromatic diamidines, such as berenil [7, 26, 28, 73, 74], DAPI [36], pentamidine [32, 33, 75, 76] and stilbamidine [25] (see chemical structures in Fig. 1), were employed as model compounds to elucidate the binding of small molecules to DNA, showing that these molecules can interact non-covalently with the minor groove of DNA. The molecular basis of these interactions has been studied by using different methods that, alone or in combination, can define the binding modalities at the molecular level.

With this aim, many research groups in the past few years have utilized (a) biophysical methods, including UV, CD and fluoresence spectroscopy [25, 36], (b) X-ray crystallographic [76] and molecular modelling [13, 32, 76] techniques, together with "in solution" ^{1}H and ^{31}P NMR one- and two-dimensional spectroscopy [28], and (c) molecular biology techniques such as DNase I and hydroxyl radical footprinting [73, 75]. By using these experimental approaches, it has been shown that polyamidines can interact with the minor groove of DNA double helix in an integrated fashion including hydrogen bonds, ionic charge attractions and van der Waals' interactions [7, 76]. These compounds display preferential affinity for AT-rich regions of DNA, at least three–four base pairs long. However, footprinting studies suggest that GC base pairs are allowed in the close proximity (or at the ends) of the binding box sequences [76]. In addition, some of these compounds, including DAPI, display significant interactions with GC sequences [76].

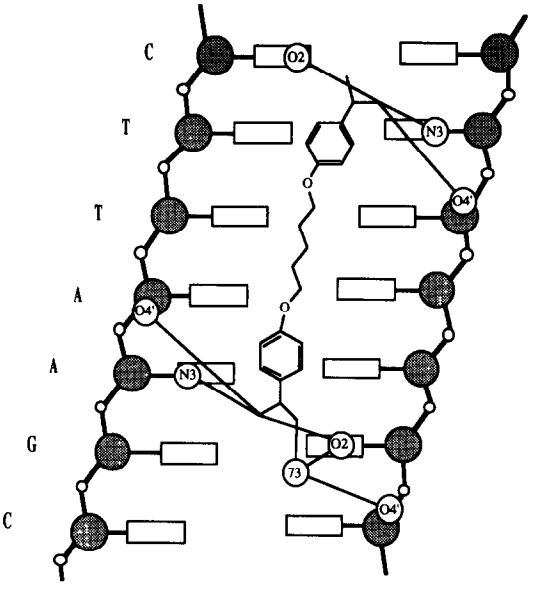

Fig. 2. Schematic representation of the interaction modalities between pentamidine and double-stranded DNA (adapted from Ref. 33).

Different factors seem to be responsible for the AT binding preferences of the positively charged amidinic compounds [76]. Indeed, it has been suggested that in a DNA molecule the deepest negative charge potential resides in the bottom of the minor groove of AT stretches [26]. In addition, AT portions, compared to the CG portions, could have a more pronounced tendency to assume conformations characterized by the narrowest minor groove, providing in this way a positive effect on the formation of van der Waals' stabilizing interactions between DNA and drugs [76]. Figure 2 is a schematic representation of the interactions occurring in the binding complex between pentamidine and DNA [76]. The fact that different DNA-binding drugs can specifically recognize and bind to different DNA conformers or sequence regions represents a very important feature. Indeed, gene expression is strictly regulated by interactions between transcriptional factors and motifs present in eukaryotic and viral DNA regions.

RELEVANCE OF PROTEIN–DNA INTERACTIONS IN THE REGULATION OF GENE EXPRESSION

In both eukaryotic cells and viruses, regulation of gene expressions at transcriptional level is a complex process that requires the concerted participation of several factors [1–6, 77–83]. The knowledge of the interaction between promoters and the large variety of nuclear proteins involved in the constitutive and induced gene expression is a prerequisite to designing DNA-binding drugs able to modify gene transcription in virtue of their sequence-selectivity. Despite the

Table 1. DNA sequences recognized by eukaryotic DNA-binding transcriptional factors

Transcription factor	Sequence bound	Tissue specificity
AP1	TGA(G/C)T(C/A)A	Ubiquitous
ATF	TGACG(C/T)(C/A)(G/A)	Ubiquitous
CRE-BO1	GGGGG	Erythrocytes
Ets-1	(G/C)(A/C)GGA(A/T)G(T/C)	B cells Resting T cells
GATA-1	(T/A)GATAR	Erythroid cells Megakaryocytes Mast cells
HIVEN86A	TGGGGATTCCCA	Activated T cells B-Lymphocytes
MBF-1	(C/T)TAAAAATAA(C/T)(C/T)(C/T)	Myocytes
NF-κB	GGGA(A/C)TN(T/C)CC	Ubiquitous
Oct-1	ATGCAAAT	Ubiquitous
Sp1	(G/T)(G/A)GGC(G/T)(G/A)(G/A)(G/T)	Ubiquitous

Partial list of eukaryotic transcriptional factors identifying DNA regions displaying different nucleotide sequences. Footprinting data [65, 67] and gel retardation experiments [41, 54, 55, 96] indicate that aromatic polyamidines interact with DNA in a sequence-selective manner, suggesting a differential effect on DNA–protein interactions.

fact that the molecular mechanisms involved in transcription are not yet completely characterized, a number of studies have shown that a large number of DNA-binding transcriptional factors are required for the correct initiation and maintenance of RNA transcription [77]. It is now well established that temporal regulation and tissue specificity of RNA transcription by RNA polymerase II require multiple interactions of nuclear transcriptional factors (TFs) with DNA. In general, TFs recognize and bind to relatively short and specific DNA sequences usually located within the 5′ region of eukaryotic genes (even distally from the transcriptional start site) leading to the positioning of RNA polymerase II with respect to the Inr (initiator) element [84–86]. It should be underlined that transcriptional factors involved with the initiation or RNA transcription could be divided into different classes. First, general transcriptional factors (GTFs) participate in the basal gene transcription by recognizing sequences (for instance the TATA-box) that are located close to the initiation of transcription. For instance, in TATA-containing genes, the preinitiation complex contains, in addition to RNA polymerase II, a number of GTFs, including TFII-D (the TATA binding protein), TFII-A, TFII-B, TFII-E, TFII-F, TFII-H and TFII-J [86]. In most of the TATA-less genes (such as the housekeeping genes), TFII-D is also involved in the formation of a preinitiation complex with the pivotal participation of other factors such as Inr-binding factors and Sp1 [87]. In addition to transcriptional factors involved in the basal gene transcription, TFs are described to be involved in tissue-restricted gene expression and in activation of selected genes by biological response modifiers (such as retinoic acid and interferons) [88].

In Table 1 an example is shown of eukaryotic TFs with their recognized DNA sequences [77]. From the analysis of the DNA binding sequences it appears evident that DNA motifs recognized by TFs are in most cases different in terms of nucleotide sequence. In addition, it should be considered that different TFs may be able to recognize the same (or similar) DNA-binding box.

Taken together, these considerations suggest that the possibility of synthesizing and characterizing DNA-binding drugs with a certain extent of sequence-selectivity could be of great interest. This would, in principle, direct the effects of such compounds on the promoters of a limited number of genes, potentially leading to a selective modulation of transcription of targeted genes.

Concerning the antiviral properties of DNA-binding drugs, the same mechanism of action (on transcription) could be responsible for antiviral activity, leading for instance to an inhibition of the life cycle of retroviruses. With respect to this latter point, due to the mimicry between viral genomic sequences and regulatory motifs present in eukaryotic genes, DNA-binding drugs could operate by preventing stabilizing interactions between the viral genomes and proteins expressed in the infected cells. Whichever these inhibitory effects, the design and production of antitumor and antiviral drugs appear to require an extensive analysis of their effects on DNA–protein interactions. Indeed, this feature is likely to influence the transcription of even endogenous genes, expressed in cells target of virus infection [15].

EXPERIMENTAL APPROACHES TO STUDYING THE EFFECTS OF DNA-BINDING DRUGS ON PROTEIN–DNA INTERACTIONS AND GENE TRANSCRIPTION

By means of techniques commonly employed in molecular biology, it is, in principle, possible to study whether DNA-binding drugs can interact with

a given promoter in a sequence-specific manner, possibly leading to alterations of the complex machinery involved in transcriptional initiation and maintenance [1–3, 77]. Footprinting analyses [89–92], gel retardation experiments [93–96], transcription assays and transfection of eukaryotic cells with reporter genes [97, 98] are currently used to study the effects of drug–DNA interactions. By using these techniques, it is possible to determine whether DNA-binding drugs (a) exhibit sequence-selectivity (this issue is mainly addressed by footprinting analysis); (b) inhibit the interaction between transcriptional factors and target DNA sequences (gel retardation assay); and (c) have any effects on transcription (*in vitro* "run on" transcription assay and transfections with reporter genes, such as the CAT assay) [97–100].

One of the most frequently used molecular biology approaches to studying the binding between transcriptional factors and target DNA elements is the electrophoretic mobility shift assay (EMSA) [93–96]. In a typical experiment, crude nuclear factors or purified recombinant proteins are incubated in the presence of 5′ ^{32}P-labelled double-stranded oligonucleotide containing the target motif(s) of DNA-binding protein(s). The binding between protein and synthetic oligonucleotide leads to an altered electrophoretic migration (see Fig. 3, C and D) of the oligomer (for this reason this technique is also known as the "gel retardation assay" or "band shift assay"). The specificity of the protein–DNA interaction is demonstrated by the maintenance of the shifted complex by increasing the amount of aspecific competitor such as poly(dI)·poly(dC), present in the reaction mixture. On the contrary, a decrease of the intensity of the [^{32}P]DNA–protein complexes after addition of the same unlabelled double-stranded oligonucleotide should be obtained, while no inhibition should be detected by competing the binding with unrelated oligonucleotides [94, 96]. Footprinting studies (see schemes in Fig. 3B) are also very useful in determining whether a given compound selectively binds to specific DNA sequences of a promoter region [89–92].

The results obtained by EMSA and footprinting experiments may help in determining whether the sequence selectivity of a DNA-binding drug is responsible for inhibitory activities on DNA–protein interaction(s), leading to drug-mediated alteration of gene transcription. This could be studied by a number of experimental approaches, including (a) reconstituted *in vitro* transcription of selected promoters [91, 92], and (b) transfection of target cells with promoters directing the transcription of reporter genes not endogenously expressed in the host cells (e.g. the bacterial chloramphenicol acetyltransferase-CAT-gene) [97, 98]. The effects of DNA-binding drugs may be studied by CAT analysis following different strategies. For instance, (a) a given promoter-CAT plasmid could be transfected into target cells expressing TFs specific for the promoter itself, and (b) an expression vector containing a TF cDNA under the control of a strong promoter is co-transfected with the promoter-CAT plasmid (in this case the host cells should not constitutively express the TF encoded by the TF cDNA plasmid) [101]. If in transfected cells, DNA-binding drug inhibits the activity of a TF acting by enhancing gene expression, transcription is expected to be blocked (see Fig. 3E, lane c).

All these techniques require expertise in molecular biology as well as the use of recombinant clones containing the promoter sequences of interest. However, it should be pointed out that the recent description of the polymerase-chain reaction (PCR) methodology gives a number of advantages, including the possibility of specifically amplifying virtually any genomic region encompassed between highly purified PCR oligonucleotide primers [102, 103]. In this case, recombinant DNA is not required for footprinting studies or gel retardation assays.

AROMATIC POLYAMIDINES ARE STRONG INHIBITORS OF THE INTERACTIONS BETWEEN NUCLEAR TRANSCRIPTIONAL FACTORS AND TARGET DNA ELEMENTS

In our laboratory, we have started a project to determine whether aromatic polyamidines inhibit the interaction between DNA and TFs. In addition, we were interested in identifying aromatic polyamidines exhibiting a differential inhibitory activity on TF–DNA binding. From the results obtained in some of our experiments [47, 63, 104], it could be concluded that aromatic polyamidines are strong inhibitors of TF–DNA interactions. By performing EMSA, we showed that the interaction between the DNA-binding proteins EBNA-1 [47], GTATA-IFN-γ [63], GATA-1/NFE-1 [63], OTF-1/Oct-1 [104] and their DNA target sequences is inhibited when aromatic polyamidines are added to the binding reaction mixture. In general, tetrabenzamidines (especially the halo-derivatives of tetra-benzamidines, TAPP-Cl, TAPP-Br and TAPP-I) display a higher activity when compared to di- and tris-benzamidines [47]. The activity of aromatic tetraamidines is comparable to that exhibited by the DNA-binding antibiotic distamycin [66, 104], known to strongly inhibit the interaction between target DNA sequences and DNA-binding proteins [105–107]. The experimental evidence produced during our research program indicates, however, that tetraamidines, when used at high concentrations, generate high-molecular weight complexes with oligonucleotides, PCR products and restriction-enzyme generated DNA fragments (see Fig. 4A), suggesting that these compounds, unlike di-benzamidines, could generate intermolecular bridges between DNA molecules (Fig. 4B). Figure 4C shows that inhibition of protein–DNA interaction by aromatic polyamidines could be analysed by footprinting experiments in which the reaction solution contains DNA–binding protein(s), target oligonucleotides and the compound of interest. In these experimental conditions, the inhibition of DNA–protein interaction is indicated by the observation that the specific protein footprinting is no longer detectable in the presence of the DNA-binding drug (Fig. 4C, lane 3).

Concerning the sequence-selectivity of polyamidines, our data demonstrate that high concentrations of these compounds inhibit not only the interaction between specific TFs and AT-rich DNA

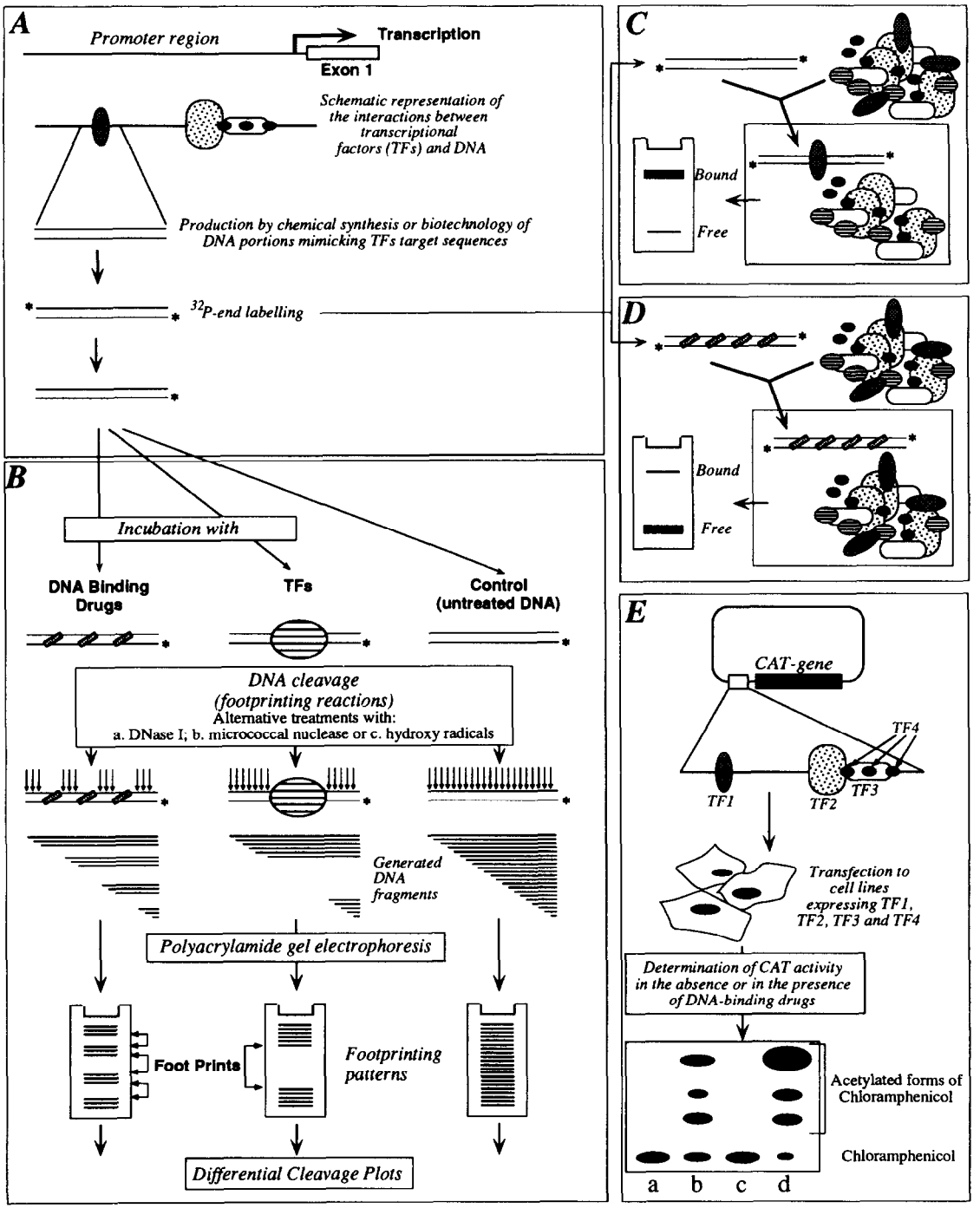

Fig. 3. Molecular biology methods currently employed for the study of the interactions between low molecular weight compounds and DNA. (A) Schematic representation of a hypothetical promoter region containing sequences specifically recognized by transcriptional factors (TFs). (B–E) Major steps in footprinting (B), gel retardation (C and D) and CAT assay (E) experimental protocols. The footprinting assay allows the identification of drug-generated protection from DNase I or chemical cleavage of DNA, indicating the sequences presumably recognized by DNA-binding drugs; gel retardation experiments allow to verify whether DNA-binding drugs interfere with TF–DNA interactions; by the use of CAT assay, information about possible effects of DNA-binding drugs on transcription of cloned promoters can be obtained. In CAT assays, following transfection, CAT activity is measured in cell lysates by determining the extent of generated acetylated forms of chloramphenicol. (a) = no CAT activity; (b–d) = CAT activity of lysates from cells transfected with the CAT plasmid (upper part of panel E) without addition of DNA-binding drugs (b) or in the presence of DNA-binding drugs interfering with the interactions of proteins with enhancer (c) or repressor (d) elements.

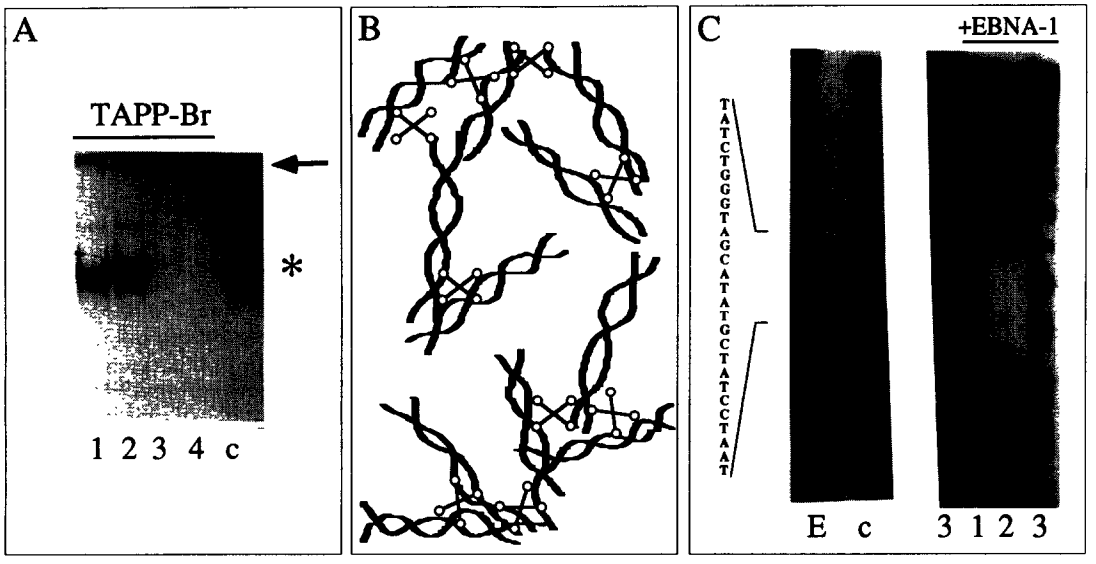

Fig. 4. Complexes generated by interactions between TAPP-Br, a tetra-benzamidine, and DNA fragments. (A) Electrophoretic mobility shift assay demonstrating the effects of TAPP-Br on the interactions between Epstein-Barr Nuclear Antigen 1 (EBNA-1) and a synthetic double-stranded oligonucleotide containing the EBNA-1 target DNA motif (lane c = control; lane 1 = TAPP-Br, 6 μM; lane 2 = TAPP-Br, 12 μM; lane 3 = TAPP-Br, 25 μM; lane 4 = TAPP-Br, 50 μM); the arrow indicates high molecular weight aggregates, tentatively interpreted as TAPP-Br/EBNA-1 oligonucleotide complexes; the asterisk indicates EBNA-1/EBNA-1 mer complexes. (B) Hypothetical representation of the interactions occurring between the tetra-benzamidine TAPP-Br and DNA fragments. (C) DNase I footprinting analysis on a double-stranded oligonucleotide containing the EBNA-1 target DNA motif. DNA fragments generated by DNase I are shown in (c). Footprints generated by EBNA-1 (lane E), TAPP-Br alone (lane 1, 25 μM) or EBNA-1 in the presence of increasing concentrations of TAPP-Br (6 μM, lane 1; 12 μM, lane 2; 25 μM, lane 3) are shown. This experiment demonstrates that 25 μM TAPP-Br inhibits the EBNA-1/EBNA-1 mer interactions, since the DNase I footprinting pattern generated in the presence of both TAPP-Br and EBNA-1 is almost superimposable to that generated by TAPP-Br alone (lanes 3 of panel C).

regions but, to some extent, also the interaction between DNA and transcriptional factors (such as Sp1) preferentially recognizing CG-rich regions [63]. Therefore, despite the fact that Fox *et al.* [75] reported that pentamidine preferentially binds to DNA sites consisting of at least five consecutive AT base pairs, no conclusive evidence indicates a sequence-specificity for di-, tris-, and tetra-benzamidine compounds on DNA–protein interactions. From these considerations, the intranuclear concentration reached after drug administration appears to be crucial to obtaining a selective action of aromatic polyamidines on different TFs. With respect to this point only few reports in the literature are available on a number of parameters that are expected to influence the biological activity of polyamidines. These include (a) cell membrane permeability and mechanism of drug uptake, (b) intracellular compartmentalization and drug metabolism, and (c) influence of delivery approaches (such as liposomes or microspheres) on intracellular steady-state concentrations of the drug.

Some other questions remain unanswered. First, do aromatic polyamidines have any effects on preformed TF/DNA complexes? This is a very important point and results performed with other DNA-binding compounds (distamycin) indicate that in some cases (*Ant*HD/DNA) [106] but not in others (NFE-1/DNA) [105] this DNA-binding drug is able to induce displacement of preformed TF–DNA complexes, possibly suggesting a differential activity on selected genes. This issue has not been conclusively investigated for aromatic polyamidines.

A second question concerns the possible affinity of aromatic polyamidines for cellular proteins, with special regard to transcriptional factors. It is indeed unclear if, in addition to DNA-binding properties, these compounds are able to bind to classes of proteins, to which transcriptional factors belong. Protein recognition by aromatic polyamidines has been demonstrated, as already pointed out, in the case of many proteolytic enzymes, including trypsin, kallikrein and factor Xa [59–62]. Therefore, interactions of these compounds to transcriptional factors is not formally excluded and could, in principle, affect the efficiency of inhibition of DNA–protein interactions.

ALTERATIONS OF THE EXPRESSION OF SELECTED GENES BY AROMATIC POLYAMIDINES: A PERSPECTIVE

In conclusion, we would like to underline that the search for sequence specific DNA-binding drugs is becoming a topic of great interest in current

molecular pharmacology research. The elucidation of possible preferential effects of a given class of DNA-binding drugs on different transcriptional factors could facilitate the design of drugs retaining enhanced potency and possibly exhibiting effects on a limited numbers of genes. Several considerations, however, must be kept in mind. First, it is reasonable to hypothesize that, after treatment with DNA-binding drugs, the inhibition of interaction between a negative transcriptional factor and its target sequence could lead to an increased expression of certain genes (see scheme in Fig. 3E, lane d). For instance, the ANF (sequence recognized CTTTATCTGG) [108], GCF (sequence recognized $^{C}/_{G}$-CG-$^{C}/_{G}$-$^{C}/_{G}$-$^{C}/_{G}$-C) [109], and PCF (sequence recognized AGAAAGGGAAAGGA) [110] factors are involved in the negative regulation of albumin, EGF and c-*myc* genes, respectively. AP1 is a positive or negative regulatory factor of various cellular and viral genes [111–113]. TIN-1 (sequence recognized AGGAAGTTCC) [114] and WT-ZFP (sequence recognized CGCCCCCGC) [115], preferentially expressed in testis (TIN-1) and developing kidney and Bowman's capsule (WT-ZFP), act as transcriptional repressors.

More importantly, LTRs of retroviruses usually display regions that are recognized by negative transcriptional factors [15–17, 116]. For instance, when the Negative Regulatory Element (NRE) is deleted from the HIV-1 LTR, an increase in transcription of the HIV-1 genome occurs [116]. The NRE regions are recognized by a number of nuclear proteins, as judged by footprinting assays [116].

When cells expressing these factors are treated with DNA-binding drugs, activation of genes that are negatively regulated could occur. In HIV-infected cells, treatment with DNA-binding drugs could lead to activation of transcription. In accord with these hypotheses, when CAT assays were performed on cells stably transfected with the CAT gene under the control of the HIV-I LTR, induction of CAT activity was obtained following treatment with DNA-binding drugs [117, 118].

These data suggest that DNA-binding drugs could, in principle, lead to an activation of silent genes that are under the negative control of nuclear proteins. Therefore, the use of aromatic polyamidines and structurally related DNA-binding drugs in anticancer or antiviral therapy should take into consideration that the inhibition of transcription of the target genes could be temporally associated with an undesired gene activation, possibly leading to side effects. In addition, an interesting possibility exists for the binding of aromatic polyamidines to double-stranded RNA. This was demonstrated for DAPI and diphenylfuranamidine, which bind to both RNA and DNA [119]. With respect to these findings, compounds exhibiting RNA selectivity could be useful for at least two reasons: (a) double-stranded RNA structures are specifically present only in defined eukaryotic mRNA subclasses, selectively expressed in cells belonging to different histotypes, therefore facilitating molecular targeting; (b) retroviral RNAs represent an appealing target for treatment with RNA-binding compounds exhibiting low affinity to DNA, minimizing the toxic effect often related to the use of antiviral drugs showing strong DNA affinity [119].

A final consideration that should be made for possible applications of aromatic polyamidines as cytotoxic antitumor drugs is that at least some of these compounds are also powerful inhibitors of the activity of a variety of proteinases [58–61]. In this respect, aromatic polyamidines are likely to be inhibitors of proteinases exerting important biological functions. This may lead to adverse effects when these compounds are used *in vivo*. Therefore, aromatic polyamidines exhibiting low antiproteinase activity but high inhibitory effects on the DNA–protein interactions could be optimal compounds for antitumour as well as aniviral therapy. With respect to this point, we demonstrated in a recent paper that N_1-substituted tetra-benzamidines retain strong inhibitory activity on tumor cell growth and DNA–protein interaction but display only a small effect on *in vitro* activity of serine proteinase [120, 121].

Taken together, these considerations suggest that the effects of aromatic polyamidines on gene expression (including long-term effects) should be evaluated carefully before considering these compounds for use in therapy of human pathologies. This concerns not only newly designed and synthesized compounds, but also those, such as pentamidine, that are under clinical trials or already accepted for clinical use.

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