Acta Biochimica Polonica

Vol. 45 No. 1/1998

127–132

QUARTERLY

Communication

Effect of DNA-interacting drugs on phage T7 RNA polymerase[*][o]

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Received: 13 October, 1997

Key words: T7 RNA polymerase, inhibition of transcription, acridines, actinomycin, netropsin, distamycin

9-Aminoacridine carboxamide derivatives studied here form with DNA intercalative complexes which differ in the kinetics of dissociation. Inhibition of total RNA synthesis catalyzed by phage T7 and *Escherichia coli* DNA-dependent RNA polymerases correlates with the formation of slowly dissociating acridine-DNA complex of time constant of 0.4–2.3 s. Their effect on RNA synthesis is compared with other ligands which form with DNA stable complexes of different steric properties. T7 RNA polymerase is more sensitive to distamycin A and netropsin than the *E. coli* enzyme while less sensitive to actinomycin D. Actinomycin induces terminations in the transcript synthesized by T7 RNA polymerase. Despite low dissociation rates of DNA complexes with acridines and pyrrole antibiotics no drug dependent terminations are observed with these ligands.

Dissociation kinetics of drug–DNA complexes and sequence specificity of a drug are two properties which can be considered as determining its cytotoxicity, anticancer potential and the molecular mechanism of inhibition of DNA-dependent RNA synthesis. Hence ethidium bromide [1], proflavine and 9-aminoacridine [2], which form with DNA complexes dissociating within a few milliseconds [3] and exhibit low sequence specificity, pref-

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[*]Presented at the 33rd Congress of the Polish Biochemical Society, September, 1997, Katowice.

[o]Supported by the State Committee for Scientific Research (KBN grant No. 4-0238-91-01).

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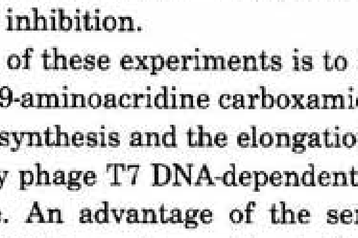
Abbreviations: pT7-7 SOD, pT7-7 plasmid bearing T7 RNA polymerase promoter ϕ 10 with human Cu/Zn superoxide dismutase; AA2C, *N*-[2-(dimethylamino)ethyl]acridine-2-carboxamide; AAC, *N*-[2-(hydroxyethylamino)ethyl]-9-aminoacridine-4-carboxamide; 2MAAC, 2-methoxy derivative of AAC; 1MAAC, 1-methoxy derivative of AAC.

erentially inhibit early steps of RNA synthesis, i.e. binding of RNA polymerase to DNA and/or initiation of RNA synthesis [1, 2, 4]. On the other hand actinomycin D, which is a ligand dissociating at a half time of the range of ten minutes [5] and exhibits d(GC) specificity [6], affects mostly elongation of synthesized polynucleotide chains [2]. Since these ligands have different structures it is not clear what is the contribution of kinetic properties and sequence specificity to the mechanisms of inhibition.

An aim of these experiments is to study the effect of 9-aminoacridine carboxamides on total RNA synthesis and the elongation of RNA chains by phage T7 DNA-dependent RNA polymerase. An advantage of the series of 9-aminoacridine carboxamide derivatives of systematically varied structure is that they form with DNA intercalative complexes of roughly similar binding strength. Considerable differences however are observed within the series in the kinetics of dissociation of their complexes with DNA, the cytotoxicity of the compounds [7] and their effect on early steps of RNA synthesis. All these properties are well correlated [8, 9]. We used here four members from the series (Fig. 1). They vary in kinetic stability of their complexes with DNA from the rapidly dissociating acridine showing the time constant of 90 ms, to those exhibiting the time constant of 2 s (see Table 1) to assay their effect on elongation by means of gel electrophoresis of the transcripts. As a control we used actinomycin D whose effect on elongation has been well established. Two pyrrole antibiotics, distamycin A and netropsin, are also included. They form with DNA very stable complexes but, in contrast to actinomycin D, they do not intercalate and exhibit a strong affinity to d(A·T) rich regions in DNA [6].



$R = -CONH(CH_2)_2\overset{\oplus}{N}H(CH_3)_2 \cdot 2Cl^-$

$X = -H$ or $X = -OCH_3$

Figure 1. Structures of the acridine derivatives.

AAC, 1MAAC and 2MAAC, R at the 4-position; AA2C, R at the 2-position; AAC and AA2C, X = H; 1MAAC and 2MAAC, X = $-OCH_3$ at the 1-position and 2-position, respectively.

MATERIALS AND METHODS

pT7-7 SOD is an expression vector based on pT7-7 plasmid bearing T7 RNA polymerase promoter ϕ 10 [10, 11] with human Cu/Zn superoxide peroxide dismutase coding sequence [12, 13] inserted in *Nde*I and *Hin*dIII sites, i.e. 63 base pairs downstream the start site. The plasmid was obtained in the Laboratory of Genetic Engineering of Terpol, Sieradz (A. Płucienniczak & G. Płucienniczak, unpublished). The plasmid was linearized with *Hin*dIII. T7 DNA-dependent RNA polymerase (Fermentas, Vilnius, Lithuania), acridines (kindly donated by Professor W.A. Denny) and other drugs described in the earlier paper [14] were used. The effect of drugs on overall RNA synthesis by T7 RNA polymerase (100 units/0.1 ml) was assayed at 3–4 drug concentrations and expressed as percentage of the values for controls containing no inhibitor, as described previously [8, 9], except that 5 mM $MgCl_2$, 10 mM KCl and 0.4 mM nucleoside triphosphates with $[^{14}C]$CTP were used. Drug concentrations resulting in a 50% decrease in RNA synthesis (IC_{50}) were read from the inhibition curves. Electrophoretic analysis of the transcripts was performed following incubation of T7 RNA polymerase as described above but the enzyme concentration was 400 units/0.1 ml, RNasin (60 units/0.1 ml) and $[\alpha\text{-}^{32}P]$CTP as a labelled substrate were used and the incubation time was 2 h instead of 10 min. The products of the transcription were analysed on non-denaturing polyacrylamide gels then subjected to autoradiography. The dried gels were cut into 0.3 cm sections and the radioactivity measured with a liquid scintillation counter.

RESULTS AND DISCUSSION

The effects of a ligand on overall RNA synthesis *in vitro* is a result of drug interference with different events in the transcription process. Four different steps of RNA synthesis can be experimentally distinguished: binding, initiation, elongation and termination. In order to evaluate the contribution of a dissociation rate of acridine-DNA complexes in inhibition of RNA synthesis we previously assayed the effect of the series of 9-aminoacridine carboxamide derivatives in an *E. coli* DNA-dependent RNA polymerase *in vitro* system. They are highly polar and their association constants for binding to poly d(A–T) and poly d(G–C) are broadly similar [7]. Analysis of the stability of complexes of these 9-aminoacridine carboxamides with calf thymus DNA revealed considerable differences within the class. Two, three or four transients can be distinguished in the dissociation kinetics [7]. Most interestingly the drugs which exhibited the fourth transient showed high cytostatic effect [7]. We previously found that inhibition of total RNA synthesis, binding of the enzyme to the promoter and the initiation correlate with the formation of this relatively slowly dissociating ligand-DNA complex of time constant 0.3–2.2 s [8, 9]. We assayed in these experiments four acridines of the series (Fig. 1), one of them, AA2C, exhibiting only three transients, the longest of 90 ms while the others show a fourth one (ref. [7] and Table 1). Actinomycin D formed long living complex with time constant of several minutes [5]. Distamycin A and netropsin bind to the minor groove of the DNA double helix, interacting with d(A·T) rich regions [6]. No dissociation data are available for these antibiotics but the necessity of using a high concentration of sodium perchlorate [15] to wash out these drugs from their complexes with DNA points to their extremely tight binding.

To characterize the system we assayed effects of the ligands on total RNA synthesis by T7 RNA polymerase and pT7-7 SOD. Drug concentrations resulting in a 50% decrease in RNA synthesis (IC_{50}) are shown in Table 1. The ocurrence of the longest living complex with the time constant t_4 (ref. [7] and Table 1)

Table 1. Inhibition of RNA synthesis by DNA-interacting drugs.

IC_{50} is micromolecular concentration of drug which inhibits to 50% of control value the RNA synthesis by *E. coli* RNA polymerase on calf thymus (CT) DNA or by phage T7 RNA polymerase on plasmid pT7-7 SOD.

Drug	Time constant (s)	IC_{50} (μM)	
		E. coli pol/CT DNA	T7 pol/pT7-7 SOD
AA2C	0.09[a]	33.4 ± 8.0[d]	32.5 ± 8.3
AAC	0.43[b]	8.7 ± 0.1[d]	10.8 ± 0.8
2MAAC	2.05[b]	9.4 ± 0.1[d]	4.6 ± 2.0
1MAAC	2.27[b]	14.1 ± 0.4[d]	6.8 ± 1.4
ACT	735[c]	0.17 ± 0.2[e]	0.77 ± 0.59
DST	not known	16 ± 0.7[e]	2.2 ± 0.9
NT	not known	9.1 ± 0.3[e]	1.9 ± 1.0

[a] Only three transients are observed, the longest one, t_3 is given [7]; [b] The fourth transient (t_4) displayed by the acridine on its dissociation from calf thymus DNA is shown [7]; [c] The time constant for actinomycin D is given for the slowly dissociating complex [5]; [d,e] For comparison with *E. coli* RNA polymerase the data previously published [d][8] and [e] [4] are presented. ACT, actinomycin D; DST, distamycin A; NT, netropsin.

contribute most significantly to inhibition of overall RNA synthesis. This relationship seems to be even more pronounced than that found previously for the same acridines with *E. coli* RNA polymerase and calf thymus (Table 1) and T7 DNA [8]. AA2C which does not show the fourth transient inhibits T7 RNA polymerase to 50% at a concentration which is five- and seven-fold higher than that of the slowly dissociating ligands, 1MAAC and 2MAAC, respectively. Phage RNA polymerase is more sensitive to distamycin A and netropsin than the bacterial enzyme while relatively less sensitive to actinomycin D, which decreases the synthesis catalyzed by T7 RNA polymerase to 50% only at a fourfold higher concentration (Table 1). Similar differences have been observed for distamycin and actinomycin assayed with *E. coli* and phage T3 RNA polymerase [16].

As found by White & Phillips [17], in the presence of actinomycin D we have observed a number of discrete bands corresponding to site-specifically terminated RNA chains (Fig. 2, lane 2). In preliminary experiments we found that optimal blocking of RNA synthesis was observed by actinomycin D at a concentration 2-5 fold higher than IC_{50}. Hence the effect of other ligands on the elongation was assayed at their concentrations similarly related to the corresponding IC_{50} values. No distinct blocking by acridines and pyrrole antibiotics were detected either under these conditions (Fig. 2, lanes 3-8) or at other concentrations (not shown). Radioactivity measurements indicate that the amount of RNA roughly corresponded to the expected inhibition of the overall RNA synthesis, and, except for actinomycin D, 60-75% of the radioactivity of RNA synthesized in the presence of acridines and pyrrole antibiotics, like in the control gel (Fig. 2, lane 1) was found in the full length transcript band.

Two mechanisms of inhibition of RNA synthesis *in vitro* by DNA-interacting compounds have been postulated [1]: i) the drug can prevent the enzyme from binding to the template or initiating synthesis but once RNA polymerase is attached to DNA it displaces the bound molecules of the ligand; ii) the effect of the ligand depends on its interference with the enzyme moving along the template.

As found before [2, 17, 18], actinomycin D follows the latter mechanism although its effect on the open promoter complex formation was also observed [4]. The acridines studied here, despite considerable differences in the kinetics of dissociation of their complexes with DNA [7] appear to follow the former mechanism. The lack of distinct termination observed here (Fig. 2, lanes 3-6) suggests that their recently detected effect on the open promoter complex formation [9] is primary with respect to the other effects, i.e. inhibition of the initiation and effect on the overall RNA synthesis.

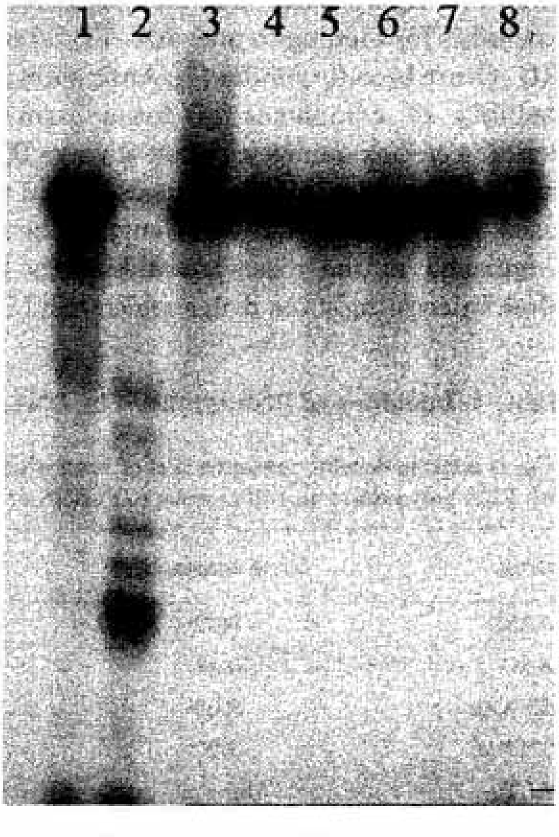


Figure 2. Autoradiogram of the transcript of plasmid pT7-7 with T7 RNA polymerase.

Lane 1, control; lane 2, actinomycin D (3 μM); lane 3, AA2C (125 μM); lane 4, AAC (40 μM); lane 5, 1MAAC (20 μM); lane 6, 2MAAC (20 μM); lane 7, distamycin A (3.5 μM); lane 8, netropsin (4.25 μM).

More surprisingly, distamycin A and netropsin which bind very tightly to DNA [6] were found to have no effect on elongation (Fig. 2, lanes 7, 8). At a low binding level distamycin A induced dissociation of bacterial RNA polymerase DNA complex [4, 19] while at a higher drug concentration a decrease in average chain length was observed. The effect of distamycin and presumably that of netropsin on the binding or initiation may be ascribed to their affinity to d(A·T) base pairs which are overrepresented in the promoter region. On the other hand, the lack of effect on elongation in our study could be due to a bypass of the tightly bound drug by the enzyme as suggested for covalent adducts to DNA [20].

In the case of 9-aminoacridine carboxamides it can be concluded that unwinding of DNA, which occurs when RNA polymerase approaches the acridine binding site, induces rapid dissociation. Thus the elongation which occurs at a rate of 50 nucleotides per second is not affected.

It can be concluded from this study that the effects of 9-aminoacridine carboxamides on the overall RNA synthesis catalyzed by T7 RNA polymerase depend on kinetics of dissociation of the drug-DNA complexes. T7 RNA polymerase is more sensitive to distamycin A and netropsin than the *E. coli* enzyme while less sensitive to actinomycin D. The transcript synthesized in the presence of actinomycin show on electrophoresis discrete bands of low molecular mass which correspond to the drug induced terminations. Despite low dissociation rates of DNA complexes with acridines and pyrrole antibiotics no drug-dependent terminations are observed with these drugs.

We are indebted to Professor W.A. Denny for the acridines. The technical assistance of Ms M. Affeltowicz is acknowledged.

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