Inhibition of RNA Synthesis *in vitro* by Acridines – Relation between Structure and Activity

Mariola K. Piestrzeniewicz[a], Dorota Wilmańska[a], Kazimierz Studzian[a], Janusz Szemraj[b], Małgorzata Czyż[a], William A. Denny[c] and Marek Gniazdowski[a]

[a] Department of General Chemistry and
[b] Department of Biochemistry I, Institute of Physiology and Biochemistry, Medical University of Łódź, Lindleya 6, 90–131 Łódź, Poland
[c] Cancer Research Laboratory, The University of Auckland, Private Bag 92019, Auckland 1000, New Zealand

Z. Naturforsch. **53c,** 359–368 (1998); received December 2, 1996/February 4, 1998

Acridines, Amsacrine, Anticancer Drugs, RNA Polymerase, DNA-Ligand Interaction

The effects of acridine derivatives (proflavine and 2,7-dialkyl derivatives, diacridines and triacridines, 9-aminoacridine carboxamides, and 9-anilinoacridine, amsacrine and its congeners) on overall RNA synthesis *in vitro*, on synthesis of initiating oligonucleotides and the binding of the enzyme to DNA were studied. The primary mechanism of action is related to inhibition of the enzyme binding to DNA. The acridines (intercalating or non-intercalating and bis-intercalating ligands) assayed here differ in the properties of their complexes with DNA. Correlation is generally observed between inhibition of RNA synthesis *in vitro* and cytotoxicity in cell cultures for di- and triacridines and 9-aminoacridine carboxamide derivatives. No relationship was found between the effect on RNA polymerase system and biological effects for amsacrine and its derivatives in contrast to the other series of acridines studied here. The aniline ring seems to decrease the inhibitory potency of a ligand. The discrepancy between the biological effect and RNA synthesis inhibition may be due to a different mechanism of cytotoxicity action of amsacrine which is a potent topoisomerase II poison.

Introduction

DNA-interacting ligands affect DNA structure and its function in various DNA-dependent enzymatic systems. Analysis of ligand effects includes the mode of ligand interactions, i.e. intercalative or non-intercalative binding; equilibrium binding strength, DNA sequence specificity and the rates of both association and dissociation of ligands (Gale *et al.*, 1981). An assay responsive to all of these effects is DNA dependent RNA synthesis, which can be reproduced with high fidelity in cell-free systems. *E.coli* or phage DNA-dependent RNA polymerase may specifically initiate RNA synthesis at their natural promoters and terminate polynucleotide chains at the proper site in the absence of other protein factors. The different sequential steps of the transcription process can be distinguished and inhibition at distinct steps, namely enzyme binding to template, initiation of polynucleotide chain and elongation may be observed (Sarris *et al.*, 1977, Straney and Crothers, 1987). In many cases correlations have been shown between inhibition of RNA synthesis *in vitro*, cytotoxicity and both the mode and kinetics of

Abbreviations: EB, ethidium bromide; PF, proflavine; DMP, 2,7-dimethyl-, DEP, 2,7-diethyl-, DIP, 2,7-diisopropyl- and DBP, 2,7-ditertbutylproflavine; DA_2 and DA_6, diacridines diamides (see Fig. 1 for the formulae); TA_2 and TA_6, triacridine diamides (see Fig. 1 for the formulae); MAPA, 9-[3-(dimethylamino)propylamino]acridine; AAC, N-[2-(dimethylamino)ethyl]-9-aminoacridine-4-carboxamide; AACH, N-[2-(hydroxyethylamino)ethyl]-9-aminoacridine-4-carboxamide; AA2C, N-[2-(dimethylamino)ethyl]-9-aminoacridine-2-carboxamide; 1MAAC, 2MAAC, 3MAAC and 8MAAC, 1-, 2-, 3- and 8-methoxyderivatives of AAC; DACA, N-[2-(dimethylamino)ethyl]acridine-4-carboxamide; DACAH, N-[2-(hydroxyethylamino)ethyl]acridine-4-carboxamide; *m*-AMSA, amsacrine, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide and o-AMSA, *o*-anisidide; AMSAAC, 9-[[2-methoxy-4-(methylsulfonylamino)phenyl]amino]-N-[2-(dimethylamino)ethyl]acridine-4-carboxamide; AMSAEC, 9-[[2-methoxy-4-methylsulfonylamino)phenyl]amino]-N-[2-(hydroxyethylamino)ethyl]acridine-4-carboxamide; CT, calf thymus; T7, phage T7; IC_{50}, IA_{50} and IO_{50} are drug concentrations resulting in a 50% decrease in RNA, pppApU synthesis and RNA polymerase to promoter binding.

Reprint requests to Prof. Marek Gniazdowski.
Fax: (48–42) 322 347, 784 277.
E-mail: chemgen@psk2.am.lodz.pl.

0939–5075/98/0500–0359 $ 06.00

DNA binding (Atwell *et al.*, 1986, Wakelin *et al.*, 1987, Markovits *et al.*, 1989, Gniazdowski and Cera, 1996). We present here experimental data on the effects of different families of acridines on overall RNA synthesis *in vitro*, on synthesis of initiating oligonucleotides and on the binding of the enzyme to DNA. Preliminary results on overall RNA synthesis were published previously (Piestrzeniewicz *et al.*, 1990, 1993). The representative ligands for each groups have been reassayed here. All the ligands except ethidium bromide (EB) belong to the acridine class of compounds. They may be divided into four subclasses: i/ proflavine (PF) and its 2,7-dialkyl derivatives (Baguley *et al.*, 1982), two of which interact with DNA without intercalation; ii/ diacridines and triacridines (Atwell *et al.*, 1986) which form bisintercalative complexes with DNA; iii/ 9-aminoacridine carboxamides which form intercalative complexes with DNA of varying kinetics of dissociation (Wakelin *et al.*, 1987), and corresponding acridine carboxamide derivatives lacking the 9-amino group (Crenshaw *et al.*, 1995); iv/ 9-aniline acridine derivative successfully used in cancer therapy, amsacrine (mAMSA), its inactive isomer, oAMSA and carboxamide derivatives of mAMSA (Wilson *et al.*, 1981, Crenshaw *et al.*, 1995), see Fig. 1 for the structures.

Materials and Methods

Acridine derivatives were synthesized in the Cancer Research Laboratory, University of Auckland, New Zealand. Phage T7 DNA and *Escherichia coli* DNA dependent RNA polymerase were isolated as described by Wilmańska *et al.* (1984). T7 DNA dependent RNA polymerase was purchased from MBJ Fermentas, Vilno. pDR 540 and pKK 223–3 plasmids were donated by Dr G. Duval-Valentin (Paris) and Professor A. Płucienniczak (Łódź) respectively. DNA fragments of 266 and 391 base pairs, bearing the *tac* promoter, were excised with Bam HI from pKK 223–3 and with Eco RI and Bam HI restriction enzymes from pDR 540, and isolated by polyacrylamide gel electrophoresis (Maniatis *et al.*, 1982). Restriction enzymes and DNA polymerase, Klenow fragment (Pharmacia LKB, Uppsala), ATP, CTP, GTP, UTP, spermidine (Novabiochem, Lucerne), [^{14}C]ATP, [^{14}C]GTP, [^{14}C]UTP (Radiochemical Centre, Amersham), agarose, heparin (Sigma, St.Louis), acrylamide, N,N'-methylene-bis-acrylamide, TEMED (N,N,N'N'-tetramethylethylenediamine) and bovine serum albumin (Serva, Heidelberg) were used. α[^{32}P]dATP was kindly donated by Professor A.Płucienniczak.

The effect of acridine derivatives on overall RNA synthesis and on pppApU synthesis in the presence of phage T7 DNA were assayed as described by Gniazdowski *et al.* (1988) except that the incubation time was 10 min. RNA or the initiating dinucleotide syntheses were assayed at 2–4 drug concentrations and expressed as a 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 read from the inhibition curves.

DNA fragments bearing the *tac* promoter, obtained either from pKK 223–3 or pDR 540 (see above) and labelled with α[^{32}P]dATP using Klenow fragment of DNA polymerase, were used to follow open promoter complex formation according to Straney and Crothers (1985). Open promoter complexes of RNA polymerase were formed at different drug concentrations in 37°C for 15 min. Following addition of saccharose/bromophenol blue/xylene cyanol and heparin, to trap the unbound enzyme, and incubation for 2 min, they were subjected to polyacrylamide gel electrophoresis under non-denaturing conditions. The relative amounts of the promoter-enzyme complexes were estimated by the radioactivity measurements and expressed as a percentage of the corresponding controls. Drug concentrations resulting in a 50% decrease in complex formation (IO_{50}) were read accordingly.

Results and Discussion

The acridines studied here differ in the properties of their complexes with DNA. The following aspects of the acridine-DNA interaction with respect to inhibition of RNA synthesis *in vitro* are considered here: i/ intercalative or non-intercalative binding; ii/ bis-intercalation versus monointercalation; iii/ positive charge(s) of the ligands and equilibrium binding strength; iv/ dissociation rates of acridine-DNA complexes. Three systems are used to approach a contribution of the interactions: i/ binding of the enzyme to the template; ii/ abortive initiation and iii/ overall RNA synthesis.

Intercalation and transcription

The inhibitory effects of acridines on RNA synthesis have been related to their ability to intercalate. Thus structural distortion of the acridine in 1,2,3,4-tetrahydroacridine derivatives and steric hindrance between the 1-nitro and 9-aminoalkyl groups in nitracrine derivatives have been shown to reduce inhibition of RNA polymerase (Gniazdowski *et al.*, 1982) presumably by impeding intercalation. PF and its 2,7-dialkyl derivatives (Fig. 1) are acridine derivatives monoprotonated at pH 7 (pK_a for PF=9,3) and ideal for the purposes of studying the effect of ligand intercalating activity. They all bind to DNA but the nature of this interaction differs across the series. PF, DMP and DEP have comparable binding constants, and form intercalative complexes. However DBP interacts with DNA without intercalation, as the bulky t-butyl substituents sterically hinder insertion of the chromophore, leaving the ligand to bind in one of the grooves. DIP is able to intercalate DNA, but only to very small extent and has a binding mode more closely related to that of DBP. DIP and DBP bind less strongly to DNA than the first three members of the series and has a preference for d(A-T) sequences while PF have no strong base specificity (Müller *et al.*, 1973, Baguley *et al.*, 1982, Ferguson *et al.*, 1988). PF forms with DNA rapidly dissociating complexes with time constant of about 1 ms (Geacintov *et al.*, 1981, Feigon *et al.*, 1984).

E.coli RNA polymerase forms the enzyme-promoter complex which can be easily separated from the uncomplexed DNA by means of gel electrophoresis (Fig. 2a,b lanes 1 and 2). Open promoter complexes of RNA polymerase (Straney and Crothers, 1985) were formed at different PF or dialkylproflavine concentrations at 37°C and sub-

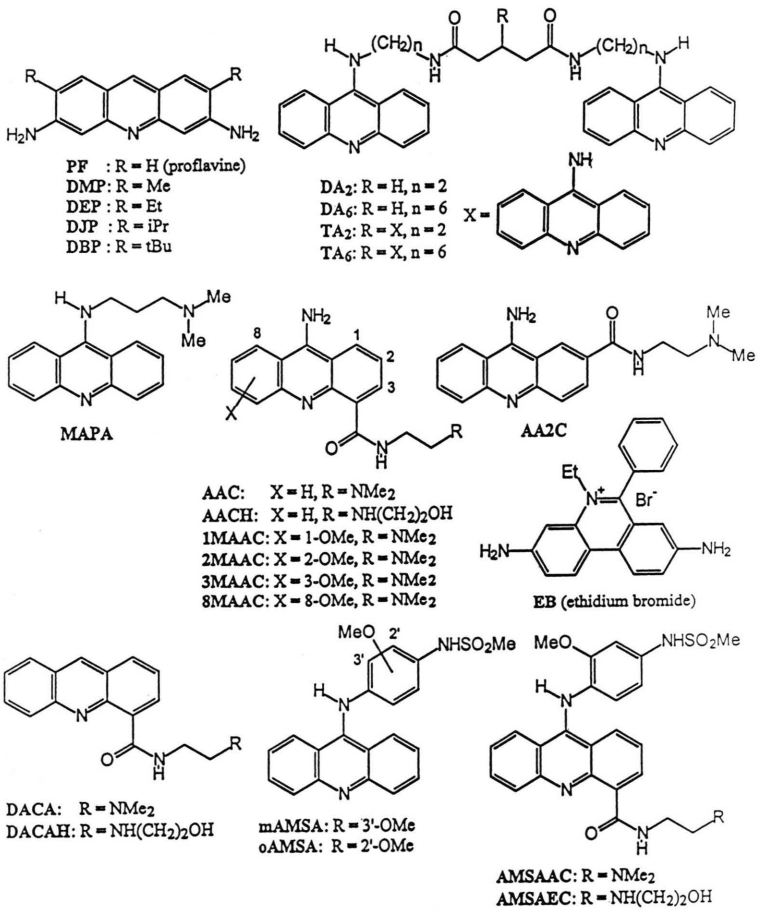

Fig. 1. Structures of ligands studied.

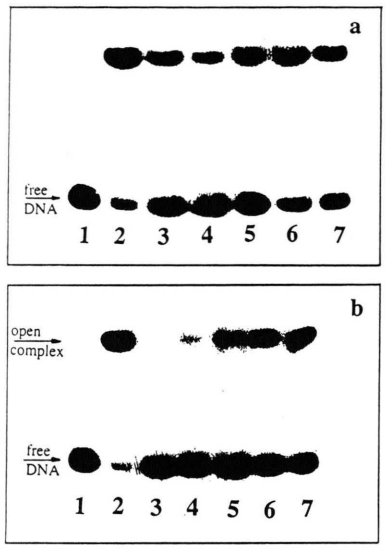

Fig. 2. Acrylamide gel electrophoresis under non-denaturing conditions at the temperature 37°C of the open complex of RNA polymerase and *tac* promoter formed in the presence of proflavine derivatives.
Examples of autoradiograms of ^{32}P labeled DNA and the enzyme-DNA complexes formed at different acridine concentrations 391 base pairs fragment bearing the *tac* promoter excised from pDR 540 plasmid was used. Lanes 1, free DNA, lanes 2, RNA polymerase/DNA complex and the complex formed in the presence of: lanes 3, PF, a/ 8.4 μM; b/ 19.7 μM; lanes 4, DMP, a/ 7.2 μM; b/ 16.3 μM; lanes 5, DEP, a/ 9.0 μM, b/ 20.3 μM; lanes 6, DJP, a/ 34.2 μM, b/ 79.0 μM; lanes 7, DBP, a/ 37.8 μM, b/ 87.3 μM.

jected to electrophoresis (Fig. 2). The amount of the complex formed considerably decreases in the presence of these drugs, although higher concentrations of DIP and DBP (Fig. 2 a,b, lanes 6, 7) than of PF, DMP and DEP (Fig. 2 a,b, lanes 3–5) are needed. The relative amounts of the complex formed at different drug concentrations were estimated by the radioactivity measurements, and expressed as a percentage of the corresponding controls, i.e. the assays without any drug added (Fig. 2a,b, lane 2). The results were plotted (Fig. 3), and IO_{50} values were read (Table I).

To study drug effects on the initiation, the assay of abortive initiation was used. RNA polymerase in the presence of ATP and UTP repetitively synthesizes pppApU on A1 and A3 promoters of T7 DNA (see Kriebardis *et al.*, 1987 and Gniazdowski *et al.*, 1988 for references). The product was quantitated by the radioactivity measurements and expressed as a percentage of the corresponding controls (Fig. 3). These data allowed determination of IA_{50} values. These results are very close to those obtained in the assay of inhibition of the overall RNA synthesis by the proflavine analogues (Fig. 3). IC_{50} values read from Fig. 3 are presented in Table I. In comparing IA_{50}, IO_{50} and IC_{50} values it should be remembered that the concentrations of DNA in these assays were comparable in terms of promoters. A twentyfold higher DNA concentration in terms of mass unit (and hence in terms of potential acridine binding sites) was used in overall RNA synthesis (IC_{50}) and abortive initiation assays (IA_{50}) than in the binding experiments.

PF and its intercalative derivatives inhibit the binding of RNA polymerase to DNA at about tenfold lower concentration than DIP and DBP (Table I). The binding efficiency versus drug concentration dependency falls below the pppApU and total RNA synthesis curves for the intercalating drugs while it is above these curves for the non-intercalating derivatives (Fig. 3). A relationship between IO_{50}, IA_{50} and IC_{50} for DIP and DBP indicates that their external binding to DNA specifically affects the enzyme interactions with the template although to a lesser extent.

DA_2 , DA_6 (Fig. 1) belong to a series of dicationic diacridines which bind to DNA much more tightly than monomeric 9-aminoacridine and PF. However they do not bind much more tightly than the more closely related dicationic monomer bearing a dimethylaminoalkyl chain in the position 9 (MAPA). Introduction of a third acridine ring to the molecule somewhat increases the binding to DNA. TA_2, TA_6 and other triacridines of this series exhibit bis-intercalative binding to DNA (Atwell *et al.*, 1986), suggesting that the increase in binding of the triacridines is due rather to the third positive charge than the additional intercalating moiety.

Inhibition of pppApU and overall RNA synthesis by diacridines and triacridines are shown in Fig. 4, together with data for MAPA and for EB. Except for TA_2 the polyacridines exhibit very high inhibition of initiation (IA_{50} values 6–7.5 μM, see Table I) and of overall RNA synthesis (IC_{50} values 7.1 μM for DA_2 and about 4 μM for DA_6 and TA_6). The latter values are considerably lower than those of MAPA and EB and other monointerca-

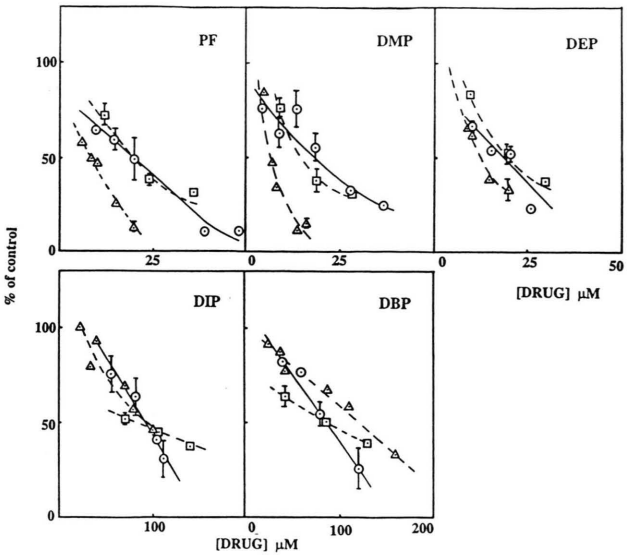

Fig. 3. Dependence of RNA, pppApU syntheses and open promoter – RNA polymerase formation on proflavine derivatives concentration.
100% of RNA, pppApU syntheses or the complex formation corresponds to the uninhibited controls. ⊙ RNA synthesis; ⊡ pppApU synthesis; △ open promoter complex formation. Error bars denote range or standard deviation for experimental points which are averages of two or three independent experiments.

Table I. Inhibition of binding and RNA synthesis by acridines. Drug concentrations (μM) resulting in a 50% decrease.

No.	Acridine	IO_{50}	IA_{50}	IC_{50}
1	PF	8.5 ± 0.8 (2)	19.7 ± 1.1 (3)	19.3 ± 2.4 (4)
2	DMP	6.5 ± 0.7 (2)	6.3 ± 1.3 (3)	18.5 ± 2.8 (4)
3	DEP	13.7 ± 1.4 (2)	20.3 ± 1.7 (3)	9.4 ± 2.7 (3)
4	DIP	98.5 ± 9.9 (2)	78.3 ± 13.7 (3)	93.3 ± 3.3 (3)
5	DBP	130.1 ± 18.9 (2)	87.3 ± 6.5 (3)	83.6 ± 6.8 (3)
6	DA_2	–	6.0	7.1 ± 0.8 (5)[a]
7	DA_6	–	7.4	4.3 ± 0.8 (4)[a]
8	TA_2	–	33.0	29.9 ± 4.8 (5)[a]
9	TA_6	–	6.6	3.6 ± 0.7 (3)[a]
10	MAPA	–	10.0	8.5 ± 1.2 (3)[a]
11	AAC	10.4 ± 0.5 (2)	15.0 ± 0.4 (2)[b]	13.6 ± 1.4 (8)[b]
12	AACH	–	13.5	9.1 ± 0.1 (3)
13	AA2C	32.2 ± 2.8 (2)	26.5 ± 2.8 (2)[b]	31.2 ± 3.7 (5)[b]
14	1MAAC	6.9 ± 1.4 (2)	10.3 ± 1.4 (3)[b]	9.4 ± 0.8 (3)[b]
15	2MAAC	3.2 ± 0.1 (2)	3.6 ± 0.1 (3)[b]	8.1 ± 0.8 (5)[b]
16	3MAAC	12.3 ± 4.0 (2)	17.6 ± 2.0 (3)[b]	16.2 ± 0.2 (3)[b]
17	8MAAC	6.8 ± 0.7 (2)	8.4 ± 0.4 (2)[b]	11.3 ± 2.2 (3)[b]
18	DACA	–	60.0	35.8 ± 3.2 (4)
19	DACAH	–	46.5	20.2 ± 5.8 (4)
20	mAMSA (amsacrine)	–	134	124 ± 17.0 (5)
21	oAMSA	–	–	215 ± 30.0 (2)
22	AMSAAC	–	70.0	51.4 ± 14.5 (4)
23	AMSAEC	–	67.4	34.0 ± 5.6 (3)
24	EB	–	7.8	9.1 ± 2.6 (8)

IO_{50}, IA_{50} and IC_{50} are drug micromolar concentrations resulting in a 50% decrease in open promoter complex formation, pppApU and RNA synthesis respectively. Where the mean values of two or more independent experiments (as indicated in parentheses) are given ± range values or ± SD are shown. Results preliminarly published.[a] Atwell *et al.* (1986); [b] Piestrzeniewicz *et al.* (1990).

lating acridines studied here (Figs. 3 and 4) but typical for other polyacridines previously screened (Atwell *et al.*, 1986). TA_2 exhibits considerably lower inhibition of total RNA synthesis (IC_{50} = 30 μM) and abortive initiation (IA_{50} = 33 μM). As the distance between the acridine rings in TA_2 is 6 and

8 Å (Atwell *et al.*, 1986) which does not allow them to span even a single base pair, it was proposed that the ligand bound by bisintercalation with the central acridine moiety interacting outside the helix. This locally high concentration of intercalating and non-intercalating acridine systems and positive charges may facilitate initial binding of the enzyme to the template. An increase of the amount of RNA synthesized was reproducibly observed at the 10 μM TA_2 concentration as well as with EB at 1 μM (Fig. 4) and with mAMSA and its congeners (see below).

Stability of the acridine – DNA complexes

An advantage of the series of 9-aminoacridine carboxamide derivatives of systematically varied structure (Fig. 1) is that they form intercalative complexes of roughly similar binding strength. In general they are highly polar, bear two positive charges, and association constants (above 10^7 M^{-1} for poly d(A-T) and poly d(G-C)) are broadly similar (Wakelin *et al.*, 1987). There are considerable differences in both the cytotoxicity of the compounds and in the kinetics of dissociation of their complexes with DNA, and these features are well correlated. Analysis of the stability of complexes of these 9-aminoacridine carboxamides with calf thymus DNA revealed that three or four transients can be distinguished. The relationship between the structure of the particular compounds and the kinetics data allowed a model of their highest stability complex with DNA to be proposed (Wakelin *et al.*, 1987).

The effects of 9-aminoacridine carboxamide derivatives on open promoter complex formation, abortive initiation and total RNA synthesis are shown in Fig. 5. As shown before for a series of 13 carboxamide derivatives comprising AAC, AA2C and the methoxy isomers (1,2,3 and 8MAAC), inhibition of both RNA and pppApU syntheses correlates with the formation of relatively slowly dissociating ligand-DNA complexes of time constants 0.3–2.2 s. (Piestrzeniewicz *et al.*, 1990). AAC, 1MAAC, 2MAAC (Wakelin *et al.*, 1987) and presumably AACH form DNA complexes of the latter type and show higher inhibitory effects on total RNA synthesis (IC_{50} below 10 μM, except AAC, see Table I). Inhibition of open promoter complex formation is similar to the inhibitory effect on the pppApU and overall RNA syntheses (Fig. 5). The rapidly dissociating AA2C exhibits lower inhibitory effects in the systems (IA_{50} = 27 μM; IC_{50} = 31 μM and IO_{50} =32 μM, see Table I). For this class of compounds IO_{50} correlates with their association binding constants to poly d(A-T), and on the formation of a slowly dissociating complex with DNA (manifested for AAC, 1MAAC and 2MAAC by the existence of a slow fourth transient in the dissociation kinetics, see Wakelin *et al.*, 1987). Carrying out forward stepwise multiple

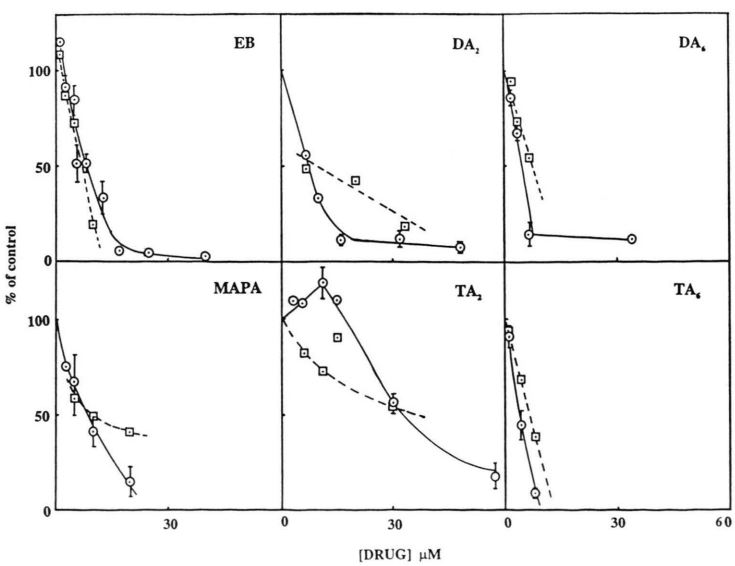

Fig. 4. Dependence of RNA and pppApU syntheses on diacridines and triacridines concentration.
EB and the monomer MAPA [9-(3-dimethylamino)propylamino)acridine] are included to this figure ⊙ RNA synthesis; ⊡ pppApU synthesis. See legend to Fig. 3 for further details.

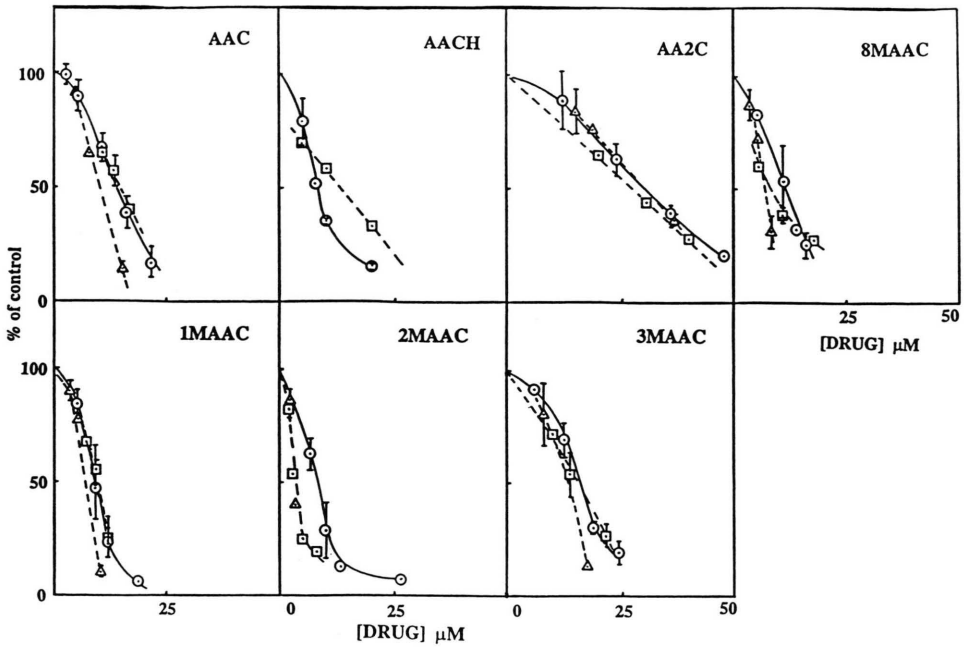

Fig. 5. Dependence of RNA and pppApU syntheses and open promoter-RNA polymerase formation on 9-aminoacridine carboxamides concentration.
266 base pairs bearing the *tac* promoter excised from pKK 223–3 plasmid was used in the open promoter complex formation assay. ⊙ RNA synthesis; ⊡ pppApU synthesis ; △ open promoter complex formation. See legends to Figs. 2 and 3 for further details.

linear regression of log IO_{50} against log K_{AT} and the indicator variable I (I=1 if the exhibiting the fourth transient complex is observed, i.e. for AAC, 1MAAC and 2MAAC and I=O for AA2C, 3MAAC and 8MAAC) we obtain the best fit equation:

$$\log IO_{50} = -0.89\ (\pm\ 0.21)\ \log K_{AT} - 0.20\ (\pm\ 0.11)\ I + 7.61$$
$$n = 6 \qquad r = 0.95 \qquad s = 0.11$$

where n is the number of compounds, r is the multiple correlation coefficient, s is a standard error of the estimate and the numbers in parentheses are the 95% confidence limits of the coefficients. Similar dependence was previously found for the series of 13 9-aminoacridine carboxamide derivatives – in pppApU and the overall RNA synthesis assays (Piestrzeniewicz *et al.*, 1990). The six drugs studied here belong to this series.

Although inhibition of total RNA synthesis is a result of ligand interactions at the level of the enzyme binding to DNA we expected that the differences in the kinetics of dissociations between the ligands may be reflected by different effects on elongation. The polyacrylamide gel electrophoresis analysis of the transcript formed with *E.coli* RNA polymerase in the presence of acridine carboxamide derivatives showed a shift to the lower molecular weight region, although no specific bands corresponding to enzyme arrests due to ligand binding were observed (not shown). Hence rapidly dissociating acridines (time constants 10–100 ms i.e. PF and its congeners, some of 9-aminoacridine carboxamide derivatives) and those of time constants of 2 s as well inhibit RNA synthesis primarily through the inhibition of the open complex formation between the enzyme. This mechanism could be postulated for diacridines and triacridines which exhibit similar inhibitory effect on abortive initiation and on overall RNA synthesis.

This conclusion is consistent with the data of Sarris *et al.* (1977) who found that 9-aminoacridine and its spermine-linked dimer affect transcription of T7 DNA by T7 RNA polymerase by inhibition of initiation of RNA chains. Although the repetitive synthesis of the initiating dinucleotide is not fully equivalent to the initiation of polynucleotide

chain (Kriebardis *et al.*, 1987, Kriebardis and Aktipis, 1988) there is a coincidence between drugs which were found to inhibit primarily initiation and/or binding, and those which inhibit abortive initiation and overall RNA synthesis to a similar extent. Similarly EB, a model monointercalating ligand exhibiting strong effect on the enzyme binding (Richardson, 1973) exhibits similar inhibition of pppApU and RNA syntheses (Fig. 4, Table I). On the other hand actinomycin D which is a classic inhibitor of elongation (Sarris *et al.*, 1977, Gale *et al.*, 1981, White and Phillips, 1989a,b), under our conditions has IC_{50} = 0.45 μM and IA_{50} = 20 μM (unpublished observation). The effect on elongation depends considerably on the dissociation rate of the ligand-DNA complexes. Hence with T7 RNA polymerase distinct stops are seen with ligands which form DNA complexes of half-lives at least 7 s (White and Phillips, 1989a,b). Similarly considerably higher inhibition of RNA synthesis than the initiating dinucleotide was seen with anthracycline antibiotics, daunomycin and marcellomycin (Kriebardis *et al.*, 1987, Kriebardis and Aktipis, 1988). Covalent adducts of psoralen with DNA (i.e. a ligand with essentially an infinite residence time) strongly inhibited RNA synthesis while pppApU synthesis was less sensitive (Gniazdowski *et al.*, 1988).

Effect of AMSA derivatives

A correlation is generally observed between inhibition of RNA synthesis *in vitro* (Table I) and cytotoxicity in cell cultures for proflavine derivatives di- and triacridines and 9-aminoacridine carboxamide derivatives (Atwell *et al.*, 1986, Wakelin *et al.*, 1987, Piestrzeniewicz *et al.*, 1990), However, no such correlation is seen with mAMSA and its derivatives. These are all intercalative ligands that differ considerably in biological activity. mAMSA a potent anticancer drug showing high cytotoxic effects, exhibits the lowest inhibitory effect in total RNA synthesis system of all the acridines studied here except that of its inactive isomer oAMSA (Table II). Attachment to mAMSA of carboxamide side chains (Fig. 1) leads to intercalative drugs which form DNA complexes with the bulky anilide ring and carboxamide substituents interacting in the major and minor groove of DNA helix, respectively (Wilson *et al.*, 1981, Wakelin *et al.*, 1990, Bailly *et al.*, 1992, Searcey *et al.*, 1996). The carboxamide chains increase the binding strength of the ligands to DNA and the inhibitory effect of AMSAAC and AMSAEC (Table II). IC_{50} are 51.4 μM and 34.0 μM respectively (Table I). However these compounds are less effective than those lacking aniline rings (DACA and DACAH). DACA exhibits considerably lower inhibition than the 9-amino analogue AAC, which at pH 7 has two positive charges (one in the ring one in the side

Table II. Dependence of RNA and pppApU syntheses on amsacrine and acridine-4-carboxamide (DACA) derivatives concentration.

Acridine concentration μM	mAMSA		oAMSA		AMSAAC		AMSAEC		DACA		DACAH	
	RNA	pppApU	RNA	pppApU	RNA	pppApU	RNA	pppApU	RNA	pppApU	RNA	pppApU
5	102±10 (4)	91	88±1 (2)	–	132±14 (4)	72	127±21 (3)	90	98±11 (4)	79	96±9 (4)	73
10	109±13 (3)	82	88	–	129±3 (2)	59	123±22 (2)	112	87	70	73	–
20	108±3 (3)	–	90	–	127±27 (4)	–	129±10 (2)	–	72±5 (3)	–	50±7 (2)	57
50	83	–	–	–	52±18 (2)	59	20	79	30	60	16	49
100	65±3 (2)	77	91	–	19±10 (3)	39	10±1 (2)	27	15±4 (2)	23	15±4 (2)	32

The results are expressed as a percentage of the corresponding controls. Where the mean values of two or more independent experiments (as indicated in parantheses) are given ± range values or ± SD are shown. Some results at drug concentrations not listed here are not shown – they were however used to compute data in Table I.

chain). As noted above in contrast to the other series of acridines studied here there is no relationship between the effect on RNA polymerase system and biological effects for mAMSA and its derivatives. The discrepancy between the biological effect and RNA synthesis inhibition may be due to a different mechanism of cytotoxicity. mAMSA is a potent topoisomerase II poison. As recently found an important factor influencing cytotoxicity of acridine-4-carboxamides is their transport rate through the cell membrane (Pastwa *et al.*, 1998).

As with TA_2 (Fig. 4) an increase in the synthesis of RNA up to 110–130% of the control (Table II) is observed at lower concentrations of mAMSA, and its 4-carboxamide congeners (AMSAAC and AMSAEC). Some increase of abortive initiation is occasionally observed with EB (Fig. 4) and AMSAEC (Table II) while no stimulation is seen with mAMSA and AMSAAC (Table II). An increase of the total RNA synthesis at low acridine concentration was observed previously (Wolfe *et al.*, 1971), but not discussed. We observed the stimulation reproducibly particularly with ligands having either additional aromatic ring which was supposed to interact in the groove of DNA (EB, mAMSA and its congeners, TA_2) or with bisacridines which for steric reasons were supposed to exhibit perturbed intercalation complex (Markovits *et al.*, 1989). One possibility is that these ligands preferentially interact with nonspecific binding sites which are transcriptionally non active, hence increasing the amount of the enzyme binding to effective initiation sites. This does not seem however to be the case since the phenomenon was observed with T7 DNA but not with calf thymus DNA (Markovits *et al.*, 1989 and unpublished observation). In the latter template, containing much more single strand breaks and other lesions which might serve as transcriptionally non-active binding sites this stimulation was negligible. Moreover if the amount of the enzyme was changed, the stimulation persisted (data not shown).

The stimulatory effect does not seem to occur at the level of the initiation since except for slight stimulatory effect of AMSAEC on pppApU synthesis, a plateau of the dinucleotide synthesis at the corresponding drug concentration is observed. Hence any specific inhibition by these ligand on initiation from promoters directing the synthesis of short RNA chains is also not probable. There is a possibility that at the low ligand concentration their weak interactions with DNA affects recognition of the termination sites by the enzyme (Richardson, 1973). The evidence of this mechanism is presently lacking.

Conclusions

The effect on RNA synthesis *in vitro* of acridines primarily depends on their binding strength to DNA which in turn depends on intercalation and on ligand positive charge. The primary mechanism of action is related to inhibition of the enzyme binding to DNA. Later steps of RNA synthesis are less sensitive to the drugs. This is different to the effect of covalently binding drugs. Correlation between the cytotoxic activity and RNA synthesis inhibition is not observed with topoisomerase II inhibitors of the AMSA series, but all other compounds (polyacridines, proflavine and its congeners and 9-amino-4-carboxamide acridine derivatives) show correlation between RNA synthesis inhibition and cytotoxicity. Comparison of the inhibition of initiation and of total RNA synthesis may provide simple tests a drug effect differentiating between drug effects on initiation and elongation.

Acknowledgments

This work has been supported by Grant No. 4–0238–91–01 from the State Committee for Scientific Research (Poland).

Atwell G. J., Baguley B. C., Wilmańska D. and Denny W. A. (1986), Potential antitumor agents. 45. Synthesis, DNA-binding interaction, and biological activity of triacridine derivatives. J. Med. Chem. **29**, 69–74.

Baguley B. C., Ferguson L. R. and Denny W. A. (1982), DNA binding and growth inhibitory properties of a series o 2,7-di-alkyl-substituted derivatives of proflavine.Chem.-Biol. Interact. **42**, 97–105.

Bailly C., Denny W. A., Mellor L. E., Wakelin L. P. G. and Waring M. J. (1992), Sequence specificity of the binding of 9-aminoacridine and amsacrine-4-carboxamides to DNA studied by DNase footprinting. Biochemistry **31,** 3514–3524.

Crenshaw J. M., Graves D. E. and Denny W. A. (1995), Interactions of acridine antitumor agents with DNA: Binding energies and groove preferences. Biochemistry **34**, 13682–13687.

Feigon J., Denny W. A., Leupin W. and Kearns D. R. (1984), Interactions of antitumor drugs with natural DNA: ^{1}H NMR study of binding mode and kinetics. J. Med. Chem. **27**, 450–465.

Ferguson L. R., Denny W. A. and Feigon J. (1988), „Petite" mutagenesis in *Saccharomyces cerevisiae* by a series of 2,7-di-alkyl-substituted derivatives of proflavine with differing DNA-binding properties. Mutat. Res. **201**, 213–218.

Gale E. F., Cundliffe E., Reynolds P. E., Richmond M. H. and Waring M. J. (1981), Inhibitors of nucleic acid synthesis. In: The Molecular Basis of Antibiotic Action. Wiley, New York, 258–401.

Geacintov N. E., Waldmeyer J., Kuzmin V. A., Kolubayev T. (1981), Dynamics of the binding of acridine dyes to DNA investigated by triplet excited state probe techniques. J. Phys. Chem. **85**, 3608–3613.

Gniazdowski M., Szmigiero L. and Wilmańska D. (1982), Thiol-dependent inhibition of RNA synthesis *in vitro* by acridines. Structure-inhibition relationships. Cancer Lett. **15**, 73–79.

Gniazdowski M., Czyż M., Wilmańska D., Studzian K., Frasunek M., Płucienniczak A. and Szmigiero L. (1988), Inhibition of DNA dependent RNA synthesis by 8-methoxypsoralen. Biochim. Biophys. Acta **950**, 346–353.

Gniazdowski M. and Cera C. (1996), The effects of DNA covalent adducts on *in vitro* transcription. Chem. Rev. **96**, 619–634.

Kriebardis T., Meng D. and Aktipis S. (1987), Inhibition of the RNA polymerase-catalyzed synthesis of RNA by daunomycin. Effect of the inhibitor on the late steps of RNA chain initiation. J Biol. Chem. **262**, 12632–12640.

Kriebardis T. and Aktipis S. (1988), Inhibition of the RNA polymerase-catalyzed synthesis of RNA by marcellomycin. J. Biol. Chem. **263**, 6960–6963.

Maniatis T., Fritsch E. F. and Sambrook J. (1982), in: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, New York.

Markovits J., Wilmańska D., Lescot E., Studzian K., Szmigiero L. and Gniazdowski M. (1989), DNA binding, cytotoxicity and inhibitory effect on RNA synthesis of two new 1-nitro-9-aminoacridine dimers. Chem.-Biol. Interact. **70**, 73–87.

Müller W., Crothers D. M. and Waring M. J. (1973), A non-intercalating proflavine derivative. Eur. J. Biochem. **39**, 223–234.

Piestrzeniewicz M. K, Czyż M., Denny W. A. and Gniazdowski M. (1990), Inhibition of RNA synthesis *in vitro* by 9-aminoacridine carboxamide antitumor agents. Effects on overall RNA synthesis and synthesis of the initiating dinucleotide. Acta Biochim. Polon. **37**, 299–307.

Pastwa E., Ciesielska E., Piestrzeniewicz M. K., Denny W. A., Gniazdowski M. and Szmigiero L. (1998), Cytotoxic and DNA-damaging properties of N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA) and its analogues. Biochem. Pharmacol, in the press.

Piestrzeniewicz M.K, Szemraj J. and Gniazdowski M. (1993), Effect of proflavine and its 2,7-dialkyl substituted derivatives upon initiation of transcription. Acta Biochim. Polon. **40**, 80–82.

Richardson J. P. (1973), Mechanism of ethidium bromide inhibition of RNA polymerase. J. Mol. Biol. **78**, 703–714.

Sarris A. M., Niles E. G. and Canellakis E. S. (1977), The mechanism of inhibition of bacteriophage T7 RNA synthesis by acridines, diacridines and actinomycin D. Biochim. Biophys. Acta **474**, 268–278.

Searcey M., Martin P. N., Howarth N. M., Madden B. and Wakelin L. P. G. (1996), DNA threading agents – effect of side chain bulk on DNA binding and cytotoxicity of 9-anilinoacridine-4-carboxamides. Bioorg. Med. Chem. Lett. **6**, 1831–1836.

Straney D. C. and Crothers D. M. (1985), Intermediates in transcription. Initiation from *E.coli lac* UV5 promoter. Cell **43**, 449–459.

Straney D. C. and Crothers D. M. (1987), Effect of drug-DNA interactions upon transcription initiation at the *lac* promoter. Biochemistry **26**, 1987–1995.

Wakelin L. P. G., Atwell G. J., Rewcastle G. W. and Denny W. A. (1987), Relationships between DNA-binding kinetics and biological activity for the 9-aminoacridine-4-carboxamide class of antitumor agents. J. Med. Chem. **30**, 855–861.

Wakelin L. P. G., Chetcuti P. and Denny W. A. (1990), Kinetic and equilibrium binding studies of amsacrine-4-carboxamides: a class of asymmetrical DNA-intercalating agents which bind by threading through the DNA helix. J. Med. Chem. **33**, 2039–2044.

White R. J. and Phillips D. R. (1989a), Sequence dependent termination of bacteriophage transcription *in vitro* by DNA-binding drugs. Biochemistry **28**, 4277–4283.

White R. J. and Phillips D. R. (1989b), Drug-DNA dissociation kinetics. *In vitro* transcription and sodium dodecyl sulphate sequestration. Biochem. Pharmacol. **38**, 331–334.

Wilson W. R., Baguley B. C., Wakelin L. P. G. and Waring M. J. (1981), Interaction of the antitumor drug m-AMSA(4'-(9-acridinylamino)methanesulphono-m-anisidide) and related acridines with nucleic acids. Mol. Pharmacol. **20**, 404–414.

Wilmańska D., Małagocka E., Szmigiero L. and Gniazdowski M. (1984), Effect of intercalating and groove-binding ligands on formation of covalent complexes between nitracrine (Ledakrin, C-283) or 8-methoxypsoralen and DNA. Biochim. Biophys. Acta **782**, 285–294.

Wolfe A. D., Cook T. M. and Hahn F. E. (1971), Antibacterial nitroacridine, Nitroacridin 3582: binding to nucleic acids *in vitro* and effects on selected cell-free model systems of macromolecular biosynthesis. J. Bacteriol. **108**, 1026–1033.