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Inhibition of T4 Bacteriophage Yield by 9-Anilinoacridines; Comparison with *in Vivo* Antitumour Activity*

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Abstract—*A series of 9-anilinoacridine derivatives, substituted in the anilino-ring, has been tested in a simple bacteriophage T4 inhibition assay. Corresponding derivatives of 9-anilino-3-aminoacridine and 9-anilino-3, 6-diaminoacridine have also been tested to provide a series of compounds with a wide range of DNA binding characteristics. Anti-tumour assays using the murine L1210 leukaemia system have also been carried out and the results compared in terms of various physicochemical parameters. When biological activity is expressed as a function of DNA binding (estimated using an ethidium displacement assay) and molar refractivity values of the anilino-substituents, two conclusions can be made. Firstly, there is a requirement for a minimum degree of DNA binding in each system. Above this minimum, biological activity is only slightly altered by the magnitude of DNA binding. Secondly, anilino-substituents of small size or polarisability are necessary for optimal activity in the phage system, whereas substituents of larger size or polarisability (e.g., methanesulphonamide) are necessary for optimal activity in the L1210 leukaemia system. Thus the acridine moiety of these compounds plays the major role in DNA binding, whereas, the anilino-moiety may determine the biological selectivity.*

INTRODUCTION

As PART of a programme to develop novel antitumour agents, Cain and co-workers have prepared and tested a large number of derivatives of 9-anilinoacridine [1, 2]. Many of these compounds show high *in vivo* activity against a range of animal tumours, including the L1210 leukaemia. Some of these compounds have also been shown to bind strongly to DNA by intercalation [3, 4] and it has been suggested that cellular DNA may be the site of action. Recent (unpublished) studies in this laboratory of quantitative relationships between antitumour activity and DNA binding for a large group of 9-anilinoacridine derivatives lends support to this hypothesis.

Studies of drug effects on simpler bacterial systems may provide clearer information about the precise mode of action of these compounds. In addition, the simpler bacterial tests may well be suitable predictors of *in vivo* antitumour activity for compounds which act at the level of cellular DNA. Previous work [5] with a series of bisquaternary ammonium heterocyclic compounds showed a clear relationship between antibacteriophage lambda activity in an *Escherichia coli* system, and anti-leukaemia L1210 activity in the mouse. In the case of the anilinoacridine derivatives, the phage lambda system is unsuitable because *E. coli* has a permeability barrier to acridines. However, T-even phage-infected *E. coli* are known to respond to simple aminoacridines [6] and this system was therefore chosen for study. A simple phage inhibition assay has been used, and the activity of a number of simple derivatives compared with that in the L1210 leukaemia system. The results have been analysed in terms of DNA binding and various other physicochemical parameters.

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MATERIALS AND METHODS

Agents

All agents were synthesised at the Cancer Chemotherapy Laboratory, Auckland. Synthetic details are either published [1, 2, 7] or will shortly be published. Samples were pure as judged by thin-layer chromatography. 9-Aminoacridine, ethidium bromide, proflavine sulphate and poly d(AT) were obtained from Sigma Chemical Co., U.S.A. DNA binding C_{50} values [8] in 0.01M ionic strength buffer (9.3mM NaCl, 2mM sodium acetate buffer, pH 5 and 100 μM EDTA) were estimated as described previously using an ethidium displacement technique and poly d(AT) (1 μM in nucleotides) except that the assays were conducted at pH 5.0.

Assay for anti-T4 phage activity

E. coli B was used as both host and indicator strain with phage T4B, and was kindly provided by Professor P. L. Bergquist, Department of Cell Biology, University of Auckland. Phage lysates were prepared using standard methodology and were stored at 4°C over chloroform. Logarithmic phase cultures of *E. coli* in L broth (1% Difco bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.6) were diluted to 10^8 cells/ml in medium, equilibrated at 37°C for 3 min, and infected at a multiplicity of infection of 0.22. After adsorption for 5 min the cells were diluted 10^4-fold into flasks with appropriate dilutions of test drugs. Cultures were incubated for 60 min at 37°C in a shaking water bath, then put on ice and the phage yield was determined by plating appropriate dilutions in L broth on H agar [1% bactotryptone, 0.5% NaCl, 0.2% sodium citrate, 0.2% glucose added separately, and either 1.1% (bottom layer) or 0.6% (top layer) agar]. Reproducibility in repeat determinations was $\pm 16\%$.

Drug concentrations were tested 2–2.5-fold concentration intervals and the concentration for 90% inhibition (I_{90}) was either interpolated, or in some cases extrapolated using the mean slope of the dose-response curves. Over the range 70–90% inhibition, a 2-fold increase in drug concentration produced an approximately 10-fold increase in phage inhibition. Above 99% inhibition, background levels of unabsorbed T4 phage prevented accurate determination.

RESULTS

Table 1 lists a total of 43 simple 9-anilinoacridines which were tested for their ability to inhibit phage T4 production in infected *E. coli*. The measure of activity chosen was the micromolar concentration required for 90% inhibition of phage yield (I_{90}). To avoid problems of drug precipitation from the medium, the maximum concentration of drug used was 70μM. Also listed in Table 1 are two measures of antileukaemic activity for the compounds, the maximum drug-induced increase in lifespan, and the dose producing a 40% increase in lifespan. The methods for the *in vivo* antitumour testing have been described in detail previously [1, 2], and a modified screening evaluation method was used [9]. C_{50} values, as previously defined [7], have been used before as an indicator of DNA binding in the derivation of quantitative relationships between antileukaemic activity, lipophilic/hydrophilic balance and DNA binding for a large series of bisquaternary ammonium heterocycles [10]. In Table 1 the C_{50} values for the binding of 9-anilinoacridines to poly d(AT) at pH 5 measure the DNA binding ability of the drug cations. The decision to use pH 5 values rather than pH 7 values was based firstly on the problem of the solubility of some of the weaker bases at pH 7, which led to less reliable results, and secondly because the C_{50} value then became a pH-independent parameter. *pK* values, determined as before [2] are shown in Table 1. The C_{50} values of agents with *pKa* values greater than 7.5 were the same, within error, at pH 5 and pH 7. The C_{50} value at pH 7 for the weakest base (compound 25, $pKa = 5.48$) was 2.6-fold higher than that at pH 5, and the ratios for other weak bases were intermediate between 2.6 and 1.0. The use of pH 5 rather than pH 7 values did not affect the conclusions discussed below.

Physicochemical parameters listed in Table 1 are σ_p and MR (molar refractivity) values for the 1′-substituents [11], measuring respectively electronic and steric (or polarisability) effects of these groups, and Rm values, which are chromatographically-derived measures of the lipophilic/hydrophilic balance of the agents.

Initially, a group of 24 1′-substituted 9-anilinoacridines was examined, but only 11 of these were active in the phage assay. To extend the range of active compounds, further series of 3-amino substituted (compounds 25–38) and 3, 6-diamino substituted (compounds 39–44) derivatives of 9-anilinoacridine were tested. These compounds were all active in the T4 phage assay (Table 1).

The activities of 9-aminoacridine, ethidium

and proflavine were also measured (Table 1) for reference purposes. These compounds were active in the same range as were the substituted anilinoacridines.

Relationship between DNA binding and physicochemical parameters

For the series of 24 1′-substituted compounds in Table 1, it can be shown that the dominant physicochemical parameter related to DNA binding (as measured by C_{50} values) is the electronic parameter σ_p:

$$\log C_{50} = 0.71\ (\pm 0.27)\ \sigma_p + 0.59$$
$$r = 0.75;\ P < 0.001. \qquad (1)$$

Similarly, for the series of 14 compounds which have a 3-amino substituent on the acridine moiety:

$$\log C_{50} = 0.65\ (\pm 0.21)\ \sigma_p + 0.17$$
$$r = 0.87;\ P < 0.001. \qquad (2)$$

Equations (1) and (2) can be combined by inserting an indicator variable representing the effect of the 3-amino group on log C_{50} values [I $(3-NH_2) = -0.42$]

$$\log C_{50} = 0.80\ (\pm 0.13)\ \sigma_p - \mathrm{I}\ (3-NH_2) + 0.62$$
$$r = 0.90;\ P < 0.0001. \qquad (3)$$

The series of 3, 6-diaminoacridines is too small to calculate a separate indicator variable, but the points lie close to the line described by equation (3).

Relationship between biological activity and physicochemical parameters

For the 30 compounds in Table 1 active in the T4 phage assay, MR was found to be the most important physicochemical parameter relating to activity. If $(-\log I_{90})$ is taken as an index of activity,

$$-\log I_{90} = -0.67\ (\pm 0.25) \log \mathrm{MR} - 0.82$$
$$r = 0.71;\ P < 0.001. \qquad (4)$$

Since DNA binding is thought to be important in the biological action of the acridines, C_{50} values were chosen as the other parameter, and the results for all compounds are shown in Fig. 1.

In the case of the antitumour data, both MR and Rm (as a measure of lipophilic–hydrophilic balance) were important in describing the activity of the 22 active compounds. If $(-\log D_{40})$ is taken as an index of activity,

$$-\log D_{40} = -1.36\ (\pm 0.46)\mathrm{Rm} + 0.74(\pm 0.57) \log \mathrm{MR} + 3.84$$
$$r = 0.82;\ P < 0.0001. \qquad (5)$$

Equations (4) and (5) do not adequately predict the properties of the corresponding inactive compounds (Table 1). Regression equations incorporating a non-linear function of C_{50} may be necessary to predict the activity of both groups of compounds. Because of the limitations of the data we have not attempted to formulate a mathematical model. The notable feature of equations (4) and (5) is that anti-phage T4 activity is negatively related to MR, whereas anti-leukaemia L1210 activity is positively related.

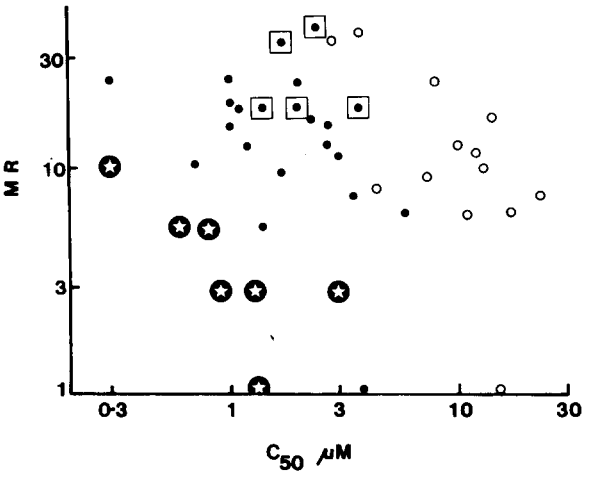
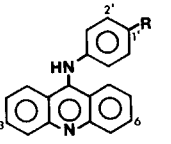


Fig. 1. Relationship between molar refractivity (MR values), C_{50} values as a measure of DNA binding capacity, and biological activity in the 1′-substituted anilinoacridines listed in Table 1. Open circles represent compounds inactive in the T4 phage assay, closed circles represent active compounds, and large starred circles represent the most active compounds (concentration for 90% inhibition less than 20 μm). The boxed circles represent the most active compounds in leukaemia L1210 assays (greater than 100% increase in life span).

DISCUSSION

The plotting of C_{50} values (as a measure of DNA binding) vs molecular refractivity values (as a measure of substituent polarisability and/or size) in Fig. 1. represents a type of cluster analysis for the class of 1′-substituted anilinoacridines. Of the subgroup of 12 compounds with C_{50} values greater than 4.0, 10 are not significantly active in the phage assay (compound 22 shows 70% inhibition of phage yield at 60μM) and 11 are inactive in the L1210 murine leukaemia assay system. Among the compounds with low C_{50} values, those with low MR values are the most active in the phage assay, whereas those with the

Table 1. *Physicochemical and biological data for derivatives of* 9-*anilinoacridine*



No.	R*	I_{90}† (μM)	C_{50}‡ (μM)	*pKa*§	Rm‖	σ_p¶	MR**	ILS††	D_{40}‡‡ (mg/kg/day)
(a) Derivatives of above structure									
1	OH	8	2.9	7.91	0.32	−0.37	2.85	80	75
2	$NH(CH_2)_2CH_3$	23	1.0	8.25	0.74	−0.55	19	0	0
3	$NHCH_2CH_3$	36	1.0	8.30	0.51	−0.61	15	0	0
4	NH_2	39	1.4	8.36	−0.08	−0.66	5.42	69	21
5	$NH(CH_2)_3CH_3$	43	1.0	8.18	0.86	−0.51	24	0	0
6	$NHCOCH_3$	44	2.7	7.51	0.26	0.0	15	60	20
7	$NHCH_3$	50	0.7	8.42	0.24	−0.84	10.3	53	42
8	$NHSO_2PhNH_2$	55	2.4	7.35	−0.04	0.01	40	110	0.3
9	$NHCONHCH_3$	56	2.0	7.77	0.25	−0.25	18	114	15
10	$NHSO_2CH_3$	64	3.7	7.19	0.0	0.03	18	131	15
11	$N(CH_3)$	64	0.9	8.46	0.66	−0.83	16	55	50
12	NHCOPh	—	2.8	7.39	0.64	−0.19	35	35	0
13	$NHSO_2Ph$	—	3.7	7.09	0.45	0.01	38	75	0
14	OCH_3	—	4.4	7.94	0.52	−0.27	7.87	0	0
15	Br	—	7.3	7.00	0.60	0.23	8.88	0	0
16	$N(CH_3)SO_2CH_3$	—	7.9	6.95	0.12	0.15	23	65	80
17	SO_2NH_2	—	10	6.11	−0.47	0.57	12.3	0	0
18	Cl	—	11	7.06	0.60	0.23	6.03	0	0
19	$COCH_3$	—	12	6.12	0.35	0.5	11.2	0	0
20	$CONH_2$	—	13	6.47	−0.25	0.36	9.81	0	0
21	SO_2NHCH_3	—	14	6.02	−0.03	0.57	16	0	0
22	H	—	15	7.46	0.43	0	1.03	0	0
23	CN	—	17	5.93	0.24	0.66	6.33	0	0
24	NO_2	—	23	5.58	0.27	0.78	7.36	0	0
(b) 3-Amino derivatives									
25	OH	4.1	1.3	9.60	−0.01	−0.37	2.85	nd	nd
26	NH_2	12	0.6	9.90	−0.47	−0.66	5.42	93	2.2
27	$NHCH_3$	13	0.3	9.95	−0.07	−0.84	10.3	nd	nd
28	H	31	3.9	9.12	0.35	0	1.03	62	24
29	SO_2NH_2	34	2.7	7.98	−0.65	0.57	12.3	63	1.9

30	CN	35	6.0	7.88	0.11	0.66	6.33	79	36
31	SO_2NHCH_3	41	2.3	7.92	−0.13	0.57	16	55	2.9
32	$NH(CH_2)_3CH_3$	44	0.3	9.96	0.54	−0.51	24	nd	nd
33	$COCH_3$	59	3.0	8.01	0.20	0.50	11.2	53	35
34	$N(CH_3)SO_2CH_3$	62	2.0	8.85	−0.07	0.15	23	99	2.5
35	NHCOPh	65	1.7	9.4	0.41	−0.19	35	115	20
36	Br	65	1.7	8.72	0.42	0.23	8.88	30	0
37	$NHSO_2CH_3$	70	1.4	9.8	−0.14	0.03	18	135	0.8
38	NO_2	79	3.5	7.59	0.14	0.78	7.36	35	0
(c) 3, 6-Diamino derivatives									
39	H	2.6	1.6	10.29	−0.11	0	1.03	nd	nd
40	OH	9.3	0.9	10.70	−0.51	−0.37	2.85	nd	nd
41	NH_2	12	0.8	10.99	−0.65	−0.66	5.42	nd	nd
42	SO_2NH_2	31	1.2	9.05	−1.06	0.57	12.3	63	2
43	$NHSO_2CH_3$	34	1.1	10.85	−0.74	0.03	18	76	0.47
(d) Reference compounds									
44	9-aminoacridine	4.5	8.1						
45	Ethidium	9.3	(1.1)						
46	Proflavine	14	2.2						
47	Acridine orange	—	3.9						

*Substituent in formula; Ph = phenyl; $PhNH_2$ = *para*-aminophenyl.

†Concentration for 90% inhibition of T4 phage yield under standard conditions. Dashed compounds were inactive.

‡Concentration for 50% decrease in DNA-ethidium fluorescence in binding assay with poly d(AT) [7].

§*pKa* measured in 20% dimethylformamide-H_2O at 25°C. Values supplied by G. J. Atwell (personal communication).

‖Chromatographic mobility function related to lipophilic–hydrophilic balance [2].

¶Hammet's σ constants [11] for *p*-substituents.

**Molar refractivity, taken from the compilation of Hansch [11].

††Anti-leukaemia L1210 activity. Maximal increase in life span (%), 0 = not significant (less than 25%) n.d. = not determined.

‡‡Anti-leukaemia L1210 activity; dose required (q.d. for 5 days) for 40% increase in life-span. 0 = dose not reached. nd = not determined. Calculated as detailed previously [9].

highest activity in the L1210 system are distributed in a region of high MR values.

The C_{50} values are not directly related to the corresponding association constants of the compounds in Table 1. Reduction of ethidium fluorescence is a consequence of both displacement of ethidium from DNA and quenching of the fluorescence of DNA-bound ethidium [12]. The latter can be estimated in an assay where the poly d(AT) is in excess and the effect of bound drug on ethidium fluorescence is measured in the absence of significant displacement. A correction for quenching enables calculation of binding constants, assuming competitive binding between intercalating species (B. C. Baguley, W. A. Denny and B. F. Cain, manuscript in preparation). Although not shown here, the use of these derived binding constants does not alter the conclusions of this study, and in fact gives a better separation of active and inactive compounds.

The choice of C_{50} values as one of the axes in Fig. 1 is arbitrary since *pKa* values are also highly correlated with σ_p values [2], and it could be suggested that weakly basic compounds in Table 1 are inactive because they are not taken up by bacteria. Although this argument cannot be refuted in the case of bacteria, it does not apply to antitumour activity, since several weakly basic compounds are active [2]. In an analysis of acridine-substituted derivatives of compound 10 (Table 1) using cultured L1210 cells, DNA binding rather than *pKa* is the parameter which best distinguishes biologically active from inactive congeners (B. C. Baguley and R. Garnham, unpublished).

Molar refractivity (MR) values are measures of the polarisability of substituents (i.e., the ability to form an induced electrical dipole). Since, for the series considered, MR is highly correlated with substituent mol wt ($r=0.92$) the dependence of activity on MR may reflect either steric or polarisability considerations. This suggests that the 1′-substituent makes important contacts, either with DNA or with a second macromolecule, which are critical for activity, and which are different in the two systems studied.

Lipophilic–hydrophilic balance, as measured by Rm values, is not important in describing either C_{50} values or T4 phage activity. However, it becomes important in describing L1210 leukaemia activity, presumably reflecting the important role of lipophilic–hydrophilic balances in the pharmacologic behaviour of the agents in mice.

In conclusion, a comparison of the structural requirements for biological activity in two grossly different biological test systems suggests that whereas a minimum degree of DNA binding is important in both cases, the requirements for the 1′-anilino substituent are different. In the case of phage T4, 9-aminoacridine is known to have multiple effects in the phage growth cycle, affecting for example the timing of early events, the frequency of recombination and mutation, the size of the DNA replication complex, the kinetics of head assembly and the timing of lysis [13]. It is probable that the anilinoacridine derivatives affect at least one of these steps and possible that the requirements for 1′-substituent size and shape are different for optimal activity at each step. Mutagenicity has not been examined in the phage T4 system, but in frameshift mutagenesis data gathered using the Ames tester strain TA1537, no correlation has been found between mutagenic activity and T4 phage activity ([7] and L. R. Ferguson, unpublished data).

In the case of mammalian cells, a similar situation may apply, and several processes important for cellular proliferation may be affected. It is possible that normal and tumour cells are differentially affected in one or more of these processes. Hence the choice of an appropriate 1′-substituent in this series may form the basis for antitumour selectivity. A question raised by this concept is whether the same 1′-substituent will be optimal in other mouse tumours as well as in the L1210 leukaemia, and, more importantly, whether the same substituent would be optimal in human tumours.

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