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A Comparison of the Requirements for Antitumour Activity and Antibacteriophage Lambda Activity for a Series of Non-intercalative DNA-binding Agents*

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Abstract—*A series of non-intercalative DNA-binding agents, comprising mainly bisquaternary ammonium heterocyclic compounds, has been found to inhibit strongly the production of bacteriophage lambda following its induction in* Escherichia coli. *The inhibition is much greater than that found with a number of DNA intercalating agents, including 9-aminoacridine, ethidium and Daunorubicin. The inhibition correlated significantly with antitumour effect, as measured in a life extension assay with L1210 leukaemia. Activity in both biological systems demanded the presence of strongly charged groups and a rigid co-planar aromatic skeleton, these requirements being almost identical to those needed to displace ethidium efficiently from DNA in a simple assay system. It is suggested that biological activity is associated with the ability of these agents to bind in the minor groove of the DNA double helix. Data on the antibacteriophage action of one of these agents suggests possible models for antitumour activity.*

INTRODUCTION

IN A series of papers (reviewed in refs[1, 2]), Cain and co-workers have reported the synthesis of a series of bisquaternary ammonium heterocyclic compounds structurally related to the antitrypanosomal phthalanalides (reviewed by Bennett [3]; see compound **9**, Fig. 1 for representative structure). Many of these compounds have shown high activity in the murine leukaemia L1210 system, and there is a significant correlation between antitumour activity and DNA binding, the latter being estimated using an ethidium displacement assay [2].

Cain *et al.* [1] proposed a series of structural requirements which were necessary for compounds of the above type to be active antitumour agents. These included correct lipophilic–hydrophilic balance, the presence of two strongly charged cationic centres separated by a rigid, co-planar aromatic skeleton, and an overall curved conformation. On the basis of model building, they proposed that these properties, with the exception of lipophilic–hydrophilic balance, were required for lodgement of the molecules in the minor groove of the DNA double helix. Subsequent work [4] has shown that the DNA-binding properties of representative members of this series are entirely consistent with this mode of binding.

A series of bisquaternary ammonium heterocyclic antitumour compounds [based on compound **10** (Fig. 1)] has been shown to suppress the production of bacteriophage lambda following infection, or following induction of a heat-inducible mutant [5]. Since it is possible that the mode of antibacteriophage action is in some ways analogous to that of antitumour action, we have examined the structural requirements proposed by Cain *et al.* [1] to be necessary for antitumour activity, in order to determine whether they are also required for antibacteriophage activity.

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MATERIALS AND METHODS

Agents

Compounds **6**, **10–24**, **26–32** (Table 1) were synthesised in the Cancer Research Laboratory, Auckland, as previously described [1, 2], and were provided by Professor B. F. Cain. The compounds were pure as judged by thin-layer chromatography in two solvent systems (B. F. Cain, personal communication). Compounds **5** and **8** were provided by May and Baker Ltd., U.K. Compound **8** was purified and converted to the dichloride salt (by G. J. Atwell, Cancer Chemotherapy Laboratory) before use. Compound **9** was provided by Dr. H. B. Wood, National Institutes of Health, U.S.A. Compound **7** was purchased from Boehringer Mannheim, Germany, and compounds **3**, **4** and **25** were from the Sigma Chemical Company, U.S.A.

Biological tests

Bacteriophage λ cI857, the *E. coli* strain PB624λ and the indicator strain PB151 were provided by Professor P. L. Bergquist of the Department of Cell Biology, University of Auckland. The λ cI857 lysogen was derived from a pure *E. coli* K12 strain and differed from that used by Counsilman *et al.* [5], which was a hybrid strain of *E. coli* B and K12. The procedure followed was similar to that described previously [5]. The agents, dissolved in aqueous solution at 1 mg/ml, were added to

Table 1. *DNA binding and biological data*

No.	Name or ref. No.	Anion	Interchange distance (Å)	C_{50} Values Poly (dA–dT) (μM)	C_{50} Values Poly (dG–dC) (μM)	Phage log *I*	L1210 ILS_{max}
1	9-Aminoacridine	Cl^-	—	10	9.1	0.7	n.t.
2	Proflavine	SO_4^{2-}	—	4.7	5.3	0.5	n.t.
3	Ethidium	Br^-	—	—	—	0.0	n.t.
4	Acridine Orange	Cl^-	—	6.0	8.2	0.1	n.t.
5	Daunorubicin	Cl^-	—	—	—	0.2	n.t.
6	*m*-AMSA	$CH_3SO_3^-$	—	13	19	0.1	111
7	Distamycin A	Cl^-	—	0.07	36	1.8	n.t.
8	Berenil	Cl^-	13	0.9	5.7	0.3	<25
9	NSC-57153	Cl^-	19	2.2	11	2.6	272
10	NSC-101327	TsO^-	26.5	0.4	0.7	5.9	174
11	NSC-125028	TsO^-	22.5	0.14	1.8	5.8	214
12	SN 4094	TsO^-	18	1.5	2.1	5.4	110
13	SN 6999	Br^-	18.5	0.17	2.8	5.3	170
14	NSC-176319	Br^-	18.5	0.09	1.05	3.3	239
15	SN 6134	TsO^-	24.5	0.3	0.52	1.9	86
16	SN 6135	I^-	24.5	0.77	1.7	4.9	176
17	SN 6136	I^-	21.5	0.91	2.1	7.0	170
18	SN 7003	TsO^-	21	2.0	4.5	0.4	25
19	SN 7108	TsO^-	20.5	3.5	5.0	0.2	<25
20	SN 5673	TsO^-	19.5	7.7	22	1.9	<25
21	SN 6631	TsO^-	28.5	0.22	0.23	4.5	98
22	SN 6752	TsO^-	27	0.11	0.38	5.7	76
23	SN 6050	TsO^-	22.5	0.18	0.60	4.6	145
24	SN 13232	Br^-	25	22	77	0.4	<25
25	Spermidine	Cl^-	10.5	46	32	0.1	—
26	SN 16814	TsO^-	26	5.3	11	1.4	100
27	SN 8518	TsO^-	25	6	10	0.1	<25
28	SN 8305	Br^-	18	2.5	6.5	0	<25
29	SN 6058	TsO^-	20	2.8	8.8	1.3	<25
30	SN 9592	Br^-	18.5	4.5	15	0	<25
31	SN 6330	TsO^-	26	5.4	12	2.4	45
32	SN 6324	TsO^-	26	8	14	0	27

Chemical structures are shown in Fig. 1; the compounds are all salts with the indicated anion (TsO^- = *p*-toluenesulphonate). The interchange distance was measured from Courtald models [1]. C_{50} values were measured for the two double-standard DNA polymers poly (dA–dT) and poly (dG–dC). Daunorubicin could not be assayed because of interference with ethidium fluorescence. The inhibition of phage lambda yield was determined at a drug concentration of 10 μg/ml and given by the expression log $I = \log_{10}$ (control titre/experimental titre). The standard standard deviation of determinations was $\pm\log_{10} 0.1$. The life extension data (ILS_{max}) for mice with L1210 leukemia is quoted from previous work from this laboratory where available [1, 2, 10]. n. t. = Note tested.

cultures of the lysogen at 30°C, 5 min before temperature shifts to 42°C for 15 min, followed by 37°C for 75-min. Phage λ was then liberated by shaking the cultures with chloroform, and dilutions were plated on *E. coli* PB151. The initial added drug concentration, unless otherwise stated, was 10 μg/ml, a concentration which still allowed continued growth of the uninduced bacteria [5–7]. For several derivatives where dose–response relationships were determined, the drug concentration required for 90% inhibition of phage yield was approximately 0.5 of that required for 99% inhibition and 0.3 of that required for 99.9% inhibition.

DNA-binding assays

C_{50} values, defined as the drug concentration required to halve the observed fluorescence due to DNA-bound ethidium, were obtained by drug displacement of ethidium from either poly (dA–dT) or poly (dG–dC) (both obtained from Sigma, U.S.A.), and were measured as previously described [2]. C_{50} values for native calf thymus DNA were also determined, but are not shown since they lay between those for poly (dA–dT) and poly (dG–dC).

RESULTS

Antibacteriophage activity of DNA intercalating compounds

Table 1 lists results with six compounds (**1–6**) known to bind to double-stranded DNA by intercalation. None are strong inhibitors of phage λ production. 9-Aminoacridine (**1**) and proflavine (**2**), which are known to possess antibacterial activity, inhibited by 80 and 70% respectively. Ethidium bromide (**3**) and acridine orange (**4**), which are capable of 'curing' bacteria of sex factors and other episomes [8], showed no significant inhibition at this concentration. The two antitumour agents (**5** and **6**), daunorubicin [9] and *m*-AMSA (compound **6**; 4′-(9-acridinylamino) methanesulphon-*m*-anisidide; NSC 249992[10]), also caused no significant inhibition.

Antibacteriophage activity of non-intercalative DNA-binding compounds

Table 1 lists antitumour and antibacteriophage results, and also ethidium displacement data (C_{50} values) for a variety of non-intercalative DNA-binding agents. Distamycin A (**7**) is well-known for its selective binding to adenine–thymine rich DNA and is thought to bind in the minor groove of the DNA double helix [11]. At the same concentration as that used for the DNA intercalators, it caused a 94% inhibition of phage lambda production. The trypanocidal agent berenil (**8**), which, like distamycin, does not unwind closed circular duplex DNA [12], inhibited by only 50%. In contrast, the trypanocidal and the antitumour phthalanilide (**9**) and the antitumour bisquaternary salt (**10**), which are non-intercalative DNA-binding compounds by the same criteria as used for berenil [4], inhibit phage production by 99.7 and 99.9999% respectively. Bacterial growth is only slightly inhibited in uninduced cultures over the same period as used for phage assay [5–7]. Four other antitumour bisquaternary salts (**11–14**), two of which (**13** and **14**), like compounds **8–10**, appear to be non-intercalators [4], inhibit phage production. Compound **14** has been subjected to pharmacological testing as a potential clinical antitumour agent [13].

Homologous series of compounds based on the structures of **10**, **12** and **13**, but symmetrically substituted with differing *n*-*N*-alkyl quaternary functions, were examined for activity (Fig. 2). The optimum chain length was 2–3 for compounds based on structure **10** and 4 for compounds based on structure **12**. However, all the homologues caused strongly reduced bacteriophage lambda yield at a concentration of 10 μg/ml (10–15 μM). This concentration was therefore used for the remaining comparisons in Table 1.

Activity of compounds with different basic centres

The properties of compounds **9–14**, together with those of compounds containing a methylguanylhydrazone, biguanide or guanide group in combination with a quaternised pyridinium function (**15–17**), demonstrate that the combination of one type of strongly basic centre with another can provide active agents. However, the combination of a strong base with a weakly basic benzylamine or aniline function (**18**, **19**) provided compounds which showed little or no biological activity. By comparison, distamycin A (**7**) has only one strongly cationic centre, but nevertheless binds strongly without intercalation to poly (dA–dT) [11], has a low C_{50} value for poly (dA–dT) and shows significant antiphage activity.

Dependence of biological activity on the distance between basic centres

Compounds **20–23** (Table 1) are related to compounds **10–12**, but have different intercharge distances, as measured on Courtald models in co-planar configuration ([1]; B. F. Cain, personal communication). Together with compounds **8–17**, they cover a range from 13 to

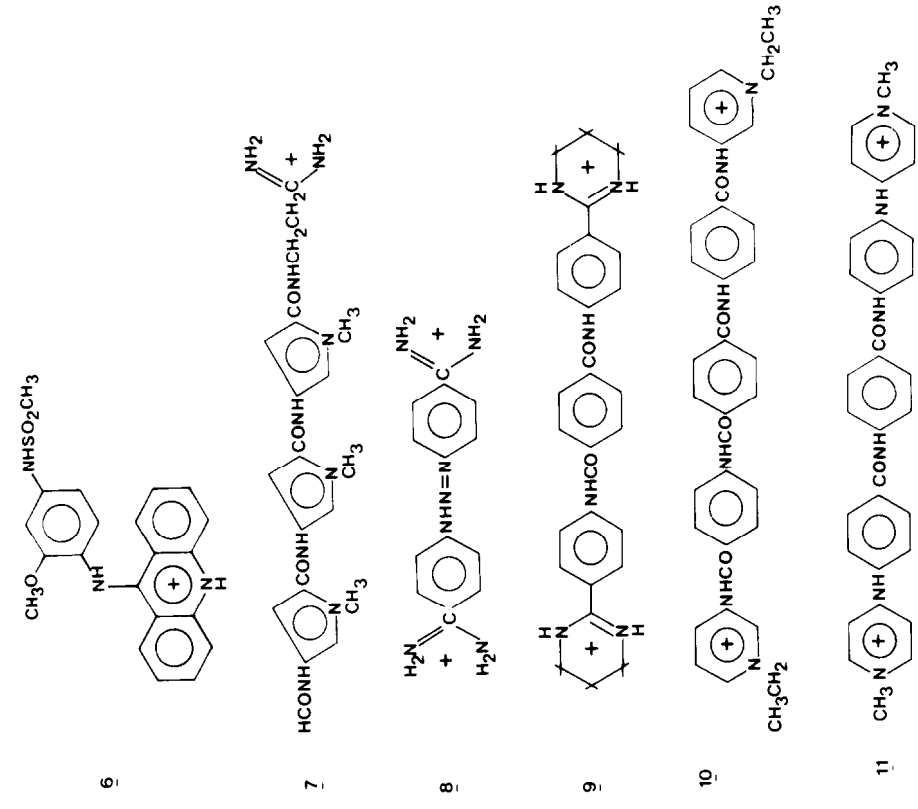

Fig. 1(b).

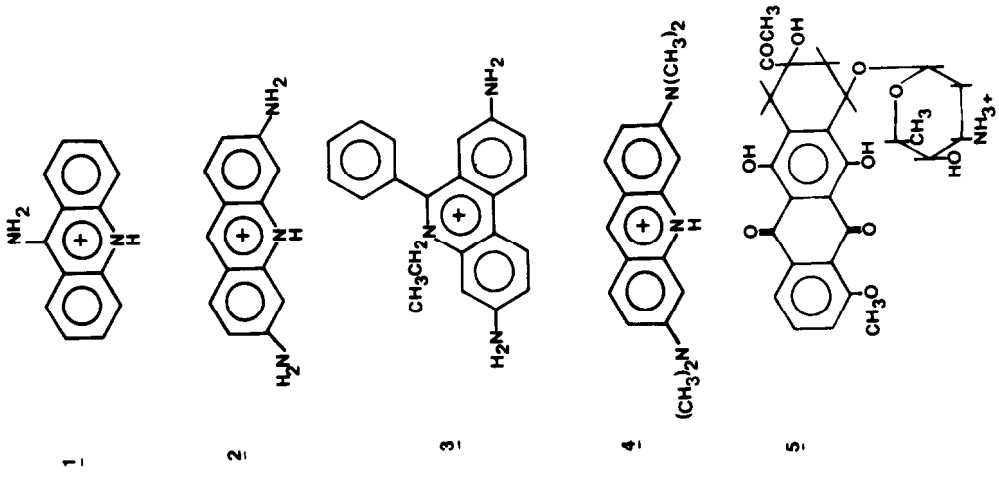

Fig. 1(a).

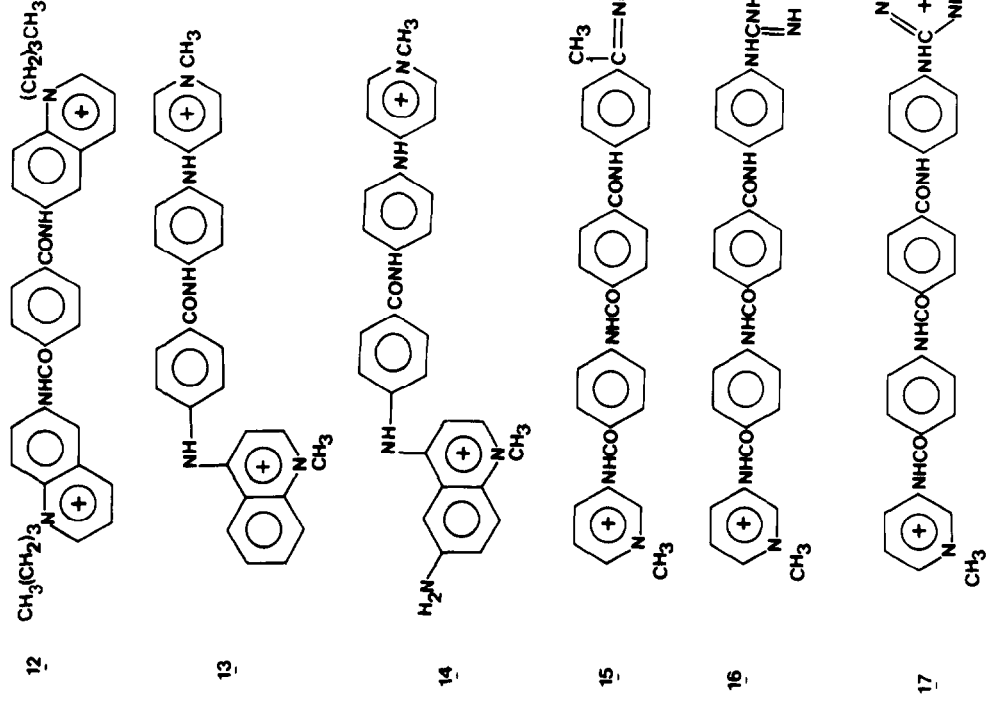

Fig. 1(*c*).

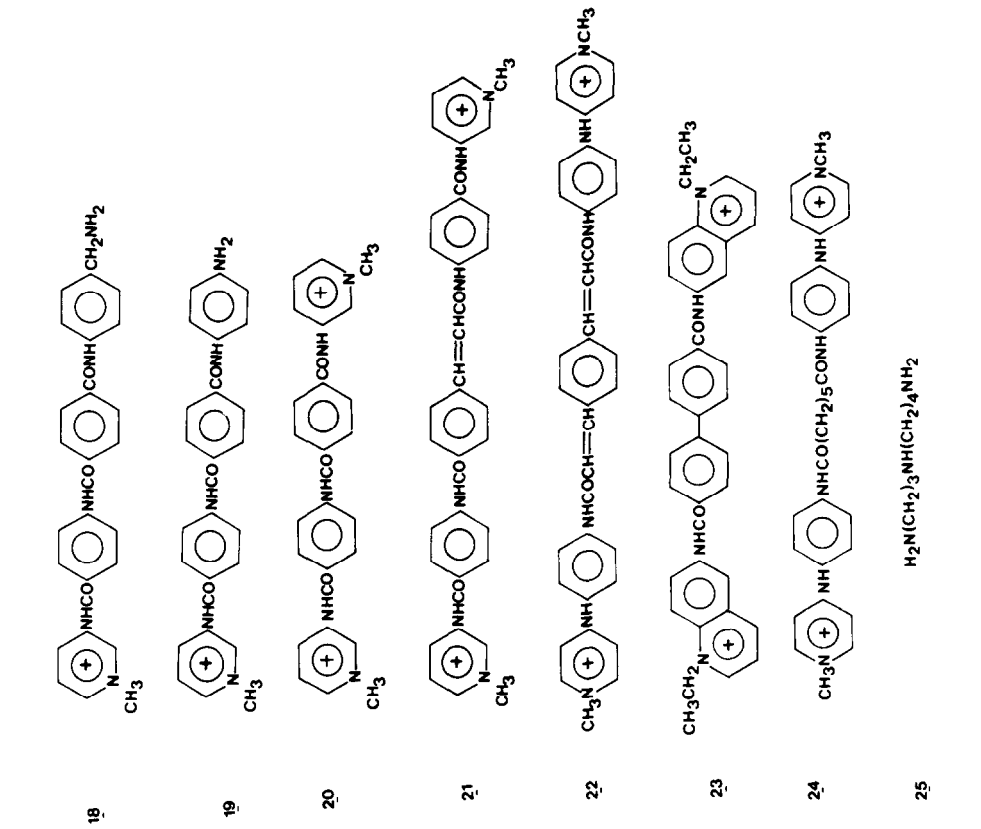

Fig. 1(*d*).

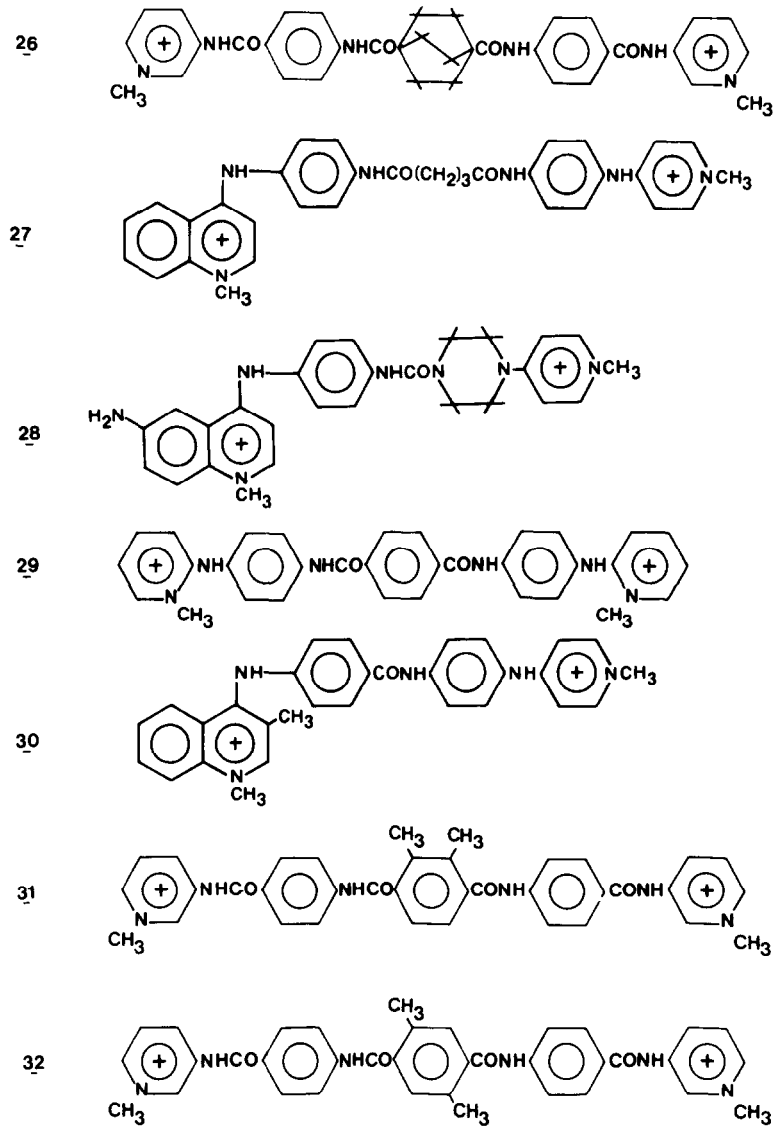

Fig. 1(*e*).

Fig. 1. *Structures of compounds described in Table* 1.

28.5 Å. Compound **8** was the least active in both systems, but no clear relationship between biological activity and intercharge distance was observed.

Dependence of biological activity on the rigidity of the aromatic skeleton

Compound **24** (Table 1) has the central aromatic moiety replaced by a flexible aliphatic chain. It has a relatively high C_{50} value for poly (dA–dT), comparable to that of the polyamine spermidine (compound **25**), and lacks biological activity in the two assays. On the other hand, compound **26**, where the central aromatic residue has been replaced by a rigid bicyclo-octyl group, has a lower C_{50} value and shows activity in both phage and L1210 systems. In the quinolinium series, replacement of a central aromatic residue of **14** by an alkyl chain (compound **27**) or a piperazine linkage group (compound **28**) provides compounds with no biological activity in either system. Molecular rigidity therefore appears to be important for activity.

Requirement for co-planarity of the aromatic skeleton

Compound **26**, although not constrained to be co-planar, can nevertheless adopt a conformation where the aromatic residues are all in the same plane. Compounds **29** and **30**, because of steric interference between the methyl

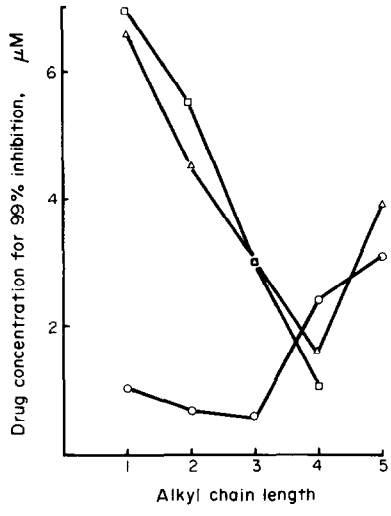

Fig. 2. Antiphage λ activity of homologues of compounds **10**(○- - - -○), **12**(△- - - -△) *and* **13**(□- - - -□)(*Table* 1) *containing* n-*alkyl* N-*quaternising functions of differing chain lengths. Each compound (the* n-*propyl homologue of compound* **12** *was not available) was tested at a variety of concentrations. The micromolar concentration required for* 99% *inhibition of phage yield* (I_{99}) *was determined and plotted versus the carbon chain length of the quaternising function.*

group and the linkage of the quaternary centre to the rest of the molecule, cannot adopt a completely co-planar conformation. They are inactive towards L1210 leukaemia, although compound **29** shows some activity in the phage system.

Requirements for an overall curved structure

The design of compounds to test this requirment is much more difficult. Following the example of Cain *et al.* [1], compounds **31** and **32** were compared. *Trans* di-methyl substitution of the central aromatic group would be expected to inhibit the ability of the molecule to fit a curved site of radius 20 Å (corresponding to the DNA minor groove). However, both **31** and **32** may be hindered in the adoption of a co-planar conformation by interference between the methyl groups and the adjacent amide linkage groups (see previous section). Compound **31** shows greater biological activity than compound **32** in both assay systems.

DISCUSSION

Although ethidium displacement data is the only available evidence for the DNA binding of compounds **11**, **12** and **15–32** in Table 1, the obvious structural similarity in the series suggests that they bind to DNA in a manner analogous to that of compounds **7–10**, **13** and **14**, i.e., with a large apparent site size and in the absence of DNA unwinding [4]. This behaviour is consistent with non-intercalative DNA binding in the minor groove, previously suggested for the bisquaternary ammonium heterocycles [1], berenil [12] and distamycin A [11]. The activity observed in the phage lambda system may be a sensitive indication of non-intercalative DNA binding. This activity contrasts with the ability of some intercalating antibiotics to *induce* the production of lambda bacteriophage in lysogenic *E. coli* [14]. Studies with compound **10** have failed to show any evidence of phage induction [15], but it is possible that any inductive activity is masked by the inhibitory properties.

Ethidium displacement data do not provide a quantitative measurement for the binding of non-intercalative drugs to DNA, but nevertheless they provide a useful means for comparison. Ethidium displacement has confirmed the DNA-binding sequence selectivity of a number of agents which have been studied by classical methods [16]. In a large series of bisquaternary ammonium heterocycles, antitumour activity, as measured by increased lifespan of drug-treated leukaemic mice, is a function of C_{50} values for poly (dA–dT) and poly (dG–dC) [2]. In the limited series of quaternary or bisquaternary compounds listed in Table 1, the percentage increase in lifespan (ILS, putting ILS = 0 for inactive compounds) is significantly related to C_{50} values for poly (dA–dT) (the 95% confidence limits on the slope are shown in brackets, and s is the standard error of the regression):

$$\text{ILS} = -86.7(\pm 28.7)\log C_{50} + 91.3$$

$$(n = 22,\ r = 0.78,\ s = 51.5,\ P < 0.001).$$

It is apparent that the introduction of a flexible link in the drug molecule, or the introduction of groups which hinder the adoption of the rigid planar curved structure proposed by Cain *et al.* [1], decreases biological activity and also decreases DNA binding as measured by C_{50} values. Therefore, the structural requirements for fitting a curved site provided by the minor groove of the DNA helix and the requirements for antitumour activity appear to be similar.

For the series of quaternary and bisquaternary compounds in Table 1, antitumour activity also correlates significantly with antibacteriophage activity:

$$\text{ILS} = 26.8(\pm 8.2)\log I + 8.4$$

$$(n = 22,\ r = 0.81,\ s = 46.4,\ P < 0.001).$$

Despite the overall correlation, the activity of some compounds is not parallel in the two systems (for instance, compounds **9**, **26** and **29**). Factors related to drug distribution, metabolism and selective toxicity are certainly involved and a more detailed study is underway to compare antibacteriophage activity with antileukaemic activity both *in vitro* and *in vivo*. Other factors related to the conformation or base sequence of the DNA-binding site may also be involved.

The correlation obtained suggests that the mechanism of antibacteriophage action of bisquaternary ammonium heterocycles is relevant to understanding their antitumour effect. The action of NSC 101327 (compound **10**, Table 1) on the different stages of phage development has been investigated by Counsilman *et al.* [5] and Robertson [6]. Exonuclease, an early phage function, is expressed in the presence of the drug, and the expression of the products of genes N and O which lead to loss of viability [17] is probably not inhibited strongly since the host *E. coli* cannot be 'rescued' following heat induction. DNA synthesis occurs following phage induction [5] and evidence that at least some replication functions are intact is provided by the observation (Dr. R. C. Gardner, Department of Cell Biology, University of Auckland, personal communication) that compound **9** does not affect the replication of the plasmid λ dv, which contains the phage replication genes [18]. However, the synthesis of phage lysozyme and the production of intact intracellular phage particles is inhibited and hybridisation experiments have indicated that phage-specific RNA synthesised late (30–60 min) after heat induction is strongly inhibited, whereas host RNA synthesis continues [6]. Thus, one possible mechanism for antiphage activity is selective inhibition of RNA synthesis and selective effects on gene expression could be the basis of the antitumour effects of these compounds.

In conclusion, there is a great variety of experimental antitumour drugs now available which bind DNA by non-intercalative means. The strong inhibition of the production of phage lambda caused by some of these compounds has not been observed with other types of DNA binding agents. The immense amount of information now available for bacteriophage lambda should enable the elucidation of the molecular basis for this inhibition and provide data of relevance to understanding the antitumour action of these compounds. Conversely, it may be possible to use the bacteriophage inhibition assay to screen for new antitumour compounds of this type.

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