

Potential Antitumor Agents. 28. Deoxyribonucleic Acid Polyintercalating Agents

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DNA monointercalating agents of the 4'-(9-acridinylamino)methanesulfonanilide series provide high, broad-spectrum experimental antitumor activity. Certain consequences of a DNA intercalation site of action have been explored. For a topologically constrained, negatively supercoiled, twin-helical DNA, drug association constants in relation to those for the corresponding linear, relaxed DNA should increase exponentially as the number of equivalent intercalating nuclei in a drug increases. As a preliminary to preparing DNA polyintercalating agents for antitumor evaluation the structural features necessary for bisintercalation have been investigated. Drug binding to nucleic acids was monitored by spectrofluorometric quantitation of the displacement of ethidium from DNA; results are provided as C_{50} values, those micromolar drug concentrations necessary to displace 50% of DNA-bound ethidium. Potential bisintercalators prepared differed in the structures employed to space the intercalating nuclei and the separation of the latter. Each homologous series of potential bisintercalators provided an individual pattern of progression of C_{50} values. Viscometric studies showed selected agents of low C_{50} values to unwind the supercoiling of PM2 bacteriophage DNA in a manner characteristic of intercalating agents. A symmetrical diacridine provided a high unwinding figure (41° , in relation to ethidium at 26°), twice that of the monomeric acridine precursor and, to this criterion, is a bisintercalating agent. Although considered supralipophilic many agents containing two spaced acridine nuclei, or one acridine and one quinoline nucleus, proved active against the P-388 and L1210 leukemias; more hydrophilic examples may display greater antitumor activity. There was no clear correspondence between PM2-DNA unwinding angles or C_{50} values and level of biologic activity.

Earlier studies of the structure-antileukemic (L1210) relationships, for congeners of the DNA-intercalating 4'-(9-acridinylamino)alkanesulfonanilides, demonstrated that examples bearing a second strongly basic function [$-(CH_2)_nNH_3^+$; $-(CH_2)_nNHC(=NH_2^+)NH_2$] could provide exemplary activity in usual screening tests.¹ Unfortunately, the dictates of the relationship between agent lipophilic-hydrophilic balance and L1210 activity require that strongly basic agents be very hydrophilic for maximum activity. Such highly charged, hydrophilic agents lack the capacity to effectively distribute into pharmacologic sanctuaries (e.g., the cerebrospinal spaces), fail to eradicate tumor cells in these areas, and are therefore unlikely to have true curative potential in a clinical situation. It was then concluded¹ that such strongly basic materials should be avoided and more desirable agents might result if alternate consequences of a DNA-intercalation site of action were investigated. This publication details the preliminary investigation of one possible consequence of such a site.

A negatively supercoiled, covalently closed circular DNA duplex has free energy in excess of that of the corresponding relaxed, i.e., nonsupercoiled, DNA species.²⁻⁴ The positive free energy of supercoiling can be released by reagent treatment which unwinds or denatures the helical DNA structure.^{2,5} Consequently, the rate constants for such reagent interactions with supercoiled DNA are higher than for those observed with the corresponding relaxed (linear or "nicked" circular) forms.^{2,5} For example, hydroxyl ion, which causes denaturation, reacts more readily with the supercoiled species.^{2,5} Similarly, DNA unwinding ligands, in particular, the intercalating agents, can release some of the positive free energy of supercoiling and these bind more avidly to the supercoiled DNA than the relaxed species.³⁻⁵

Consideration of the equilibrium constants for binding of an intercalating ligand to a supercoiled DNA (K_1) and to the corresponding relaxed form (K_2) demonstrates that these should be related by the expression⁵

$$K_1/K_2 = \exp[g'(\sigma)\Delta\tau/RT] \quad (1)$$

In this equation $g'(\sigma)$ is a binomial function of the superhelical density, σ , i.e., the number of superhelical turns per Watson-Crick helical turn, and $\Delta\tau$ is the unwinding produced by the ligand, measured in fractional turns about the helical axis.

If molecules were prepared containing multiple (i) intercalating moieties, each capable of equivalent DNA unwinding by $\Delta\tau$, the ratio of the equilibrium binding constants (K_1/K_2) should increase in exponential fashion [$\exp(i)$] as the number of intercalating residues increases. Such binding selectivity must be considered of topological origin and deriving from the tertiary structures since the primary sequences of the DNA species considered are identical.

Covalently closed, circular duplex DNA is relatively widely distributed in nature. The rapidly growing list of such DNAs includes those from mitochondrial DNA, the tumor viruses polyoma,^{6,7} SV40, and rabbit and human papilloma,⁸⁻¹⁰ certain bacteriophage DNAs, the intracellular form of ϕ X174, M13,¹¹ and λ ,¹² and bacterial episomes;^{13,14} and the colicinogenic factor E₁, a bacterial plasmid.¹⁵

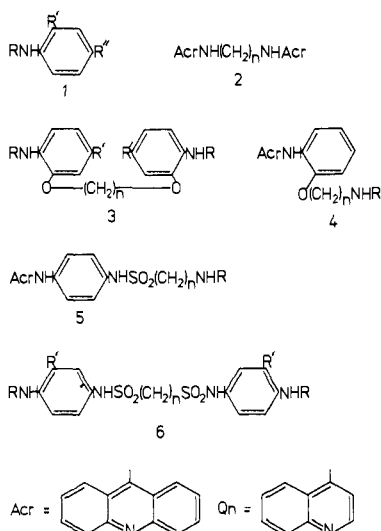
Induction of animal cancers by tumor viruses is well proven and similar viral etiology is suspected for man. Chemotherapeutic eradication of virally induced animal cancers need not eliminate the causative virus; later reinfection can occur.¹⁶ Polyintercalating agents, by binding selectively to such viral DNAs when these are of superhelical nature, might permit selective abrogation of viral multiplication and function. Such agents might then have valuable prophylactic properties for reducing both initial tumor incidence and recurrence following treatment.

Additionally, many existing antitumor agents both clinically employed (actinomycin D,^{17,18} daunorubicin,^{18,19} and adriamycin²⁰) and, as yet, experimental [ethidium,²¹ fagaronine,²² ellipticine,²³ coralyne,²⁴ tilorone,²⁵ and 4'-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA)^{26,27}] have been shown to be monointercalating agents, and this property has been implicated in their mode of action. From earlier discussion it could be conjectured that polyintercalating agents might well display even more favorable antitumor selectivity than monointercalating drugs. Certain tumor-inhibitory members of the quinoxaline antibiotics, in particular, echinomycin, have already been demonstrated to be bisintercalators.²⁸⁻³⁰ While the well-known DNA monointercalator 9-aminoacridine (14, Table I) does not inhibit growth of the P-388 leukemia, members of a series of bis(9-aminoacridines), alkyl chain linked through their 9-amino groups (15-24, Table I), significantly inhibit this leukemia.^{31,32} The tumor-active members of this series

have recently been demonstrated to be DNA bisintercalators.³³

The potential rewards, attendant on development of successful DNA polyintercalators, have prompted us to study the problems of preparing and characterizing such materials. This communication details a preliminary study of the structural requirements necessary for DNA bisintercalation in a series of model compounds.

Approach and Methods. From our earlier series³⁴ of tumor-active, monointercalating acridine derivatives, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA, 10; Table I) has been selected as a clinical trial candidate.^{26,27} To obtain polyintercalating agents that are readily synthetically accessible, the side-chain component of *m*-AMSA offers advantages in that additional intercalating nuclei can be readily appended via the 3'-oxygen atom or the alkanesulfonamide residue. This potential difunctionality, of the *m*-AMSA side chain, could possibly permit the synthesis of polyintercalating agents, and the excellent antitumor^{26,35} and antiviral³⁶ activity of this agent could prove advantageous when probing further for biologic activity. Simple probes based on the 9-anilinoacridine skeleton (cf. 4 and 5) can be employed to gauge the ac-



ceptability of further intercalating nuclei (R of 4 and 5), attached at these two alternate positions, and also the most effective range of alkyl chain length (*n* of 4 and 5) compatible with bisintercalation, provided adequate assessment criteria for the latter are available. Classical assessment criteria depend on changes in hydrodynamic behavior attendant on the unwinding of supercoiled, closed-circular DNA duplexes (e.g., PM2 bacteriophage DNA)^{18,28} or on the lengthening of small rod-like segments of DNA helices.^{17,37-39} Unwinding of PM2 DNA can be monitored by viscometry or sedimentation analysis,^{18,28} and the drug/DNA phosphate ratio (D/P), when added agent just unwinds all superhelical turns (equivalence point, Figure 2), allows the mean unwinding angle ($\Delta\tau$) per DNA-bound drug molecule to be calculated. Demonstration that a symmetrically structured, putative bisintercalator has a D/P equivalence ratio half that of the individual nuclei alone provides supportive evidence for bisintercalation.²⁸ Increase in length of helical DNA segments (sonicated DNA) attendant on agent intercalation can also be readily monitored viscometrically. The relative helix extension L/L_0 is approximated by the cube root of the ratio (reduced viscosity of complex)/(reduced viscosity of uncomplexed DNA).^{39,40} The theoretical slope of the straight line plot of L/L_0 vs. D/P for a monointercalating agent is 2 and for a bisintercalator 4.^{39,40}

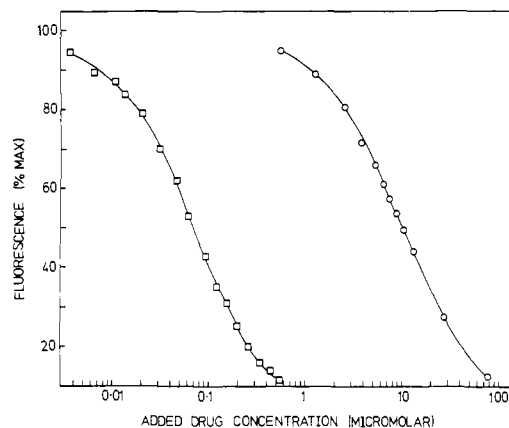


Figure 1. Decrease in ethidium-DNA fluorescence following addition of agents 10 (O) or 58 (□). Initial ethidium and calf thymus DNA concentrations were 1.26 and 1 μ mol, respectively. To provide values for the decrease in the fluorescence of the ethidium-DNA complex alone, that of free ethidium has been subtracted. Fluorescence values are provided as percentages of the maximum. The micromolar drug concentration providing 50% decrease in fluorescence (C_{50} value) is directly available from such plots.

Unfortunately, both of the above diagnostics are relatively laborious for application to an extensive set of molecular probes.

When both nuclei of a symmetrical bisintercalator bind to DNA, there is a considerable increase in association constant over that observed with each nucleus alone. While there may be many other possible modes of drug binding to DNA, besides intercalation, the high occupancy of intercalation sites, by simple acridine derivatives, suggests that these latter sites are energetically favored. It would therefore be expected that if DNA binding constants were monitored, while lengthening an alkyl chain linking two acridine nuclei, when chain dimensions and stereochemical features became such as to just permit both nuclei to intercalate into a DNA stack there would be a sharp increase in binding constant. In developing quantitative structure-activity relationships for our monointercalating,²⁷ tumor-active acridines a modified DNA binding assay, based on the work of Le Pecq and Paoletti,^{41,42} has been employed.⁴³ This assay utilizes competition of an added drug with ethidium for DNA intercalation sites. The marked fluorescence enhancement seen when ethidium intercalates into DNA^{41,42} permits ready spectrofluorometric quantitation of ethidium displacement from such sites. Ethidium displacement assays provide rapid, readily reproducible measures of drug-DNA binding and require only milligram quantities of drug and microgram quantities of DNA. For successful examination any drug must absorb neither the exciting radiation employed (546 nm) nor the emitted fluorescence (595 nm). None of the compounds examined (Table I) absorbed or fluoresced at critical wavelengths. Further, there is some fluorescence quenching of bound ethidium, depending on DNA occupancy level, and this must first be determined before accurate drug-DNA association constants can be derived.

From C_{50} values for ethidium displacement, the micromolar concentration of added drug necessary to displace 50% of the DNA-bound ethidium (Figure 1), it is possible to deduce drug-DNA binding constants. In the simplest case, when all DNA sites are equivalent to both drug and ethidium, if there is a free ethidium concentration (E_F) in equilibrium with bound drug (E_B) and free DNA sites (S_F), then the equilibrium constant for ethidium binding

Table I. Intercalating Agents. Analytical Data and Physicochemical Properties

No.	Type	Substituents	Mp, °C	Formula	Analyses ^a	C ₅₀ ^b	R _m ^c	O.D. ^d	ILS, % ^e P-388	ILS, % ^e L1210
7	1	R = Acr, R' = H, R'' = NHSO ₂ CH ₃ ^f				5.9	0.00	45	223 (3)	107
8	1	R = Qn, R' = H, R'' = NHSO ₂ CH ₃ ^g				490	-0.39	200	-	-
9	1	R = Acr, R' = R'' = H ^h				13	0.43	105	-	-
10	1	R = Acr, R' = OCH ₃ , R'' = NHSO ₂ CH ₃ ⁱ				10.8	0.18	6.7	(5)	114 (2)
11	1	R = Acr, R' = OCH ₂ CH ₃ , R'' = NHSO ₂ CH ₃ ^j				19	0.37	60	218	71
12		Chloroquine dihydrochloride				11	-0.50	40	-	-
13		Mepacrine dimethanesulfonate salt				0.51	-0.13	90	-	-
14		9-Aminoacridine hydrochloride				8.1	-0.08	12.5	-	-
15	2	n = 2 ^k				0.70	-0.44	5	-	-
16	2	n = 4				0.30	-0.26	10	-	-
17	2	n = 5				0.21	-0.06	7.5	36	-
18	2	n = 6				0.15	0.13	2.5	53 ^l	-
19	2	n = 7				0.10	0.27	5	67	-
20	2	n = 8				0.11	0.44	2.5	55 ^m	-
21	2	n = 10				0.09	0.65	2.5	33	-
22	2	n = 12				0.11	0.79	5	-	-
23	2	n = 14				0.14	0.82	-	-	-
24	2	n = 16				0.23	0.88	-	-	-
25	3	n = 2, R = Acr, R' = H	216-218	C ₄₀ H ₃₀ N ₄ O ₂ ·2HCl·2H ₂ O	C, H, N, Cl	0.40	0.17	125	-	-
26	3	n = 3, R = Acr, R' = H	282-284	C ₄₁ H ₃₂ N ₄ O ₂ ·2HCl	C, H, N, Cl	0.60	0.28	50	-	-
27	3	n = 4, R = Acr, R' = H	329 dec	C ₄₂ H ₃₄ N ₄ O ₂ ·2HCl	C, H, N, Cl	0.80	0.53	>500	-	-
28	3	n = 5, R = Acr, R' = H	332 dec	C ₄₃ H ₃₆ N ₄ O ₂ ·2HCl	C, H, N, Cl	1.4	0.60	>500	-	-
29	3	n = 6, R = Acr, R' = H	346 dec	C ₄₄ H ₃₈ N ₄ O ₂ ·2HCl	C, H, N, Cl	2.1	0.71	>500	-	-
30	3	n = 7, R = Acr, R' = H	317 dec	C ₄₅ H ₄₀ N ₄ O ₂ ·2HCl	C, H, N, Cl	3.8	0.75	>500	-	-
31	3	n = 8, R = Acr, R' = H	347 dec	C ₄₆ H ₄₂ N ₄ O ₂ ·2HCl	C, H, N, Cl	9.0	0.82	>500	-	-
32	3	n = 9, R = Acr, R' = H	315 dec	C ₄₇ H ₄₄ N ₄ O ₂ ·2HCl	C, H, N, Cl	3.3	0.85	>500	-	-
33	3	n = 10, R = Acr, R' = H	328 dec	C ₄₈ H ₄₆ N ₄ O ₂ ·2HCl	C, H, N, Cl	8.0	0.92	>500	-	-
34	3	n = 4, R = Acr, R' = NHSO ₂ CH ₃	359 dec	C ₄₄ H ₄₀ N ₆ O ₆ S·2HCl	C, H, N, Cl	0.22	0.01	>500	-	-
35	3	n = 4, R = 4-CONH ₂ -Acr, R' = NHSO ₂ CH ₃	292 dec	C ₄₆ H ₄₂ N ₈ O ₈ S ₂ ·2HCl·2H ₂ O	C, H, N, Cl	0.72	-0.87	275	-	-
36	3	n = 4, R = Qn, R' = H	304-305	C ₃₄ H ₃₄ N ₄ O ₂ ·2HCl	C, H, N, Cl	9.2	-0.10	25	-	-
37	4	n = 3, R = Qn	97-98	C ₃₁ H ₂₆ N ₄ O·0.5H ₂ O	C, H, N	2.7	-0.19	50	-	-
38	4	n = 4, R = Qn	192 dec	C ₃₂ H ₂₈ N ₄ O·2HCl·2H ₂ O	C, H, N, Cl	2.6	-0.07	20	-	-
39	4	n = 5, R = Qn	178-180	C ₃₃ H ₃₀ N ₄ O·2HCl·H ₂ O	C, H, N, Cl	1.9	0.23	17	-	-
40	4	n = 6, R = Qn	288-290	C ₃₄ H ₃₂ N ₄ O·2HCl·0.5H ₂ O	C, H, N	0.99	0.42	15	-	-
41	4	n = 7, R = Qn	129 dec	C ₃₅ H ₃₄ N ₄ O·2HCl·0.5H ₂ O	C, H, N	0.71	0.52	25	-	-
42	4	n = 8, R = Qn	170-172	C ₃₆ H ₃₆ N ₄ O·2HCl	C, H, N, Cl	0.60	0.59	31	-	-
43	5	n = 2, R = Qn	242-244	C ₃₀ H ₂₅ N ₅ O ₂ S·2HBr·2H ₂ O	C, H, N, Br	0.12	-0.54	90	36	-
44	5	n = 3, R = Qn	314 dec	C ₃₁ H ₂₇ N ₅ O ₂ S·2HBr	C, H, N, Br	0.12	-0.45	375	-	-
45	5	n = 4, R = Qn	301 dec	C ₃₂ H ₂₉ N ₅ O ₂ S·2HBr	C, H, N, Br	0.18	-0.31	75	76	29
46	5	n = 5, R = Qn	319-320	C ₃₃ H ₃₁ N ₅ O ₂ S·2HCl·0.5H ₂ O	C, H, N, Cl	0.40	-0.24	200	37	-
47	5	n = 6, R = Qn	190-192	C ₃₄ H ₃₃ N ₅ O ₂ S·2HBr·1.5H ₂ O	C, H, N, Br	0.65	-0.09	75	-	-
48	5	n = 2, R = Acr	269 dec	C ₃₄ H ₂₇ N ₅ O ₂ S·2HCl·3H ₂ O	C, H, N, Cl	0.09	-0.17	60	31	-
49	5	n = 3, R = Acr	228-230	C ₃₅ H ₂₉ N ₅ O ₂ S·2HCl·3H ₂ O	C, H, N, Cl	0.10	-0.25	300	38	-
50	5	n = 4, R = Acr	214-218	C ₃₆ H ₃₁ N ₅ O ₂ S·2HCl·3H ₂ O	C, H, N, Cl	0.09	-0.10	37	-	-

51	5	n = 5, R = Acr	208-212	C ₃₇ H ₃₃ N ₅ O ₅ S ₂ ·2HCl·3H ₂ O	C, H, N, Cl	0.17	-0.04	250	63	35
52	5	n = 6, R = Acr	218-224	C ₃₈ H ₃₅ N ₅ O ₅ S ₂ ·2HCl	C, H, N, Cl	0.21	0.08	150	-	-
53	5	n = 2, R = 4-quinazoliny	268-270	C ₃₉ H ₃₄ N ₆ O ₅ S ₂ ·2HCl·0.5H ₂ O	C, H, N, Cl	0.67	-0.53	50	100 (3)	57
54	6	n = 3, R = Acr, R' = H	263 dec	C ₄₁ H ₃₄ N ₆ O ₄ S ₂ ·2HCl·0.5H ₂ O	C, H, N, Cl	0.064	-0.16	250	29	-
55	6	n = 4, R = Acr, R' = H	352 dec	C ₄₂ H ₃₆ N ₆ O ₄ S ₂ ·2HCl	C, H, N, Cl	0.053	-0.11	>500	-	-
56	6	n = 5, R = Acr, R' = H	236-238	C ₄₃ H ₃₈ N ₆ O ₄ S ₂ ·2HCl·H ₂ O	C, H, N	0.070	-0.02	210	49	28
57	6	n = 3, R = Acr, R' = OCH ₃	171 dec	C ₄₃ H ₄₀ N ₆ O ₅ S ₂ ·2MsOH·0.2H ₂ O	C, H, N, S	0.20	0.22	50	66	32
58	6	n = 4, R = Acr, R' = OCH ₃	263-264	C ₄₄ H ₄₀ N ₆ O ₅ S ₂	C, H, N, S	0.057	0.27	>500	39	26
59	6	n = 5, R = Acr, R' = OCH ₃	154 dec	C ₄₅ H ₄₄ N ₆ O ₅ S ₂ ·2MsOH·H ₂ O	C, H, N, S	0.14	0.35	400	71 (2)	36
60	6	n = 6, R = Acr, R' = OCH ₃	242-247	C ₄₆ H ₄₄ N ₆ O ₅ S ₂ ·2HCl·4H ₂ O	C, H, N, Cl	0.15	0.42	270	-	-
61	6	n = 4, R = Qn, R' = OCH ₃	235-237	C ₃₆ H ₃₆ N ₆ O ₅ S ₂ ·2HCl·H ₂ O	C, H, N, Cl	7.5	-0.28	12	-	-
62	6	n = 4, R = 4-CONH ₂ -Acr, R' = OCH ₃	254 dec	C ₄₆ H ₄₂ N ₈ O ₈ S ₂ ·2HCl·1.5H ₂ O	C, H, N, Cl	0.068	-0.64	11	-	-

^a Analyses for the indicated elements were within ±0.4% of the theoretical figures for the formulas provided. ^b C₅₀, micromolar drug concentration necessary to displace 50% of DNA (calf thymus) bound ethidium; see the Experimental Section and text. ^c R_m values, measure of lipophilic-hydrophilic balance from reversed-phase chromatography; see ref 62. ^d O.D. = optimum intraperitoneal (ip) dose in mg/kg/day in antitumor assays. Treatment daily qd 1-5. P-388 inoculum 10⁶ cells ip; L1210 10⁵ ip. For tumor-inactive agents approximate maximum tolerated dose. ^e ILS % = percentage increase in life span in L1210 and P-388 tests = T/C% - 100. -- signifies significant (>25%) ILS was not observed at doses employed. ^f Reference 34. ^g Reference 34. ^h Reference 34. ⁱ Reference 26. ^j Reference 62. ^k Doses and ILS % P-388 for entries 15-24, where available, are by courtesy of Dr. E. S. Canellakis, Department of Pharmacology, Yale University. Dosing in P-388 tests, for these entries, was ip daily for 7 days starting 24 h after tumor (10⁵ cells ip) implantation. ^l Mean of replicate ILS values of 60 and 47%. ^m Mean of results of 60, 56, and 62% ILS. ⁿ 4-Carboxamido-9-acridinyl in place of 9-acridinyl. ^o MsOH = methanesulfonic acid.

$$K_E = E_B/E_F S_F \tag{2}$$

Similarly, the equilibrium binding constant for the added drug competing for the same sites

$$K_D = D_B/D_F S_F \tag{3}$$

In this equation D_B is the concentration of bound drug, S_F is that of the free DNA sites, and D_F is the free drug concentration. It follows from eq 2 and 3 that

$$K_D = D_B E_F K_E / E_B D_F \tag{4}$$

By measuring the degree of displacement of ethidium, the values of E_F and D_F can be calculated, and utilizing the DNA-ethidium association constant K_D can be computed. Equation 4 shows that C₅₀ values should be inversely related to the drug-DNA association constants.

Use of the ethidium displacement technique with a series of known monointercalating agents provides drug-DNA association constants which are in excellent agreement with those found by more conventional techniques.⁴⁴

Nonintercalating DNA binding drugs will also compete with ethidium. The antileukemic bisquaternary salts originating in this laboratory⁴⁵ bind avidly to DNA but do not remove the supercoiling of PM2 bacteriophage DNA. To this latter criterion these agents are not intercalators and it has been suggested they lodge in the minor groove of DNA.⁴⁵ However, these agents will reversibly displace DNA-bound ethidium. Other nonintercalating DNA binding agents, such as berenil and other chemotherapeutic bisamidines, as well as spermine and spermidine, will also displace ethidium from DNA.⁴³ Most conventional techniques for examining DNA binding provide gross figures for equilibration of drug among all possible binding sites. Ethidium appears no better in this regard and, even though an intercalating agent, its use as a DNA binding probe does not permit distinction of the various sites available for drug lodgement.

The simple treatment leading to eq 4 must be modified further when the sites for ethidium and added drug are of different sizes, as when examining bisintercalators, and allowance made for the fact that some sites on partially filled DNA will be large enough for ethidium but not for drug when site occupancy is high. Additionally, DNA intercalation of an ethidium molecule effectively excludes drug (or ethidium) entry into the two immediately adjacent sites lying to either side ("nearest neighbor exclusion").⁴⁶ Development of necessary mathematical equations, methods for obtaining site size, and application to determination of accurate drug-DNA association constants, for agents of this series, will be dealt with more fully elsewhere.^{43,44} The conclusion that C₅₀ values for ethidium displacement are inversely related to drug-DNA association constants is not altered by the more rigorous treatments.

C₅₀ values, for ethidium displacement, have alone provided a valuable perspective on the binding properties of our probes without necessary recourse to the absolute binding constants. For example, with the series 15-24 (Table I) all agents have lower C₅₀ values than 9-aminoacridine (14). In passing from 17, with n = 5, to 18, with n = 6, the C₅₀ figures reach a nadir value which remains sensibly constant until n reached 14 (23). Independent studies of the ability of agents 15-23 to unwind PM2 DNA, and increase the viscosity of sonicated DNA fragments, show that 14-16 have the properties of typical monointercalators whereas 18-23 are, to these criteria, bisintercalators.³³ Agent 17, with n = 5, proved to have intermediate properties between those expected for a mono- (16, n = 4) and bisintercalator (18, n = 6).³³ Examination

of C_{50} values alone would have directed attention to the first member of this series fully capable of bisintercalation. Our planned approach was then to monitor the C_{50} values for members of any single, congeneric series of probes and, on the basis of these figures, select examples for examination by the more rigorous hydrodynamic assays.

Chemistry. The terminal synthetic step for preparation of type 3-6 structures was normally acid-catalyzed coupling of either a 9-chloroacridine or 4-chloroquinoline component with the requisite aromatic amine. In aqueous media, due to co-occurring hydrolysis of the γ -chloro heterocycle, it was difficult to ensure complete reaction and difficultly separable mixtures of mono- and dicoupled products resulted when diamine substrates (e.g., 3, $R = R' = H$) were employed. Such partial reaction was more evident with shorter chain length isomers (e.g., 3, $n = 2$), presumably the first attached heteroaromatic nucleus hindering approach of the second. While use of excess γ -chloro heterocycle partially circumvents this problem, use of anhydrous media, conveniently *N*-methyl-2-pyrrolidone, is more satisfactory. This solvent also appeared to augment coupling rates with reactions being complete in minutes at room temperature. A standard coupling method employing a moderate excess of γ -chloro heterocycle ($1.1 \times$ theory), in this solvent, provided close to theoretical yields of products, which often crystallized directly from the reaction mixtures in essentially pure condition.

Interaction of sodium 2-nitrophenate and α,ω -dibromoalkanes in dimethylformamide, containing trace quantities of KI, provided high yields of the symmetrical α,ω -di(2-nitrophenoxy)alkanes which, on nitro group reduction (H_2 , Pd/C), provided the diamines necessary for generation of type 3 agents when $R' = H$. Under equivalent conditions sodium 2-acetamido-5-nitrophenate provided similar symmetrical diethers (3, $R' = NO_2$; $R = CH_3CO$). Nitro group reduction, in the latter, mesylation of the formed amine functions, and then hydrolytic removal of masking acetamido groups provided the diamines (3, $R' = -NHSO_2CH_3$; $R = H$) necessary for terminal coupling.

Side-chain components for type 4 agents were prepared by etherification of 2-nitrophenol with *N*-(ω -bromoalkyl)phthalimides, hydrazinolytic removal of the phthaloyl protecting group, and reaction of the liberated amine group with the requisite γ -phenoxy heterocycle in phenol. A following reduction (Fe/H^+)⁴⁷ of the nitro function then produced aromatic amine functions which were reacted with 9-chloroacridine by the standard coupling procedure.

Reaction of 4-phenoxyquinoline with the amine function of the ω -amino(4'-nitrophenyl)alkanesulfonanilides¹ provided the corresponding 4-quinolinylamino derivatives. Nitro group reduction (Fe/H^+) then provided aromatic amines which with 9-chloroacridine provided variants 5 ($R = 4$ -quinolinyl). The corresponding diacridinyl congeners (5, $R = 9$ -acridinyl) could be conveniently prepared in a single pot reaction sequence. The aromatic amino group of the requisite ω -amino(4'-aminophenyl)alkanesulfonanilide was first reacted with 9-chloroacridine in anhydrous phenol employing acid catalysis and then sufficient NaH added, followed by further 9-chloroacridine, and the 9-phenoxyacridine so generated in situ reacted with the remaining aliphatic amine function.

Acylation of either 4-acetamidoaniline or 3-methoxy-4-butanamidoaniline³⁴ with the alkane- α,ω -disulfonyl chlorides, and following hydrolytic removal of alkanamido functions, provided amines 6 ($R = R' = H$ and $R = H$; $R' = OCH_3$) necessary for generation of variants 54-62. The isomeric diamines, with chain lengths of two methylene

groups, i.e., 6, $n = 2$ ($R = H$; $R' = H$ and OCH_3), were unavailable by this route. Ethane-1,2-disulfonyl chloride on reaction with organic bases liberates SO_2 and provides amide derivatives of ethylenesulfonic acid.^{48,49}

Results

C_{50} Values. C_{50} values for a series of purported DNA intercalating agents (7, 9-14; Table I) are provided for perspective purposes. Values for these compounds are in qualitative agreement with expected behavior; 4-aminoquinoline derivatives (8, 12) are not bound as firmly as corresponding 9-aminoacridine derivatives (7, 13), and a subsidiary basic function increases binding to the anionic DNA (cf. 14, 13).

Examination of molecular models, of DNA intercalated agents, suggests that appending a 3'- OCH_3 function to a 9-anilinoacridine, as in 10, could impede total entry of the acridine nucleus into the nucleic acid stack. Possibly this is one reason why 10 has a higher C_{50} value than the precursor 7. In agreement, providing a further increase in the bulk of the 3'-OR function provided an additional increase in C_{50} value (cf. 10, 11). The C_{50} values of the symmetrically coupled diethers 25-33 are then best compared with the more sterically demanding 11, rather than 10 or the series parent 7. Early members of this diether series (25-27) have low C_{50} values and the relationship of these to that of the 3'- OC_2H_5 variant (11) is not disparate with the relationship seen between 9-aminoacridine (14) and the demonstrable bisintercalators 18-23. However, the progression of C_{50} values, on alkyl chain homologation in the diethers 25-33, is quite different from that seen in the bis(9-aminoacridines) 15-24. In the latter a relatively constant value is seen over chain lengths of 6 to 14 methylene groups (18-23), while there is a rapid increase in the diethers 25-31. From the added span contributed by the 9-anilino function a type 3 diether, linked by three methylene groups (26), could provide equivalent separation of the acridine nuclei to that in the $-(CH_2)_7-$ linked bis(9-aminoacridine) 19. There was a discontinuity in C_{50} progression between variants 30 and 33. A similar discontinuity was not encountered in any other homologous series.

With 9-anilinoacridine (9) addition of a 1'- $NHSO_2CH_3$ function, furnishing 7, provides a 2.2-fold decrease in C_{50} value. Similarly, in the symmetrical diether 27 appending two functions, to provide 34, affords a cumulative decrease of 3.6-fold.

Continued use of acridine nuclei, as the intercalating chromophore in polyintercalating agents, would inevitably result in the generation of high molecular weight, difficultly formulable agents. To attempt to alleviate such problems the acceptability of the smaller quinoline nucleus, quoted to DNA intercalate with the antimalarial chloroquine 12,⁵⁰ was investigated employing a type 3 structure. The resulting agent (36) clearly binds more strongly to DNA than does the monomeric precursor 8. The more desirable solubility properties of this quinoline analogue prompted adoption of this nucleus for later studies.

The type 4 quinoline derivatives 37-42 demonstrated a continual decrease in C_{50} values as chain length increased up to eight methylene units (42). Separation of the heteroaromatic nuclei in 42 should be comparable, from models, to that in the C_{10} -bisacridine 21. The reverse progression of C_{50} values was seen in the type 5 quinoline variants 43-47, with values increasing with lengthening alkyl chain, and there was similar progression with the corresponding acridine derivatives 46-52.

Members of the symmetric, dimeric acridine derivatives 54-56 and 57-60 provided the lowest C_{50} values encoun-

Table II. Drug-Induced Unwinding of Bacteriophage PM2 DNA

Agent	D/P ^a at equivalence	Normalized D/P ratio ^b	Helix unwinding angles, deg
10	0.082	1.00	20.5 ^c
26	0.072	0.89	23.0
43	0.12	1.46	14.0
45	0.091	1.10	18.6
48	0.078	0.95	21.6
50	0.066	0.80	25.6
58	0.041	0.50	41.0
61	0.15	1.83	11.2

^a Drug per nucleic acid phosphate ratio at equivalence; determined viscometrically by the methodology detailed for Figure 2. ^b Normalized with respect to 10 which provides equal unwinding to the parent agent 7; ref 27. ^c Employed as internal standard. Initially based on the unwinding produced by ethidium (26°); see ref 27.

tered, with a $-(\text{CH}_2)_4-$ link chain (55, 58) providing optimal binding. A bis(quinoline) analogue, utilizing this same C_4 link chain (61), had a C_{50} value lower than that of the monomeric, biologically active acridine 10.

Each different structural unit, employed to link two heteroaromatic nuclei, provided a different pattern of progression of C_{50} values, as the separation between those two nuclei was increased. There was no duplication of the trend in C_{50} values seen with the simple diacridine bis-intercalators 18–23. The molecular components linking the intercalatable heteroaromatic nuclei cannot be considered merely as convenient spacing devices for those nuclei; the link structures employed must themselves influence either the manner and/or the extent of DNA binding.

PM2-DNA Unwinding Studies. Representative members of the agents in Table I have been examined viscometrically for their capacity to remove the supercoiling in the DNA helix of PM2 bacteriophage DNA. Examples were selected on the grounds of low C_{50} values (Table I) and adequate solubility in the buffers employed in the unwinding assays. All examples studied (Table II) showed the characteristic rise-and-fall response (Figure 2) reflecting removal and reversal of the supercoiling attributable to the drug-induced unwinding of the DNA helix, as required by the intercalation hypothesis.^{50,51} The agents of Table II, to this criterion, are DNA intercalating agents. The drug to nucleic acid phosphate ratios (D/P) at equivalence point, where the drug-induced unwinding of the helix exactly balances the initial number of superhelical turns in the DNA (Figure 2), are provided in Table II along with the normalized ratios of these in respect to that of agent 10. Preliminary investigation²⁷ showed the added 3'-OCH₃ function to provide no significant change in helix unwinding angles; the unwinding produced by the series parent 7 is identical with that of 10 within the limits of experimental error.

The D/P ratios at equivalence point reflect both the capacity of the agents to unwind the helical structure as well as the DNA association constant of the drug. It is the intercalated fraction of the DNA-bound molecules alone which provides unwinding, and the relative proportion of bound molecules is provided by the association constants. Studies of the influence of PM2 DNA concentration of D/P ratios at equivalence point²⁷ demonstrated that, under the experimental conditions employed here, less than 3% of 10 remains unbound at the equivalence point. As evidenced by the measured C_{50} values (Table I) all agents of Table II bind more strongly to calf thymus DNA than does 10. Provided that these C_{50} figures also reflect binding

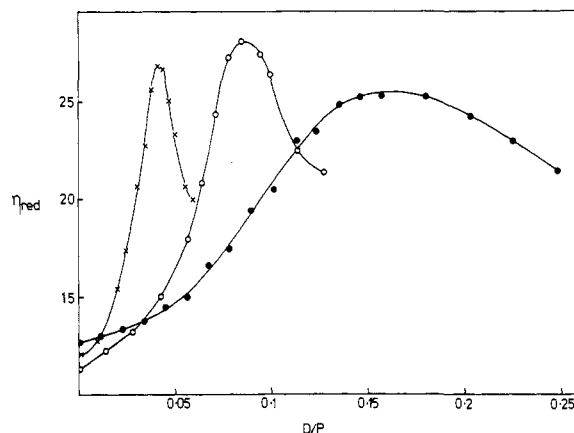


Figure 2. Effect of three different types of agent [10 (O), 58 (X), and 61 (●)] on the viscosity of bacteriophage PM2, closed-circular duplex DNA. Reduced viscosity (η_{red}) at 25 °C was determined as described in the Experimental Section. The molar ratio of drug to DNA-phosphate (D/P) is provided on the abscissa.

to the unwound PM2 DNA, as exists at equivalence point (Figure 2), then the corrections necessary to convert the drug per phosphate figures to true drug bound per phosphate are sufficiently small to be reasonably ignored. Helix unwinding angles provided by a test drug ($\Delta\tau_T$) can then be obtained directly from the drug per phosphate ratio at equivalence point ($[\text{D}/\text{P}]_T$) by comparison with that of a standard agent ($[\text{D}/\text{P}]_S$) producing a known unwinding value ($\Delta\tau_S$), determined by independent means,⁵² using the relationship²⁷

$$[\text{D}/\text{P}]_T/[\text{D}/\text{P}]_S = \Delta\tau_S/\Delta\tau_T \quad (5)$$

While the absolute magnitude of $\Delta\tau$ figures so determined is dependent on the correctness of that of the standard agent,⁵² the relative unwinding angles provided in Table II are correct and provide a valid basis for comparison of the various drugs.

The symmetrical diacridine 58 has an equivalence point exactly half that of the corresponding monomeric acridine 10 (Figure 2), strongly indicative that 58 is a bisintercalating agent. The congener of 58 in which the acridine nuclei are replaced by quinoline rings (61) is, by the unwinding criterion, also an intercalating agent (Figure 2). The analogous structures of 58 and 61 lead to the expectation that the latter would also be a bisintercalating agent. The unwinding data for 61 (Table II, Figure 2) do not support this viewpoint. The $\Delta\tau$ for 61 (11.2°) is appreciably less than that for simple quinoline derivatives such as chloroquine (12, $\Delta\tau = 24^\circ$)^{53,54} which is in turn comparable to that for ethidium (26°).⁵² Unwinding studies with closed circular PM2 DNA have provided unwinding figures, for a wide and diverse range of monointercalators, ranging from 3 to 26°.^{50,51,55} However, DNA intercalation of a planar aromatic nucleus of usual thickness (3.4 Å) would be expected to provide a relatively constant degree of unwinding. To explain this discrepancy it has been suggested that there is more than one type of DNA binding site available and that the ratio of the unwinding angle produced by a drug, to the maximum attainable with a planar intercalating chromophore (assumed to be that provided by ethidium, 26°), provides a relative measure of the fraction of DNA-bound drug actually residing in intercalation sites.⁵³ The low helix unwinding produced by 61 suggests that the bulk of this drug binds to DNA at other than intercalation sites. It is not possible from the data provided to know if the small fraction of this drug in intercalation sites is either singly or doubly intercalated. It is instructive that loss of planar

ring area, by deleting two aromatic rings from 58, providing 61, results in a pronounced change in the fraction of drug which intercalates into DNA, presumably reflecting relative changes in the energetics of binding of these drugs to intercalation and alternative sites.

The above findings highlight a deficiency of the unwinding technique. If a drug provides an unwinding angle which is significantly greater than that possible with any monointercalating agent (i.e., $>26^\circ$), then this provides positive evidence in support of bisintercalation. However, an unwinding angle below 26° cannot be used alone as negative evidence indicating a lack of bisintercalation. When the proportion of bisintercalated drug decreases below 50% of the total DNA-bound drug, such low unwinding figures are to be expected.

Excepting 58 the agents examined in unwinding assays (Table II) have not provided unwinding figures greater than 26° and, therefore, do not provide acceptable evidence of bisintercalation.

From the above discussion it can be expected that those alternatives to the unwinding assays, which also examine changes in the hydrodynamic properties of a DNA species, will likely return a similar set of results. There is a need for alternative techniques, for examining possible DNA polyintercalation, which will not be overly influenced by the partitioning of drug between the possible DNA binding sites available.

Antitumor Activity. The dominating influence of agent lipophilic-hydrophilic balance on biologic activity in the parental 4'-(9-acridinylamino)alkanesulfonanilide series has been well documented.⁵⁶ As before²⁶ R_m values of agent cations, from reversed-phase partition chromatography, have been employed as measures of such balance.

Earlier, from a study of a series of dicationic variants containing a strongly basic aliphatic amine or guanidine function, appended to the end of the alkanesulfonamide chain, it was concluded that maximum antitumor activity required an extremely hydrophilic nature.¹ In the examples of this paper the second basic function is a heteroaromatic component; these variants will therefore be excessively lipophilic by at least the difference between the lipophilic-hydrophilic balance of amine or guanidine functions and that of the heterocyclic unit employed. Because of their supralipophilic nature it was not expected that the initial probes, prepared for determining optimum structural features providing high DNA binding and/or unwinding, would display antitumor activity. Once optimum structural features had been delineated it was expected that examples containing these would have to be made more hydrophilic, by acceptable means, before biologic activity would be encountered. However, in view of the structural novelty of the probes synthesized, these were screened but the generally more drug-sensitive P-388 leukemia was first employed. When significant life extension was encountered with the P-388, the agents were then screened against the L1210.

No example where the two intercalatable nuclei were linked via a 3'-ether function (25-42) provided significant P-388 activity. Generation of more hydrophilic examples by appending in turn acceptable $-\text{NHSO}_2\text{CH}_2$ (34) or acridine 4- CONH_2 functions (35) failed to furnish active compounds.

Several type 5 agents proved P-388 active with little difference between the maximum life extensions obtained with acridine and quinoline variants. If these agents are in fact supralipophilic then it can be expected that the acridine variants, which are more lipophilic than their quinoline counterparts, would be capable of providing

higher levels of biologic activity when adjusted to optimum lipophilic-hydrophilic balance. To check that these probes are in fact excessively lipophilic, the more hydrophilic 4-quinazolinylnyl counterpart (53) of the P-388 active 4-quinolinyl congener (43) was examined and found to have significantly higher activity. Despite the marked reduction in base strength on replacing 4-quinolinyl (4-aminoquinoline, $\text{p}K_a = 9.17$)⁵⁷ with a 4-quinazolinylnyl unit (4-aminoquinazoline $\text{p}K_a = 5.70$),⁵⁸ there was not a corresponding increase in C_{50} values.

There was marked alternation in optimum doses in the acridine (48-52) and quinoline (43-47) subseries as the linking alkyl chain was lengthened. There was also alternation in the life extensions observed, and in the measured C_{50} values, for the diacridine congeners 48-52.

Certain of the bis-AMSA analogues 54-60 displayed convincing antileukemic activity. Again there was alternation in both toxicity and activity as the linking alkyl chain was lengthened. From earlier arguments these, as the most lipophilic congeners prepared, should provide even higher levels of antitumor activity than possible with previous examples, if lipophilic-hydrophilic balance was optimally adjusted. Despite this view an attempt to obtain a more hydrophilic example by appending acridine 4- CONH_2 groups, these earlier found acceptable in the parent AMSA series,⁵⁹ furnished a much more toxic and inactive variant 62. The use of a $-(\text{CH}_2)_4-$ linking chain in the preparation of the latter, and also the tumor-inactive bisquinoline 61, was dictated by the early observed nadir in C_{50} values at this chain length in the bis-AMSA series (55, 58). When animal screening data later became available, a $-(\text{CH}_2)_5-$ alkyl chain length appeared optimal for antitumor activity (56, 59). Despite this deficiency 58, with a tetramethylene linking chain, is biologically active and the more hydrophilic 62 would then have been expected to be more active.

We have previously encountered a subgroup of the 9-anilinoacridines, those bearing anionic functions, where the effects of acridine substituents on antileukemic activity proved markedly different from those seen in the parent 4'-(9-acridinylamino)methanesulfonanilide series.^{60,61} It was then suggested that repulsion between agent and site anionic functions might provide different orientation of the intercalated acridine nucleus from that adopted by agents not bearing such groups. If orientation of the intercalated acridine nucleus changed, then the substituent space adjacent to that nucleus can also be expected to change. Within series of bisintercalating agents, such as 18-23, similar differences in orientation of the acridine ring system are to be expected. The principle of neighboring-site exclusion would predict that there should be a minimum of two purine-pyrimidine base pairs between the two nuclei of a bisintercalator. Molecular model fitting to this scheme would correctly predict the minimum alkyl chain length compatible with bisintercalation in these bis(acridines). When the alkyl chain linking the two acridine nuclei is of just sufficient dimensions to permit bisintercalation, the restraints imposed by that chain will severely limit the orientations that can be adopted by those nuclei. As the linking alkyl chain is lengthened (e.g., 18-21) such restraints on the intercalating nuclei will be relaxed and these may then adopt orientations more closely resembling that which 9-aminoacridine itself might favor. Relative orientation of the intercalated nuclei to the adjacent base pairs will alter with changing length of the linking alkyl chain. Substituent space about those nuclei must then also vary with changing length of the alkyl chain. Such reasoning leads to the novel view that the

acceptability of various acridine substituents could change as the length of the linking alkyl chain is altered. Also, demonstration of the acceptability or desirability of certain substituents, when employed in monointercalating agents (e.g., congeners of 10), need not be directly transferable to bisintercalating examples (e.g., 58).

Discussion

From the examples of Table I there appears to be no simple, direct relationship between C_{50} values and antitumor effectiveness. There are compounds with relatively high and low values which are tumor active and examples where the reverse holds. Further, within homologous series of agents greatest antitumor activity is not displayed by those compounds with the lowest C_{50} values (43, 50, 55, 58); higher activity is provided by agents in which the heterocyclic nuclei are separated by somewhat longer alkyl chains (45, 51, 56, 59). Similarly when the DNA unwinding criterion for bisintercalation is considered, C_{50} values alone pinpointed a bisintercalator (58), in the homologous series 57–60, but this agent was not the most effective antitumor agent; greater activity was observed at longer chain length (59). As the first member (57) of this series is suggested to be supralipophilic, activity would, on this basis, be expected to decrease as lipophilic character was further increased by lengthening the linking alkyl chain. In fact such chain extension, beyond that of the demonstrable bisintercalator 58, provided a more effective agent, 59. Similarly in the bis(9-aminoacridines) 15–24, the most active example is not the shortest chain length member which can be demonstrated to be a bisintercalator;³³ higher activity is encountered at greater linking chain length. From the viewpoint developed above it might be speculated that maximum biologic activity is associated with particular orientations of the intercalating nuclei. Such orientations may not necessarily correspond with those providing the lowest C_{50} values.

Within the variants prepared there is no clear-cut relationship between antitumor effectiveness and either the level of DNA binding, as provided by C_{50} values, or capacity to unwind superhelical PM2 DNA. Adequate examination of the proposition that biologic activity is dependent on a drug–DNA interaction will require effective quantitation of the different drug interactions with the various DNA sites available.

Experimental Section

Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within $\pm 0.4\%$ of the theoretical values. Analyses were performed by Dr. A. D. Campbell, Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal melting point apparatus with the maker's supplied stem corrected thermometer; melting points are as read. NMR spectra were obtained on a Varian A-60 spectrometer (Me_4Si). IR spectra (KBr) were recorded using a Beckmann 237 Infracord. UV spectra were recorded on a Shimadzu UV-200.

To monitor the progress of reactions, purification of products, etc., TLC on SiO_2 (Merck SiO_2 , F_{254}) was used. The partition chromatographic methods used in measuring R_m values have been described earlier.⁶²

Fluorometric Determination of C_{50} Values. A Zeiss PMQ-2 spectrophotometer with a ZFM-4 fluorescence attachment was used at the maximum sensitivity. Excitation of the buffer solution (3 mL in 1-cm path length glass cuvettes) was achieved using a high-pressure mercury lamp and a 546-nm (M 546) filter. Fluorescence emission was measured at 595 nm (slit width 0.75 mm). The buffer designated 0.01 SHE was of ionic strength 0.01 and contained 2 mmol of Hepes, 10 μmol of EDTA, and 9.4 mmol of NaCl. The pH was adjusted to 7.0 with NaOH. Ethidium bromide (1.26 μmol ; Sigma Chemical Co., St. Louis, Mo.) was dissolved in the buffer and a solution of calf thymus DNA (Sigma,

highly polymerized, type 1; 150 μmol of nucleotide concentration in 0.01 SHE) was added to provide a concentration of 1 μmol , increasing the fluorescence reading of the ethidium solution from 15 to 100. The test agent in aqueous solution (0.1–1.0 μmol , depending on the compound) was added in microliter portions from a microsyringe (Agla) through a fine plastic (Portex) catheter. The C_{50} value was defined as the drug concentration required to reduce the fluorescence of the DNA–ethidium complex to 50% (scale reading 57.5). With selected agents multiple determinations of C_{50} values provided figures which have lain within an extreme range of $\pm 9\%$ of the mean value.

Viscometry. The closed-circular, bacteriophage PM2 DNA used in these experiments was kindly provided by Professor A. R. Morgan, Department of Biochemistry, University of Alberta, Canada. Viscometric measurements were performed in 0.01 SHE buffer according to the method of Revet et al.,⁶³ following the procedures used by Dr. M. J. Waring in these laboratories.²⁷ The capillary viscometer used was patterned on that of Waring but contained 1.3 mL of buffer and 212 μmol of DNA (i.e., 91 μg /1.3 mL). Two modifications of the method of Waring were made to minimize possible precipitation of these drugs in the precision microsyringe (Agla) or their adsorption to the plastic (Portex) catheter. The drugs were prepared in aqueous solution, and the first 20 μL of the drug solution in the syringe (the volume of the catheter) was discarded before each addition of drug solution to the viscometer. The temperature was maintained at 25.0 ± 0.1 °C in a water bath. The flow time for water in the viscometer was 93.0 s. DNA and drug solutions were freed from possible trace particulate contaminants by brief centrifugation in glass tubes in a bench centrifuge before use. Flow times were measured in duplicate or triplicate, and reduced viscosities, η_{red} , were calculated in units of dL/g, taking into account the small dilution caused by addition of drug solutions.

α,ω -Bis(2-nitrophenoxy)alkanes. To a suspension of sodium 2-nitrophenate (10 g, 0.062 mol) and KI (0.1 g) in dimethylformamide (DMF, 8 mL) was added the requisite α,ω -dibromoalkane (0.0295 mol) and the mixture heated on a steam bath for 2 h and then to boiling, with stirring, for 0.5 h. Addition of H_2O to the hot solution to turbidity and then cooling provided crystalline products. The collected crystals were washed with ice-cold MeOH and much H_2O and then recrystallized from Me_2CO –EtOH until homogeneous to TLC. Yields were in the range of 58–84%. The bulk of these intermediates has been previously prepared, by somewhat more cumbersome procedures, and recorded physical constants^{64,65} were in good agreement with those observed. Not formerly recorded were 1,7-bis(2-nitrophenoxy)heptane, mp 60–61 °C [Anal. ($\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}_6$) C, H, N], and 1,9-bis(2-nitrophenoxy)nonane, mp 20–21 °C [Anal. ($\text{C}_{21}\text{H}_{26}\text{N}_2\text{O}_6$) C, H, N].

α,ω -Bis(2-aminophenoxy)alkanes were prepared by catalytic hydrogenation of the above nitro compounds in EtOH solution at 60 °C at 45 psi of H_2 in the presence of 5% Pd/C catalyst. Physical constants were in agreement with those earlier described.^{64,65} Not previously reported were 1,7-bis(2-aminophenoxy)heptane, mp 62–63 °C [Anal. ($\text{C}_{19}\text{H}_{26}\text{N}_2\text{O}_2$) C, H, N], and 1,9-bis(2-aminophenoxy)nonane, mp 58–59 °C [Anal. ($\text{C}_{21}\text{H}_{30}\text{N}_2\text{O}_2$) C, H, N].

1,4-Bis(2-acetamido-5-nitrophenoxy)butane. To a stirred, ice-water-cooled suspension of 2-hydroxy-4-nitroacetanilide (10 g, 0.053 mol) in DMF (8 mL), NaH (1.22 g, 0.053 mol) was added as permitted by the resulting effervescence. The resulting thick paste was stirred until the bulk of the NaH had reacted. KI (0.1 g) and 1,4-dibromobutane (5.51 g, 0.0255 mol) were then added and the mixture was slowly heated, while stirring, until the DMF started to boil. After 0.5 h at reflux temperature the mixture was cooled and HOAc (0.5 mL) added, followed by sufficient H_2O (30 mL) to dissolve precipitated salts. The crude crystalline product, collected from the cooled reaction mixture, was suspended in a solution of KOH (5.1 g) in H_2O (50 mL) at 25 °C and the suspension was stirred until of smooth consistency. The undissolved product was collected, well washed with H_2O , and crystallized from DMF–EtOH. Pure product was obtained as colorless needles of mp 279–280 °C (6.7 g, 59%). Anal. ($\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_8$) C, H, N.

1,4-Bis(2-acetamido-5-aminophenoxy)butane. The aforementioned nitro compound (12.6 g, 0.028 mol) was suspended in 65% DMF– H_2O (100 mL), the mixture brought to the boil, and

Table III. *N*-[ω -(2-Nitrophenoxy)alkyl]phthalimides

Alkyl chain	Mp, °C	Formula ^a	Yield, %
Propyl	163-165	C ₁₇ H ₁₄ N ₂ O ₅	84
Butyl	126-127	C ₁₈ H ₁₆ N ₂ O ₅	74
Pentyl	102-103	C ₁₉ H ₁₈ N ₂ O ₅	63
Hexyl	108-109	C ₂₀ H ₂₀ N ₂ O ₅	72
Heptyl	99-101	C ₂₁ H ₂₂ N ₂ O ₅	61
Octyl	93-94	C ₂₂ H ₂₄ N ₂ O ₅	59

^a All compounds analyzed satisfactorily for C, H, and N.Table IV. ω -(2-Nitrophenoxy)alkylamine Hydrochlorides

Alkyl chain	Mp, °C	Formula ^a	Yield, %
Propyl	162-164	C ₉ H ₁₂ N ₂ O ₃ ·HCl	84
Butyl	138-139	C ₁₀ H ₁₄ N ₂ O ₃ ·HCl	81
Pentyl	124-126	C ₁₁ H ₁₆ N ₂ O ₃ ·HCl	82
Hexyl	106-107	C ₁₂ H ₁₈ N ₂ O ₃ ·HCl	77
Heptyl	92-93	C ₁₃ H ₂₀ N ₂ O ₃ ·HCl	59
Octyl	87-88	C ₁₄ H ₂₂ N ₂ O ₃ ·HCl	66

^a All compounds analyzed satisfactorily for C, H, N, and Cl.

Fe powder (30 g) added. To the well-stirred suspension HOAc (3.4 mL) was added, a vigorous reaction resulting. After 10 min of boiling the mixture was cooled to 50 °C and concentrated NH₄OH (6 mL) added. Iron oxides were filtered from the boiling solution and the filter pad was well washed with boiling DMF-H₂O. The filtrate was evaporated to dryness in vacuo, EtOH added, plus NH₄OH (0.2 mL), and the mixture boiled until the thick gum was converted to crystalline product. Recrystallization was by solution in hot DMF, clarification, and then addition to the hot solution of 4 vol of hot *n*-BuOH. Slow cooling and back-seeding provided pure product as colorless crystals of mp 219-221 °C (10.2 g, 92%). Anal. (C₂₀H₂₆N₄O₄) C, H, N.

1,4-Bis[(2-acetamido-5-methanesulfonamido)phenoxy]butane. The corresponding amine (10.1 g, 0.027 mol) was dissolved in *N,N*-dimethylacetamide (60 mL) by brief boiling and the solution stirred with cooling in an ice-water bath. When the internal temperature reached 5 °C pyridine (12 mL) was added and then methanesulfonyl chloride (4.55 mL, 0.058 mol) in dropwise fashion so that the temperature was maintained. After 1 h of further stirring at room temperature TLC demonstrated that reaction was complete. Following addition of an equal volume of boiling H₂O, the reaction mixture was heated to boiling and then hot H₂O added to incipient crystallization. Slow cooling provided pure product as colorless prisms of mp 185-186 °C (13.2 g, 89%). Anal. (C₂₂H₃₀N₄O₈S₂) C, H, N, S.

1,4-Bis[(2-amino-5-methanesulfonamido)phenoxy]butane Dihydrochloride. The aforementioned acetyl derivative (13.2 g, 0.024 mol) was suspended in boiling EtOH (75 mL) and 12 N HCl (25 mL) added to the hot solution. After a few additional minutes of boiling a homogenous solution resulted and, after ca. 25 min of further boiling, product hydrochloride started to crystallize. Boiling was continued for a further 15 min, the mixture cooled thoroughly, and the salt collected on a sintered glass filter. The hydrochloride was dissolved in the minimum quantity of boiling H₂O, the solution clarified, and 0.25 vol of 12 N HCl added to the hot solution. Pure product dihydrochloride was recovered from the cooled mixture as colorless needles of mp 281 °C dec (11.7 g, 78%). Anal. (C₁₈H₂₆N₄O₈S₂·2HCl) C, H, N, Cl.

***N*-[ω -(2-Nitrophenoxy)alkyl]phthalimides (Table III).** To sodium 2-nitrophenate (6.64 g, 0.04 mol) suspended in dry DMF (12 mL) containing KI (20 mg) the requisite *N*-(ω -bromoalkyl)phthalimide (0.04 mol) was added and the heterogeneous mixture stirred and heated on a steam bath for 0.5 h and then boiled for 5 min. HOAc was added in dropwise fashion until the red color of the solution changed to yellow; then H₂O was added to the boiling mixture until just turbid. Cooling and trituration provided crystalline products. The crystals were collected, washed well with EtOH, petroleum ether, and H₂O and then dried. Products were recrystallized by solution in EtOH-Me₂CO (1:1) at the boil; then Me₂CO was removed by distillation to incipient crystallization.

Table V. 4-[ω -(2-Nitrophenoxy)alkylamino]quinolines

Alkyl chain	Mp, °C	Formula	Analyses	Yield, %
Propyl	147-148	C ₁₈ H ₁₇ N ₃ O ₃	C, H, N	73
Butyl	114-116	C ₁₉ H ₁₉ N ₃ O ₃ ·HCl 0.5H ₂ O	C, H, N, Cl	68
Pentyl	188-189	C ₂₀ H ₂₁ N ₃ O ₃ ·HCl	C, H, N, Cl	63
Hexyl	64-65	C ₂₁ H ₂₃ N ₃ O ₃ ·HCl	C, H, N, Cl	67
Heptyl	90-91	C ₂₂ H ₂₅ N ₃ O ₃ ·HCl	C, H, N	55
Octyl	53-54	C ₂₃ H ₂₇ N ₃ O ₃ ·HCl	C, H, N, Cl	61

Table VI. 4'-Nitro[ω -(4-quinolinylamino)]alkanesulfonanilides

Alkyl chain	Mp, °C	Formula	Analyses	Yield, %
Ethyl	250-251	C ₇ H ₁₆ N ₄ O ₄ S	C, H, N	89
Propyl	162-164	C ₈ H ₂₀ N ₄ O ₄ S HCl·H ₂ O	C, H, N, Cl	84
Butyl	213-214 ^a	C ₉ H ₂₄ N ₄ O ₄ S HCl·H ₂ O	C, H, N, Cl	79
Pentyl	244-225	C ₁₀ H ₂₈ N ₄ O ₄ S	C, H, N, S	67
Hexyl	227-228	C ₁₁ H ₃₂ N ₄ O ₄ S HCl·0.5H ₂ O	C, H, N	71

^a First melted at 142-143 °C, then resolidified, and melted at the indicated figure.

ω -(2-Nitrophenoxy)alkylamines (Table IV). The precursor phthalimido derivatives (0.03 mol) were suspended in boiling EtOH (100 mL) and hydrazine hydrate (2.25 mL, 0.045 mol) was added in one portion, a homogeneous solution rapidly resulting. A short time later phthalazine-1,4-dione separated. After 0.5 h of boiling volatiles were removed in vacuo, then HOAc (3.6 mL) and H₂O (50 mL) were added, the mixture was briefly boiled and cooled to 5 °C, and the phthalazine-1,4-dione was removed and washed with 0.05 N HOAc (2 × 10 mL). The extracts were evaporated to dryness, excess 2 N NaOH was added, and the base was removed in C₆H₆. The H₂O washed and dried (Na₂SO₄) organic layer was evaporated; the resulting oil was dissolved in Et₂O and dry HCl passed through the solution at 0 °C until precipitation of the base hydrochloride was complete. All hydrochlorides crystallized as silky white needles from EtOH-*i*-Pr₂O.

4-[ω -(2-Nitrophenoxy)alkylamino]quinolines (Table V). To just molten anhydrous phenol (20 g) NaH (0.01 mol) was added cautiously portionwise and, when reacted, 4-chloroquinoline (0.01 mol) was added and the solution heated briefly to 160 °C and then cooled. The requisite ω -(2-nitrophenoxy)alkylamine hydrochloride (0.01 mol) was then added to the resulting solution of 4-phenoxyquinoline. The reaction mixture was heated to 140 °C (internal) for 1 h, cooled, and diluted with an equal volume of toluene, and dry HCl was passed through until the yellow color of the mixture bleached. On addition of excess petroleum ether, and cooling to -15 °C, crude product hydrochloride separated as a thick gum. After several washings with petroleum ether, by decantation, the product was freed of solvent in vacuo and samples were triturated with H₂O, aqueous NaCl, or HCl, as necessary, to provide seed crystals. Repeated crystallization of products from dilute HCl, sometimes with added EtOH, provided TLC homogeneous samples. Alternatively the free base, provided by treatment with excess 2 N NaOH, was crystallized from C₆H₆-petroleum ether.

Catalytic reduction (Pd/C, 45 psi of H₂, EtOH) of the nitro group in these compounds was complicated by overreduction involving the quinoline ring. Fe/H⁺ reduction, as before,⁴⁷ proceeded smoothly without complications. The resulting readily autoxidized amines were ultimately extracted into C₆H₆ from basic (pH > 10) solutions and the dry base resulting on evaporation was immediately coupled with 9-chloroacridine by the standard method.

4'-Nitro[ω -(4-quinolinylamino)]alkanesulfonanilides (Table VI). As above, 4-phenoxyquinoline (0.01 mol) was prepared in situ in phenol (20 g) solution from NaH (0.01 mol) and 4-chloroquinoline (0.01 mol). The requisite 4'-nitro- ω -aminoalkanesulfonanilide (0.01 mol) was added and the solution heated at 140 °C for 1 h. Dry HCl was passed through the cooled

Table VII.
4'-Amino[ω -(4-quinolinylamino)]alkanesulfonanilides

Alkyl chain	Mp, °C	Formula	Analyses	Yield, %
Ethyl	213-214	C ₁₇ H ₁₈ N ₄ O ₂ S	C, H, N, S	91
Propyl	120-121	C ₁₈ H ₂₀ N ₄ O ₂ S 0.5H ₂ O	C, H, N	83
Butyl	167-169	C ₁₉ H ₂₂ N ₄ O ₂ S 0.5H ₂ O	C, H, N	81
Pentyl	179-180	C ₂₀ H ₂₄ N ₄ O ₂ S	C, H, N	69
Hexyl	177-178	C ₂₁ H ₂₆ N ₄ O ₂ S	C, H, N, S	79

solution, until the yellow color bleached; then crude product hydrochloride was precipitated by addition of C₆H₆ and Et₂O. The well-washed (C₆H₆) solid was dried in vacuo and then crystallized repeatedly from EtOH-HCl until homogenous to TLC criterion. Alternatively the hydrochloride salt was dissolved in MeOH, excess NH₄OH added, and the mixture swirled at room temperature, while applying water pump vacuum, until the base crystallized from solution. Recrystallization of the bases employed DMF-MeOH-H₂O mixtures.

4'-Amino[ω -(4-quinolinylamino)]alkanesulfonanilides (Table VII) were prepared from the preceding compounds by the usual Fe⁴⁷ reductions. The free amines crystallized from EtOH-toluene or EtOH-H₂O.

4'-Nitro-2-(4-quinazolinylamino)ethanesulfonanilide. To a solution of sodium phenate, prepared by cautious addition of NaH (0.01 mol) to just molten, anhydrous phenol (20 g), were added in succession 4-chloroquinazoline (0.01 mol) and 4'-nitro-2-aminoethanesulfonanilide (0.01 mol). The 4-chloroquinazoline was conveniently prepared by the action of SOCl₂, catalyzed by a trace of DMF,⁵⁶ on 4-quinazoline. The reaction mixture was heated at 130 °C for 0.5 h and cooled, and dry HCl was passed through until acid and then product hydrochloride precipitated by excess C₆H₆. The crystals were well washed with *i*-Pr₂O and then petroleum ether and dried. Solution of this material in 0.1 N HOAc, then addition of solid NaCl at the boil to incipient crystallization, and following cooling provided pure product hydrochloride as pale yellow plates. Conversion to the free base in the usual way and crystallization from EtOH-H₂O provided pure product as light yellow needles of mp 271 °C dec (72% yield). Anal. (C₁₆H₁₅N₅O₄S·2H₂O) C, H, N.

4'-Amino-2-(4-quinazolinylamino)ethanesulfonanilide was prepared by nitro group reduction (Fe/H⁴⁷) of the aforementioned product. Crystallization from absolute EtOH provided pure product as colorless needles of mp 198-199 °C (84% yield). Anal. (C₁₆H₁₇N₅O₂S) C, H, N.

N¹,N²-Bis(4-R-3-R'-phenyl)- α,ω -alkanedisulfonamides (Table VIII). The required alkane- α,ω -disulfonyl chlorides were prepared by a route starting from the α,ω -dibromoalkanes. Reaction of the latter with thiourea in absolute EtOH provided the corresponding, relatively EtOH insoluble isothiuronium bromide salts^{66,67} which, from H₂O solutions, conveniently provided highly crystalline, relatively H₂O insoluble, bis(4-toluenesulfonate) salts on treatment with aqueous sodium 4-toluenesulfonate. Oxidation of these salts with NaClO₃-HCl as before¹ afforded a convenient preparation of the well-characterized alkane- α,ω -disulfonyl chlorides.⁶⁸⁻⁷¹ The requisite sulfonyl chloride (0.04 mol) was added slowly to a well-stirred solution of 4-acetamidoaniline (0.08 mol) in pyridine (60 mL) at -5 °C. When all sulfonyl chloride had dissolved the solution was stirred overnight at room tem-

perature and then excess pyridine removed in vacuo on a steam bath. Sufficient 2 N HCl to neutralize remaining pyridine was added and the gummy mass triturated until solid. Recrystallization from DMF-EtOH-H₂O provided pure products as colorless prisms.

Protecting acetamido functions were cleaved by hydrolysis in 2 N HCl-EtOH at reflux for 1 h. Product amines were conveniently purified by solution of the amine hydrochlorides, resulting in evaporation of the hydrolysis mixtures, in the minimum volume of boiling H₂O, clarifying, and adding 0.25 vol of 12 N HCl to the hot filtrate. On cooling pure amine hydrochlorides separated.

For synthesis of the methoxyl-substituted congeners 4-butanamido-3-methoxyaniline²⁵ was employed in place of 4-acetamidoaniline. Hydrolytic cleavage of the butanamido function required more vigorous conditions: 3 N HCl-EtOH and 2 h of reflux.

Preparation of Agents. The thoroughly dried requisite amine or amine hydrochloride (4 mmol) was dissolved in the minimum possible volume of dry *N*-methyl-2-pyrrolidone and, to this solution at 25 °C, the requisite 9-chloroacridine (4.4 mmol/amine function) was added and the mixture stirred until homogeneous. With amine precursors a trace of acid catalyst, conveniently MeSO₃H, is necessary to initiate reaction, heralded by a color change from pale yellow to deep red. The reaction mixtures were allowed to stand at room temperature until TLC monitoring demonstrated complete reaction. In many cases products crystallized directly from the reaction mixtures; in the remainder, precipitation with excess EtOAc at low temperature ensured removal of excess 9-chloroacridine. Products were dissolved in boiling 0.5 N HOAc, by addition of EtOH as necessary, solid NaCl was dissolved in the hot clarified solution, to incipient crystallization, and then the mixture was allowed to cool slowly. Product hydrochlorides separated in deep red, highly crystalline form. Recrystallization was repeated from EtOH-H₂O-HCl until products were homogeneous to TLC. In certain cases unfavorable physical characteristics, or difficulty in removing trace contaminants, required conversion of the salts to the free bases and following crystallization of these from DMF-EtOH-H₂O.

Agents 48-52 were most conveniently prepared by reaction of both aliphatic and aromatic amine functions of the requisite 4', ω -diaminoalkanesulfonanilides¹ with 9-chloroacridine by the following modified procedure. A solution of the 4'-nitro- ω -aminoalkanesulfonanilide (5 mmol) in 65% EtOH-H₂O, containing 1 molar equiv of HCl, was hydrogenated over a 5% Pd/C catalyst at 45 psi of H₂ until the theoretical hydrogen absorption for nitro group reduction had occurred. The amine hydrochloride, obtained on removal of catalyst and evaporation, was checked by TLC to ensure complete reduction and homogeneity. To this thoroughly dried (P₂O₅, vacuum) diamine monohydrochloride anhydrous phenol (10 g) and 9-chloroacridine (5.5 mmol) were added, and the mixture was heated at 100 °C for 0.5 h. After cooling NaH (16 mmol) was added cautiously in small quantities and when this had reacted a further addition of 9-chloroacridine (5.5 mmol) was made. The mixture was heated at 120 °C in an oil bath for 1 h and cooled; then dry HCl was passed through until the mixture was acid to moist litmus paper. Precipitation with C₆H₆-*i*-Pr₂O, and following thorough washing with EtOAc, provided crude product. Recrystallization was as before.

Biological Testing. BDF₁ hybrid mice were employed in tumor assays. In L1210 tests the tumor inoculum was 10⁵ cells intraperitoneally and in P-388 assays 10⁶ ip. Drug dosing (ip)

Table VIII. N¹,N²-Bis(4-R-3-R'-phenyl)- α,ω -alkanedisulfonamides

Alkane chain	R	R'	Mp, °C	Formula	Analyses
Propane	CH ₃ CONH	H	183-185	C ₁₅ H ₂₄ N ₄ O ₆ S ₂	C, H, N, S
Butane	CH ₃ CONH	H	289-291	C ₂₀ H ₂₈ N ₄ O ₆ S ₂	C, H, N, S
Pentane	CH ₃ CONH	H	249-250	C ₂₁ H ₂₈ N ₄ O ₆ S ₂	C, H, N, S
Propane	NH ₂	H	246 dec	C ₁₄ H ₂₀ N ₄ O ₄ S ₂ ·2HCl	C, H, N, Cl
Butane	NH ₂	H	259 dec	C ₁₆ H ₂₂ N ₄ O ₄ S ₂ ·2HCl	C, H, N, Cl
Pentane	NH ₂	H	249 dec	C ₁₇ H ₂₄ N ₄ O ₄ S ₂ ·2HCl	C, H, N, Cl
Hexane	CH ₃ (CH ₂) ₂ CONH	CH ₃ O	174-175	C ₂₈ H ₂₄ N ₄ O ₈ S ₂	C, H, N
Butane	NH ₂	CH ₃ O	246 dec	C ₁₈ H ₂₆ N ₄ O ₆ S ₂ ·2HCl	C, H, N, Cl
Pentane	NH ₂	CH ₃ O	97-99	C ₁₉ H ₂₈ N ₄ O ₆ S ₂ ·2HCl·2H ₂ O	C, H, N, Cl
Hexane	NH ₂	CH ₃ O	237 dec	C ₂₀ H ₃₀ N ₄ O ₆ S ₂ ·2HCl	C, H, N, Cl

commenced 24 h after tumor implantation and continued once daily for 5 days. Dose levels were arranged at 0.18 log dose intervals and ranged from the frankly toxic to the inactive. There were six animals in any one test group and one control group for every six tests.

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