Effects of Anticancer Drugs on Transcription *in vitro*

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The effects of DNA interacting drugs on: (1) total RNA synthesis catalyzed by *E.coli* and T7 RNA polymerase; (2) synthesis of the initiating dinucleotide (pppApU) by *E.coli* RNA polymerase ("abortive initiation"); (3) elongation of RNA chains synthesized by T7 RNA polymerase on pT7-7 plasmid DNA bearing T7 RNA polymerase promoter ϕ 10 with human Cu/Zn superoxide dismutase coding sequence, (4) interaction of transcription factor Sp1 and its binding site were studied. Intercalating ligands which form quickly dissociating complexes with DNA (anthracyclines, proflavine, ethidium bromide) are compared with the slowly dissociating drug of d(G·C) specificity (actinomycin D), the non-intercalating, d(A·T) specific pyrrole antibiotics (netropsin and distamycin A) and covalently binding to DNA 1-nitroacridine derivative (nitracrine). The obtained results indicate that rapidly dissociating ligands, proflavine and ethidium bromide, inhibit total RNA synthesis *in vitro* and the abortive initiation to a similar extent while they do not induce discrete elongation stops of RNA polymerase. Actinomycin D and nitracrine exhibit a high inhibitory effect on total RNA synthesis and induce stops of RNA polymerase while not affecting abortive initiation. Pyrrole antibiotics primarily inhibit the initiation, while no elongation stops are induced. Actinomycin D inhibits complex formation between nuclear proteins and the Sp1 binding site. Netropsin, ethidium bromide, proflavine and other intercalating acridines do not affect Sp1 binding. The results indicate that the effects primarily depend on sequence specificity and secondarily on the dissociation rate of ligands from their complexes with DNA.

Introduction

A number of compounds affect the biological properties of nucleic acids at the molecular level through physicochemical interactions or by covalent binding with DNA. Non-covalently interacting ligands form either intercalative or non-intercalative complexes of different equilibrium binding strength, varying in DNA sequence specificity and the rates of association and dissociation (Krakow and Kumar, 1980; Gale *et al.,* 1981; Gniazdowski and Tomasz, 1999). An assay responsive to all these effects is DNA-dependent RNA synthesis which can be performed in cell-free systems. Depending on the character of interactions of drugs with DNA they may differently affect sequential steps of the transcription process (Sarris *et al.*, 1977; Straney and Crothers, 1987). The aim of these experiments is to compare the effects of archetypal DNA-interacting ligands on (1) total RNA synthesis catalyzed by *E.coli* or phage T7 RNA polymerase; (2) synthesis of the initiating dinucleotide (pppApU) by *E.coli* RNA polymerase on early phage T7 promoter (abortive initiation) and (3) elongation of RNA chains synthesized by T7 RNA polymerase on pT7-7 plasmid DNA bearing T7 RNA polymerase promoter ϕ 10 with human Cu/Zn superoxide dismutase coding sequence (pT7-7 SOD). Ligands of low DNA sequence specificity forming rapidly dissociating complexes with DNA: proflavin (PF) and ethidium bromide (EB) are compared with slowly dissociating drugs of high sequence specificity i.e. ac-

Abbreviations: DOX, doxorubicin, trade name Adriamycin; DRB, daunorubicin; ACT, actinomycin D; DST, distamycin A; NT, netropsin; EB, ethidium bromide; PF, proflavin; NC, nitracrine; AAC, N-[2-dimethylamino)-ethyl]-9-aminoacridine-4-carboxamide; 2MAAC, 2-methoxy-derivative of AAC; DACA, N-[2-dimethylamino)ethyl]acridine-4-carboxamide; pT7-7 SOD, pT7-7 plasmid bearing T7 RNA polymerase promoter ϕ 10 with human Cu/Zn superoxide dismutase sequence; HUVEC, human umbilical vein endothelial cells; IC_{50} and IA_{50} are drug concentrations resulting in 50% decrease in RNA and pppApU synthesis.

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tinomycin D (ACT), anthracycline and pyrrole antibiotics. Effects of nitracrine (NC), a 1-nitro-9-acridine derivative which binds covalently to DNA upon reductive activation with dithiothreitol (Krakow and Kumar, 1980; Gale *et al.,* 1981; Gniazdowski and Szmigiero, 1995; Gniazdowski and Tomasz, 1999) are also assayed here. As it has been recently shown ACT in contrast to netropsin (NT), strongly inhibits binding of transcription factor Sp1 to its recognition site which is d(G·C) rich (Czyż and Gniazdowski, 1998). To evaluate the contribution of sequence specificity the effects of these drugs on protein interaction with the Sp1 target sequence are compared with the effect of EB, PF and some other acridines differing in specificity and stability of their complexes with DNA.

Materials and Methods

Proflavin and acridine carboxamide derivatives (kindly provided by Professor W. A. Denny, Cancer Research Laboratory University of Auckland), daunorubicin and doxorubicin (gifts of Drs. M. Wąsowska and I. Oszczapowicz, Institute of Biotechnology and Antibiotics, Warsaw), ethidium bromide (Calbiochem, Lucerne), actinomycin D and distamycin A (Sigma-Aldrich, St. Louis), netropsin (kindly donated by Dr F. Arcamone, Farmitalia, Milan) and nitracrine (kindly provided by Professor J. Konopa, Technical University of Gdańsk) were used. Phage T7 DNA, T7 DNA-dependent RNA polymerase, pT7-7 SOD, an expression vector bearing T7 RNA polymerase promoter ϕ 10 (kindly provided by Professor A. Płucienniczak from the Institute of Biotechnology and Antibiotics, Warsaw) and most of other reagents are the same as previously (Piestrzeniewicz *et al.,* 1998a,b). *E. coli* DNA-dependent RNA polymerase (Sigma-Aldrich, St. Louis) was used.

The inhibition of overall RNA synthesis and abortive initiation was assayed as described by Gniazdowski *et al.* (1988) and Piestrzeniewicz *et al.* (1998a). Quantification was done by trichloroacetic acid precipitation of RNA synthesized by *E. coli* RNA polymerase on phage T7 DNA (overall RNA synthesis). *E. coli* RNA polymerase in the presence of ATP and UTP repetitively synthesizes pppApU on A1 and A3 promoters of phage T7 DNA (abortive initiation). The dinucleotide was separated by paper chromatography from the substrates and quantitated. RNA or the initiating dinucleotide syntheses were assayed at 2–3 drug concentrations and expressed as percentage of the controls containing no inhibitor. Drug concentrations resulting in a 50% decrease in RNA synthesis (IC_{50}) or in pppApU synthesis (IA_{50}) were obtained from the inhibition curves. Electrophoretic analysis of the transcript synthesized on pT7-7 SOD by T7 RNA polymerase was performed as described by Piestrzeniewicz *et al.,* (1998b) but RNA polymerase concentration was 200 units/0.1 ml if not otherwise specified. 1 unit of enzyme (MBI Fermentas, Vilnius) incorporates 1 nmol of ribonucleotide into DE-81 absorbable form in 60 min at 37 °C. Non-covalent ligand-DNA complexes were assayed with the enzyme added to the reaction mixtures containing a ligand at appropriate concentration. Nitracrine-DNA covalent complexes were formed by incubation of the drug at indicated concentration with DNA (2.5 μg/0.1 ml), in the presence of dithiothreitol (5 mM) for 1h (Tołwińska-Stańczyk *et al.,* 1997) then used in the assay without purification.

Nuclear extracts isolated from human umbilical vein endothelial cells (HUVEC) were used as a source of transcription factors. Double stranded oligonucleotide: 5′-AATTCCCCGCCCCCGCCCCC-3′ containing an Sp1 binding site (underlined) was from the promoter region of the gene for the α_v subunit of human vitronectin receptor. The ^{32}P-labelled oligonucleotide containing Sp1 site incubated with nuclear extracts either in the presence or absence of drug was subjected to an electrophoretic mobility shift assay (see Czyż and Gniazdowski, 1998 for details).

Results and Discussion

The following steps of the reaction catalyzed by RNA polymerase can be distinguished: (1) binding; (2) initiation; (3) elongation and (4) termination. Inhibition of RNA synthesis is observed upon action of several DNA-interacting drugs. Two mechanisms of the inhibition have been postulated: (1) The drug can prevent the enzyme from binding to the template or from initiating. Once RNA polymerase is attached to DNA it displaces the ligand molecules and elongates the RNA chains. (2) The effect of the drug depends on its interference with the enzyme moving along the

template and eventually inducing premature termination. At least three factors influence the extent of inhibition and its mechanism. In the series of related acridine derivatives a correlation was found between inhibition of RNA synthesis *in vitro* and both the propency to form intercalative complexes with DNA and the strength of the binding (Sarris *et al.*, 1977; Atwell *et al.*, 1986; Piestrzeniewicz *et al.*, 1990, 1998a). The non-covalently interacting acridines and EB form complexes with DNA which dissociate within milliseconds (Table I). Their primary mechanism of action on RNA synthesis is related to the interference with the enzyme binding to DNA or initiation of RNA chains with a minor effect on elongation (Richardson, 1973; Sarris *et al.*, 1977; Piestrzeniewicz *et al.*, 1998a). This mechanism in our experiments is manifested by a similar inhibition of total RNA synthesis (IC_{50}) and the synthesis of the initiating dinucleotide, pppApU (IA_{50}) by PF, EB (Table I) and several other acridine derivatives which form relatively slowly dissociating complexes with DNA with time constants of 0.3–2.2 s (Richardson, 1973; Sarris *et al.*, 1977; Piestrzeniewicz *et al.*, 1990, 1998a). Contribution of strength, stability and specificity of binding to DNA is commonly illustrated by effects of ACT which forms a long-living complex with a dissociation rate of 6 min (Table I) with almost strict requirement for d(G·C) base pairs for binding (Gale *et al.*, 1981). This drug exhibits a preferential effect on elongation of RNA chains (Sarris *et al.*, 1977). A product synthesized by RNA polymerase in the presence of ACT shows a number of discrete bands corresponding to site-specifically terminated RNA chains (White and Phillips, 1989; Piestrzeniewicz *et al.*, 1998b, see also Fig. 1, panel A lanes 2 and 7 and panel B lane 2). Site-specific termination of transcription is also observed with NC (Fig. 1A, lanes 4 and 5, Fig. 1B lane 5). This highly cytotoxic 1-nitro-9-aminoalkylacridine derivative, activated by sulfhydryl compounds, binds to DNA covalently with preference to purines, particularly to guanine (see review Gniazdowski and Szmigiero, 1995). Like other covalently binding drugs, under these conditions NC exhibits a high effect on elongation (see review Gniazdowski and Cera, 1996). In our experiments this effect is illustrated by the discrete bands which are formed when DNA preincubated with the drug and dithio-

Table I. Physicochemical data for the drugs and inhibition of RNA and pppApU syntheses.

Drug	log *K* [M]	Dissociation half-life [s/ms]	IC_{50} [μM]	IA_{50} [μM]
DRB	5.90[a]		5.8	12.4 ± 5.6
DOX	6.43[a]	0.8 s[d]	6.0	14.5 ± 9.5
ACT	6.58[a]	374 s [d]	0.25 ± 0.1	⩾20
NC	–	–	4.2 ± 0.1	⩾15
DST	7.56[a]	not known	7.0 ± 0.1	3.4 ± 1.9
NT	7.63[a]	not known	7.5 ± 3.9	1.8 ± 0.8
PF	4.3[c]	1 ms[e]	19.3[f] ± 2.4	19.7[f] ± 1.1
EB	5.0[a]	25 ms[e]	9.1[f] ± 2.6	7.8[f] ± 1.1

[a] Compiled by Chaires (1997); [b] At the drug concentration of 4.2 μM the number of NC molecules covalently bound per 10^3 T7 DNA nucleotides was 0.3 (Tołwińska-Stańczyk *et al.*, 1997); [c] K for proflavin from Li and Crothers, 1969; [d] White and Phillips (1989); [e] Feigon *et al.* (1984); Wilson *et al.* (1985); [f] Piestrzeniewicz *et al.* (1998a). IC_{50} and IA_{50} result in a 50% decrease in RNA and pppApU synthesis respectively. Where the mean values of two independent experiments are given ± range values are shown.

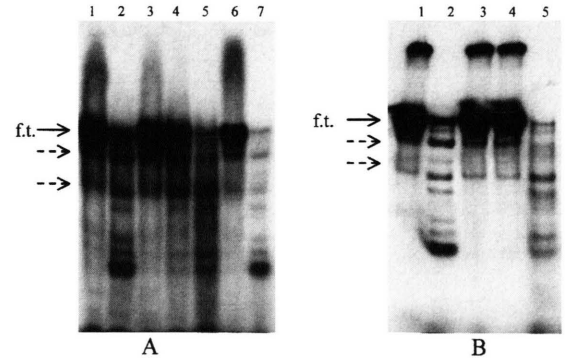

Fig. 1. Site specific stable transcriptional blockages induced by ACT and covalently binding NC.
Panel A: lanes 1 and 6 control; lanes 2 and 7 ACT (3 μM); lanes 3–5 NC (5, 15 and 50 μM). T7 RNA polymerase concentration was 200 units/0.1 ml (lanes 1–5) and 100 units/0.1 (lanes 6 and 7).
Panel B: lane 1, control; lane 2, ACT (3 μM); lane 3, DST (4 μM); lane 4, NT (4,5 μM); lane 5, NC (60 μM). Covalent complexes NC-DNA were formed by incubation of the drug with DNA in the presence of dithiothreitol. Note that non-covalent interactions of NC with DNA do not appreciably affect RNA polymerase (Gniazdowski and Szmigiero, 1995). Covalent binding of NC at 50–60 μM may be approximated to about 5–8 drug molecules per 10^3 DNA nucleotides (Tołwińska-Stańczyk *et al.*, 1997). f.t. = full transcript – Note that minor bands (broken arrows) corresponding to spontaneous transcription arrests are seen in the controls. These minor bands are enhanced and the other bands due to the drug-induced stops appear in the presence of ACT and NC.

threitol was used as a template (Fig. 1 panel A, lanes 4 and 5; panel B, lane 5). The band pattern is similar to that of ACT, a fact which is otherwise consistent with NC preference to guanine. In contrast to EB, PF, DST and NT both drugs exhibit a very low effect on abortive initiation (Table I). Low content of guanine in the promoter region, its absence in the template segment transcribed into pppApU, and drug sequence or base specificity may explain the apparent lack of sensitivity of abortive initiation to ACT and NC ($IA_{50} \gg 15$). In the latter case bulky adducts are scarcely distributed along the template. Hence probability for RNA polymerase to encounter the drug is higher for the elongating enzyme, i.e. when overall RNA synthesis is assayed, than for the enzyme bound to the promoter region repetitively synthesizing the dinucleotide.

As shown by Kriebardis *et al.* (1987), DRB exhibits a considerably lower inhibitory effect on abortive initiation than on overall RNA synthesis and similar difference is found for DOX (Table I). These effects are consistent with preference of these drugs to d(G·C) sequences. As found by White and Phillips (1989) the dissociation rate of DOX-DNA complex (a half life of 0.8 s, and presumably similar for DRB) is too high to induce specific stops of T7 RNA polymerase. Hence, in contrast to ACT and NC, in the presence of DRB or DOX no site – specific terminations of transcription are observed (Fig. 2A lanes 3,5 and 7, Fig. 2B lane 2). However, the situation differs dramatically when anthracyclines are activated with Fe(III) ions and dithiothreitol to form covalent complexes with DNA. As shown by Cullinane and Phillips (1990) an increase of inhibition and specific blockage sites of transcription are observed (Fig. 2A, lanes 2,4,6).

On the other hand pyrrole antibiotics exhibit a higher effect on the abortive initiation. IA_{50} values for DST and NT are twofold and fourfold lower respectively than corresponding values of IC_{50} (Table I). Both drugs, which are DNA-minor groove binding non-intercalating ligands, are characterized by d(A·T) specificity. Hence their relatively higher effects on pppApU synthesis has been expected and confirmed earlier observations on DST preferential inhibition of initiation of RNA chains by *E. coli* RNA polymerase (Puschendorf *et al.*, 1974). Surprisingly in contrast to

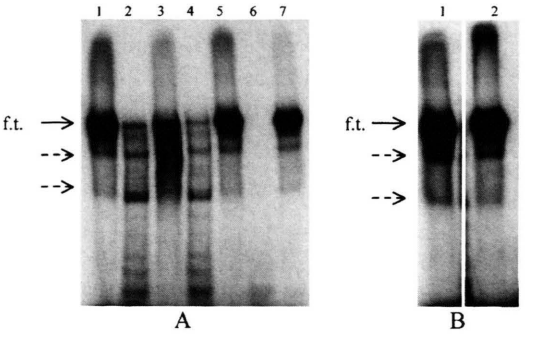

Fig. 2. Autoradiograms of transcripts synthesized in presence of anthracyclines.
Panel A: lane 1, control; lanes 2 and 3, DRB (5 μM); lanes 4 and 5 (10 μM); lanes 6 and 7 (50 μM). Lanes 3, 5 and 7, represent non-covalently interacting drug. Lanes 2, 4 and 6 – pT7-7 SOD and DRB were preincubated for 40 h in the presence of Fe(III) ions (40 μM) and dithiothreitol (7 mM) to form covalent complexes (see Cullinane and Phillips, 1990).
Panel B: lane 1, control; lane 2, DOX (50 μM). Lanes 1 and 2 originate from the same gel. Other lanes which are unrelated in this issue have been excised.
f.t. = full transcript – minor bands (broken arrows) correspond to spontaneous transcription arrests. See legend to Fig. 1 for further details.

ACT they do not induce specific stops in the transcription of DNA by RNA polymerase (Fig. 1B, lanes 3,4). NT and DST bind tightly to d(A·T) clusters consisting of four and five base pairs, respectively (Bailly and Chaires, 1998), stabilizing double helical DNA structure (Krakow and Kumar, 1980). There is no data available about dissociation rates of complexes of pyrrole antibiotics with DNA but their stability is considerably higher than that of ACT. Although there are five sequences consisting of at least five consecutive d(A·T) base pairs located downstream the promoter in pT7-7SOD plasmid DNA (Hallewell *et al.*, 1985) which are potential binding sites for the pyrrole antibiotics, no corresponding stops of RNA polymerase are observed (Fig. 1B lanes 3,4). The only explanation is that the bound drugs which interact with the minor groove are no barriers for the elongating enzyme which bypasses them reading the template without unwinding.

Several general and selective transcription factors and other regulatory proteins, in addition to RNA polymerase, are required for specific transcription to occur. Many of them exert their functions through specific contacts either in the pro-

moter region or distant sequences enhancing or attenuating the initiation. These contacts may be affected by anticancer drugs and related ligands, which then may lead to alteration of the transcription (Hurst, 1996). Sp1, a transcription activating factor, specifically recognizes the d(G·C) rich DNA sequences. Its binding to DNA is abolished by ACT and DOX, methotrexate and other drugs preferentially interacting with d(G·C) rich sequences. On the other hand two pyrrole antibiotics, DST and NT, which exhibit d(A·T) specificity, do not affect the interaction of Sp1 with DNA (Chiang *et al.,* 1998, Czyż and Gniazdowski, 1998, see also Gniazdowski and Czyż, 1999, for the review). ACT binds via intercalation to DNA preferably at the GpC sequence. It was suggested that the effect of ACT on Sp1 was due to the presence of this sequence in Sp1 binding site (Czyż and Gniazdowski, 1998). ACT- and NT-DNA complexes differ in their steric properties. To evaluate the possibility that any intercalating drug may effectively compete with Sp1 for its binding site, EB and PF and other acridine derivatives forming intercalative complexes with DNA of different base pair preferences and different dissociation rates were assayed (Fig. 3). Neither NT, EB, PF nor carboxamide derivatives of acridines whose dissociation rates of their complexes with DNA varied from few milliseconds (PF, EB) to seconds (2MAAC) and preference to d(G·C) rich sequences from negligible (PF) up to sixfold higher binding constant to poly d(G·C) over poly d(A·T) for 2MAAC (see Piestrzeniewicz *et al.,* 1990) significantly affect formation of protein-oligonucleotide complex (see Fig. 3 and the legend). Up to 10–20% of inhibition is found at a drug concentration as high as 10 μM or 20 μM (not shown). 50% of inhibition of protein binding to Sp1 site is not reached at 50 μM 2MAAC or DACA (not shown). It is worth mentioning that DACA belongs to most promising anticancer drugs presently under clinical studies. Under these experimental conditions 50% inhibition of Sp1 binding was obtained when 1 μM ACT was used (Czyż and Gniazdowski, 1998). These observations support a notion that in most cases DNA specificity of a drug plays an essential role in their effect on regulation of transcription (Gniazdowski and Czyż, 1999).

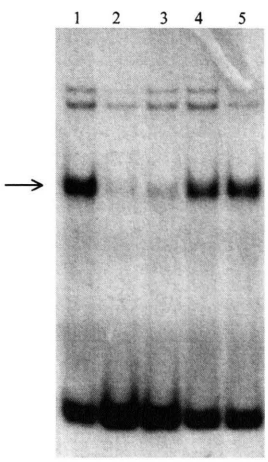

Fig. 3. Effect of drugs on interaction between nuclear extracts and the oligonucleotide bearing the dimer of the Sp1 binding site. Nuclear extracts were prepared from human umbilical vein endothelial cells (HUVEC) induced for 2 h with lipopolysacharide 100 μg/ml. A ^{32}P-labelled oligonucleotide containing two Sp1 binding sites was used in the mobility shift assay. Control binding reaction performed in the absence of DNA-binding drugs (lane 1). Competition was performed with a 100-fold excess of unlabelled oligonucleotide (lane 2). Lane 3, ACT (5 μM) and acridine carboxamide derivatives (10 μM each) which were previously characterised (see Piestrzeniewicz *et al.*, 1998a for the structures): lane 4, AAC; lane 5, 2MAAC. Similarly to acridine carboxamides, PF, EB, NT and DACA did not appreciably affect Sp1-oligonucleotide complex formation (the corresponding gels have been excised). Arrow indicates oligonucleotide-protein complex, f.p.- free oligonucleotide probe.

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