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Comparison of the *in Vivo* and *in Vitro* Antileukemic Activity of Monosubstituted Derivatives of 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide

BRUCE C. BAGULEY AND BRUCE F. CAIN

Cancer Research Laboratory, Auckland University School of Medicine, Auckland, New Zealand

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SUMMARY

The growth-inhibitory activity of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and 47 acridine-monosubstituted derivatives has been measured using cultures of L1210 murine leukemia cells grown for 3 days in the presence of each drug. The results have been compared with previously published *in vivo* antitumor activity and physicochemical properties related to DNA binding, acridine base strength, stability to chemical attack by thiols, and lipophilicity. Multiple-parameter regression equations show that both dose potency and host toxicity in mice are related to a combination of *in vitro* activity and a nonlinear (quadratic) term in lipophilicity. The *in vitro* activity can in turn be modeled as a combination of terms representing DNA binding, ability to quench the fluorescence of DNA-bound ethidium, stability to thiolysis, and lipophilicity. It is hypothesized that the terms for thiolytic stability and lipophilic-hydrophilic balance describe the availability of the drug to the cell, and that the DNA binding constant determines what proportion of the available drug is bound to DNA, the proposed target site. The remaining terms could reflect changes in the geometry of drug-DNA binding, which in turn affect the intrinsic activity of these drugs when bound at their site of action.

INTRODUCTION

The acridine antitumor agent *m*-AMSA[1] is active against a spectrum of tumors in experimental animals (1, 2) and is active clinically, especially in the treatment of acute leukemia (3). It binds to double-stranded DNA by intercalation (4) and in some ways its action resembles that of another DNA-intercalating agent, Adriamycin (1, 5). The binding of *m*-AMSA to DNA as a potential target site has therefore been a topic of intensive study in our laboratory (4, 6, 7). The synthesis of a large number of congeners of *m*-AMSA has enabled the significance of lipophilic-hydrophilic balance, acridine base strength, and DNA binding to the biological activity of these compounds to be evaluated by statistical means (8, 9). Previous studies have utilized antileukemia L1210 activity *in vivo* as a measure of biological activity. In order to separate those drug properties required for the disposition and metabolism of *m*-AMSA derivatives in the animal from those required for cytotoxic activity at the cellular level, a series of agents has now been tested using L1210 cell cultures. The series chosen (Compound 1, Table 1) was identical with that used previously to evaluate physicochemical parameters important for *in vivo* activity (8).

MATERIALS AND METHODS

Materials. All *m*-AMSA derivatives were synthesized and purified in our laboratory, and methods of preparation have been described previously (8). Poly(dA-dT) and ethidium bromide were purchased from the Sigma Chemical Company (St. Louis, Mo.).

DNA-binding assays. C_{50} values, defined as the concentration of drug required to halve the observed fluorescence due to DNA-bound ethidium at an ionic strength of 0.01 M, were determined at pH 5.0 as previously described (10). Association constants for poly(dA-dT) were calculated from C_{50} values using a correction for the quenching effect of DNA-bound drug on the fluorescence of DNA-bound ethidium (10). Assays for quenching of DNA-ethidium fluorescence were performed by stepwise addition of drug to a complex of ethidium (2 μM) and poly(dA-dT) (20 μM in base pairs) in 1 ml of buffer (9.4 mM NaCl, 2 mM sodium acetate (pH 5.0), and 0.1 mM EDTA). Under these conditions most of the decrease in fluorescence was due to quenching, and a small correction for displacement of ethidium was carried out as previously described (10).

Culture methods. L1210 cells were initially obtained

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[1] The abbreviation used is: *m*-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (NSC 249992).

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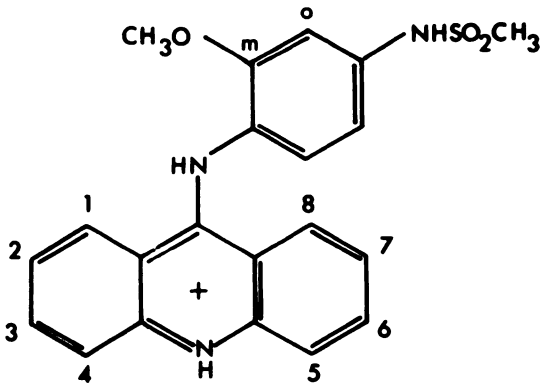
TABLE 1

Biological and physicochemical data for m-AMSA and its derivatives



Compound	R	ID_{50}[a]		D_{40}[b]		LD_{10},[c] observed	K[d] ($\times 10^{-6}$)	$Q_{0.1}$[e]	$t_{1/2}$[f]	R_m[g]	pK_a[h]
		Observed	Calculated	Observed	Calculated						
2	H	35	56	5.1	2.8	18	0.37	31	13.2	0.18	7.43
3	3-$NHCOCH_3$	71	56	1.0	3.9	41	1.74	22	23.6	0.07	7.56
4	3-Aza	710	2300	47	18	280	0.12	27	2.01	−0.14	5.53
5	3-NH_2	6	5.2	3.0	0.8	17	1.62	17	1285	0.06	9.50
6	3-$NHCOOCH_3$	39	74	3.3	3.5	21	2.34	22	35.1	0.25	7.77
7	3-$NHCH_3$	6	6	0.4	0.9	3.5	1.48	17	1956	0.17	9.50
8	3-NO_2	110	213	4.1	12	15	0.45	63	1.9	0.10	5.72
9	3-CH_3	12	63	3.0	2.9	14	0.89	22	32.6	0.44	7.70
10	3-CH_2CH_3	150	229	17	26	83	0.46	22	29	0.56	7.65
11	3-$CH(CH_3)_2$	2900	1230	400	330	890	0.29	14	28.3	0.68	7.66
12	3-OCH_3	20	44	2.2	2.5	13	0.68	18	51.3	0.29	7.81
13	3-F	150	174	9	10	56	0.35	26	6.3	0.31	7.05
14	3-Cl	70	72	3.8	6.0	26	1.15	29	4.6	0.32	6.84
15	3-Br	50	56	4.4	5.1	30	1.95	27	4.4	0.34	6.84
16	3-I	50	58	2.7	6.6	27	2.24	29	4.5	0.41	6.77
17	3-CN	1000	282	55	21	160	0.45	34	1.83	0.06	5.94
18	3-$CONH_2$	580	363	16	32	224	0.46	37	3.57	−0.41	6.66
19	3-CF_3	2900	2240	68	155	234	0.19	34	2.26	0.54	6.24
20	2-NH_2	79	98	1.3	4.5	18	0.89	33	233	−0.15	7.37
21	2-CH_3	1400	468	204	54	680	0.22	12	32.9	0.40	7.45
22	2-$CH(CH_3)_2$	1200	2800	260	173	1020	0.25	9	49.7	0.66	7.40
23	2-F	300	380	19	15	200	0.19	34	10.3	0.32	6.71
24	2-I	740	240	213	31	309	0.50	29	6.3	0.36	6.60
25	4-Aza	150	617	39	6.3	162	0.19	30	1.12	−0.07	6.09
26	4-OCH_3	40	68	3.9	3.1	10.4	0.87	13	13.4	0.19	7.39
27	4-OCH_2CH_3	115	224	16	12	58	0.59	13	13.1	0.43	7.37
28	4-OCH_2CH_2OH	25	41	3.9	2.1	23	0.55	29	14.8	0.10	7.36
29	4-$OCH_2CH(OH)CH_2OH$	100	115	2.5	5.0	45	0.79	12	11.4	−0.09	7.00
30	4-$OCH_2CONHCH_3$	220	83	3.1	7.9	>800	0.63	12	10.8	−0.02	7.35
31	4-$O(CH_2)_3CONH_2$	250	105	5.5	9.5	47	0.55	12	10.9	−0.15	7.36
32	4-$CONH_2$	350	457	22	15	83	0.30	44	2.52	−0.27	6.37
33	4-$CONHCH_3$	350	162	6.3	11	20	0.35	42	3.01	0.06	6.36
34	4-$CON(CH_3)_2$	1600	5700	18	128	47	0.11	5	3.29	0.09	6.16
35	4-$CO(NC_4H_8O)$[i]	5000	9500	123	68	340	0.09	5	3.55	−0.18	6.03
36	4-$CONH(CH_2)_3CH_3$	4200	832	42	141	110	0.25	36	2.89	0.47	6.36
37	4-$CONH(CH_2)_2OH$	300	309	7.7	11	78	0.26	37	3.29	−0.16	6.27

[a] ID_{50} values are the nanomolar drug concentrations required to inhibit the growth of cultured L1210 cells by 50% after exposure for 70 hr (11).

[b] D_{40} is the dose (micromoles per kilogram) required on a daily dose schedule (days 1–5 after inoculation of 10^5 L1210 leukemia cells) which increases the life-span of treated mice by 40% with respect to untreated controls (8).

[c] LD_{10} is the dose (micromoles per kilogram) administered as above which is lethal to 10% of treated animals (determined by a regression equation), causing them to die significantly before control leukemic animals (8).

[d] K is the DNA-binding constant for poly(dA-dT) determined at 0.01 ionic strength (10).

[e] $Q_{0.1}$ is the percentage reduction of fluorescence of DNA-bound ethidium at a drug:DNA binding ratio of 0.1 molecule per base pair (10).

[f] $t_{1/2}$ is the half-life of the agent in the presence of excess mercaptoethanol determined under standard conditions (9).

[g] R_m is a measure of lipophilic-hydrophilic balance determined from thin-layer partition chromatography (18).

[h] Acridine pK_a was measured spectrophotometrically (9).

[i] Morpholide derivative.

TABLE 1—cont.

Compound	R	ID_{50}[a]		D_{40}[b]		LD_{10},[c] observed	K[d] ($\times 10^{-6}$)	$Q_{0.1}$[e]	$t_{1/2}$[f]	R_m[g]	pK_a[h]
		Observed	Calculated	Observed	Calculated						
38	4-$CONH(CH_2)_3OH$	720	302	14	19	150	0.25	37	3.37	−0.15	6.30
39	4-$CONHCH_2CHOHCH_3$	450	550	3.2	13	110	0.22	37	2.94	0.09	6.20
40	4-$CONHCH_2CHOHCH_2OH$	710	871	30	30	200	0.18	30	2.84	−0.36	6.34
41	4-$CONHCH_2CONH_2$	780	1100	22	55	47	0.25	43	3.91	−0.50	6.18
42	4-CH_3	35	41	1.8	3.2	17	1.07	20	11.1	0.25	7.39
43	4-$(CH_2)_2CONH_2$	320	178	2.7	11	25	0.45	12	8.44	−0.17	7.15
44	4-C_6H_5	650	1100	120	40	310	0.40	7	3.20	0.45	7.02
45	4-F	140	282	7.9	7.2	50	0.45	26	4.28	0.21	6.33
46	4-Cl	410	295	37	15	90	0.58	27	2.42	0.23	6.11
47	4-Br	500	490	32	18	160	0.37	27	2.36	0.25	6.09
48	4-CN	2200	4200	83	35	210	0.10	41	3.78	−0.03	4.87
49	4-NO_2	4600	5500	125	58	190	0.16	17	2.81	0.09	5.05

from Dr. I. Wodinsky, Arthur D. Little, Inc. (Cambridge, Mass.) and were used from the same stock as that used for *in vivo* testing (8). Frozen stocks (liquid nitrogen) were thawed, and 10^6 cells were injected i.p. into DBA/2 mice. After 3 days approximately 4×10^7 cells were recovered by injection of sterile growth medium (RPMI 1640) (GIBCO, Grand Island Biological Company, Grand Island, N. Y.) supplemented with 50 μM mercaptoethanol, 10% fetal calf serum (Laboratory Services Ltd., New Zealand), streptomycin sulfate (0.05 μg/ml), and penicillin G (75 IU/ml). Cultures (4 ml) were set up at cell densities of 3×10^4, 10^5, and 3×10^5/ml in McCartney 25-ml bottles and subcultured when they reached a density of no more than 10^6 cells/ml. The cells grew with almost no lag phase, but were nevertheless passaged for 3 weeks before use in drug inhibition assays to allow the cells to adapt fully to the culture conditions. These assays were conducted between 3 weeks and 3 months after the establishment of one culture. The culture methods are described more fully elsewhere (11).

Tests were conducted in 24-well culture trays (Nunc or Linbro). Stock cultures were diluted to 3×10^4 cells/ml with prewarmed medium, and 1-ml portions were added to the wells. The trays were placed in an incubator for 1 hr, then removed for addition of drugs. Serial 2-fold dilutions of drugs in 50% aqueous ethanol (2 μl/well) were added with an S.M.I. micropettor to the center surface of each culture to minimize drug adsorption to the walls of the well. Five drug concentrations each in duplicate were set up for each agent, together with two control cultures containing 2 μl of 50% ethanol. The trays were incubated at 37° in an atmosphere of 5% CO_2 in air for 70 hr, and the cells were then counted in a Coulter Model ZF electronic cell counter. The ID_{50} concentration was defined as that drug concentration which reduced the cell density by 50% over this period. The average standard deviation for counted cell numbers in a single test (duplicate samples) was ± 7%. The standard deviation of ID_{50} determinations repeated over the 3-month period was ± 23%. Most of the compounds listed in Table 1 were assayed on at least two separate occasions.

Statistics. Multivariable, stepwise regression equations were carried out using a Burroughs B6700 computer and SPSS program. In Eqs. 1-9, r is the correlation coefficient, s is the standard error of the regression, and the limits set for the proportionality constants represent the 95% confidence values. For the 48 compounds tested, F-test values for 5% and 1% at 0.1% significance values were, respectively, 4.1, 7.4, and 12.5 (cf. Table 2). $F_{1,(x)}$ is the F-test for inclusion of each new variable, with the degrees of freedom indicated in parentheses. Stepwise development of Eqs. 5 and 8 are shown in Table 2, and

TABLE 2

Stepwise regression equations for log ($1/ID_{50}$)

Intercept log K		Log α	Log $Q_{0.1}$	I_{NH}	Log $t_{1/2}$	R_m^2	pK_a	r	s	$F_{1,(x)}$[a]
With pK_a										
−2.62	1.35						0.23	0.83	0.45	5.9 (45)
−3.36	1.09		0.91				0.38	0.86	0.41	11.1 (44)
−2.70	0.88		0.93			−1.90	0.48	0.90	0.35	15.2 (43)
−3.94	0.95		1.03	−0.62		−2.39	0.60	0.92	0.33	8.9 (42)
With log $t_{1/2}$										
−3.19	1.74							0.80	0.47	81.3 (46)
−2.03	1.49				0.26			0.82	0.45	5.1 (45)
−1.47	1.41				0.30	−1.52		0.85	0.43	7.2 (44)
−2.16	1.53			−0.82	0.54	−2.10		0.87	0.40	7.4 (43)
−3.0	1.43		0.85	−1.03	0.72	−2.14		0.91	0.35	14.0 (42)
−1.39	1.18	0.34	0.98	−0.89	0.60	−2.44		0.91	0.34	3.6 (41)

[a] Numbers in parentheses represent degrees of freedom.

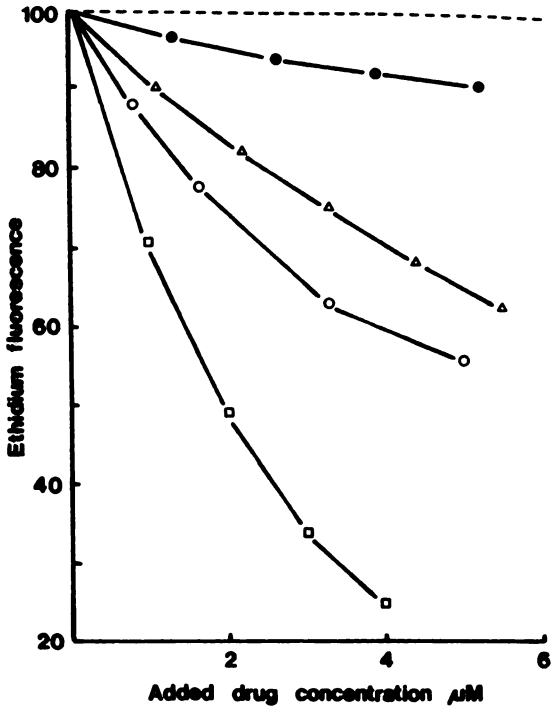


FIG. 1. *Effects of Compounds* 2 (○), 7 (△), 8 (□), *and* 44 (●) *on the fluorescence of DNA-ethidium complexes*

The expected displacement of ethidium induced by drug addition is shown by the *dashed line* (calculated for a drug association constant of 10^6 M^{-1}; see ref. 8).

a correlation matrix of the covariance between variables utilized is shown in Table 3.

RESULTS

Development of regression equations relating to in vivo and in vitro activity. Table 1 lists the physicochemical parameters of the 48 compounds in the series. The DNA-binding constants for poly(dA-dT) at pH 5.0, the measured pK_a of the acridine base nitrogen, and the measured R_m as a measure of lipophilic character have been reported previously (8). The phenomenon of quenching of ethidium fluorescence has been described previously and has been used in the determination of corrected DNA binding constants (10). Quenching varied considerably in the series in Table 1 (see examples in Fig. 1) and was not correlated with DNA-binding constants (Table 3). Since there was a possibility that it was associated with a phenomenon of biological significance, relative estimates of quenching were included in the list of variables for regression analysis.

The dose required to extend the life of leukemic mice by 40% (D_{40}) has previously been modeled in terms of DNA binding and lipophilic character (8). In order to determine whether D_{40} was related to *in vitro* cytotoxicity toward L1210 cells, ID_{50} values were determined for each of the 48 compounds. Log ($1/D_{40}$), used as a measure of *in vivo* dose potency, was modeled in terms of log ($1/ID_{50}$) and all of the physicochemical parameters. A significant equation was found which related the two terms to a parabolic term in lipophilic character (a linear term in R_m was not significant):

$$\log (1/D_{40}) = (0.64 \pm 0.15) \log (1/ID_{50}) - (1.96 \pm 1.04) R_m^2 + 0.84 \quad (1)$$

$$n = 48 \qquad r = 0.84 \qquad s = 0.39 \qquad F_{2,45} = 53.5$$

The drug dose which kills 10% of a mouse population (LD_{10}) can also be extracted from the *in vivo* antitumor data (8) and has been modeled previously in terms of DNA binding and lipophilic character. Log ($1/LD_{10}$) can be expressed by an equation analagous to Eq. 1. One compound (Compound 30, Table 1) was omitted, since a toxic dose was not reached:

$$\log (1/LD_{10}) = (0.50 \pm 0.10) \log (1/ID_{50}) - (1.36 \pm 0.88) R_m^2 + 1.01 \quad (2)$$

$$n = 47 \qquad r = 0.82 \qquad s = 0.32 \qquad F_{2,44} = 45.1$$

The ratio of doses, LD_{10}/D_{40}, is a measure of the therapeutic index, and has previously been modeled in terms of DNA binding and lipophilic character (8). A comparable, low-quality equation can be derived by using log ($1/ID_{50}$). As in the *in vivo* studies (8), R_m rather than R_m^2 entered as a significant second term:

$$\log (LD_{10}/D_{40}) = (0.17 \pm 0.10) \log (1/ID_{50}) - (0.31 \pm 0.30) R_m - 0.37 \quad (3)$$

$$n = 47 \qquad r = 0.49 \qquad s = 0.28 \qquad F_{2,44} = 7.0$$

In previous studies (e.g., ref. 9), the maximal drug-induced increase in the life-span of leukemic mice (ILS_{max}) has been used as a measure of antitumor effectiveness. For some series of compounds related to *m*-AMSA, ILS_{max} has been found to correlate with a parabolic term in lipophilic character (9). However, in the

TABLE 3

Squared correlation matrix for the dependent variables considered

	Log K	Log $Q_{0.1}$	I_{NH}	Log $t_{1/2}$	Log α	R_m	R_m^2	pK_a
Log ($1/ID_{50}$)	0.64	0.02	0.20	0.32	0.35	0.00	0.23	0.46
Log K		0.00	0.28	0.26	0.41	0.05	0.06	0.43
Log $Q_{0.1}$			0.00	0.08	0.10	0.05	0.01	0.11
I_{NH}				0.51	0.11	0.01	0.05	0.33
Log $t_{1/2}$					0.43	0.04	0.01	0.78
Log α						0.12	0.08	0.79
R_m							0.31	0.08
R_m^2								0.03

present series, the range of ILS_{max} values was small in comparison to the standard deviation of ILS_{max} determination. No significant dependence on lipophilic character was found, and only a weakly positive correlation (r = 0.44) between ILS_{max} and log ($1/ID_{50}$) was evident (data not shown).

Development of regression equations relating in vitr activity to DNA binding. Log ($1/ID_{50}$), as an index of *in vitro* antitumor activity, was analyzed in terms of DNA binding (log K and log $Q_{0.1}$), acridine base strength (pK_a), half-life for thiolytic cleavage ($t_{1/2}$), and lipophilic character (R_m and R_m^2). A highly significant equation in four variables was obtained:

$$\log (1/ID_{50}) = 0.88\ (\pm 0.41)\ \log K - 1.89\ (\pm 0.96)\ R_m^2 + 0.47\ (\pm 0.17) pK_a + 0.91\ (\pm 0.47)\ \log Q_{0.1} - 2.65 \quad (4)$$

$$n = 48 \qquad r = 0.90 \qquad s = 0.36 \qquad F_{4,43} = 45.5$$

An examination of the residuals of Eq. 4 indicated that those derivatives of *m*-AMSA having a substituted or unsubstituted amino group were less active than was predicted by the equation. In both the AMSA series (lacking the 3′-methoxy group) and the *m*-AMSA series, these compounds are distinguished by their low frameshift mutagenicity in an Ames' test[2] (12). This subclass of AMSA compounds is also distinguished from the parent by having a lower apparent unwinding angle, as measured using closed-circular duplex DNA.[3] It therefore seemed reasonable to test the significance of an indicator variable (I_{NH}), where $I \equiv 1$ for the subclass of compounds having a substituted or unsubstituted amino group and $I \equiv 0$ for all of the rest. The indicator variable entered as a significant term, and the predicted ID_{50} values in Table 1 are based on the following equation:

$$\log (1/ID_{50}) \equiv 0.95\ (\pm 0.38)\ \log K + 0.60\ (\pm 0.18)\ pK_a + 1.03\ (\pm 0.43)\ \log Q_{0.1} - 2.39\ (\pm 0.93)\ R_m^2 - 0.62\ (\pm 0.41)\ I_{NH} - 3.94 \quad (5)$$

$$n \equiv 48 \qquad r \equiv 0.92 \qquad s \equiv 0.33 \qquad F_{5,42} \equiv 54.7$$

Influence of thiolytic half-life on activity. 9-Anilinoacridine derivatives, including *m*-AMSA, are susceptible to nucleophilic attack at position 9 of the acridine by sulfhydryl groups on plasma proteins (13). The reaction rate can be quantitated by measuring the rate of reaction with 2-mercaptoethanol (8, 14). Since serum proteins and cysteine in culture medium may slowly inactivate *m*-AMSA derivatives (13, 15), a term for thiolytic half-life might be expected to appear in the regression equation. Previous studies on thiolysis rates in this series have demonstrated that it is the cation which is the reactive species, and that the rate of reaction is strongly dependent on the electron-donating or -withdrawing character of the acridine substituent (10). Since the acridine base strength is also influenced by the electron-donating or -withdrawing character of acridine substituents (8), thiolytic half-life can be expressed in terms of pK_a and α, where α is the fraction of ionized drug at pH 7.0:

$$\log t_{1/2} = 1.08\ (\pm 0.17)\ pK_a - 0.88\ (\pm 0.32)\ log\ \alpha - 6.98 \quad (6)$$

$$n = 48 \qquad r = 0.93 \qquad s = 0.25 \qquad F_{2,45} = 144.9$$

$$\log \alpha = -\log [1 + 10^{(7.0 - pK_a)}] \quad (7)$$

Log ($1/ID_{50}$) can be expressed by Eq. 8, where log $t_{1/2}$ and log α replace the pK_a term. The term in log α is significant only at the 6% level (all other terms are significant at the 5% level). Equation 7 assumes that the intracellular pH is 7.0 and that the measured pK_a is similar to the intracellular drug pK_a. Substitution with values of "operational pH" either above or below 7.0 gave regression equations similar to that shown in Eq. 8:

$$\log (1/ID_{50}) = 1.18\ (\pm 0.43)\ \log K + 0.60\ (\pm 0.27)\ \log t_{1/2} - 2.44\ (\pm 1.01)\ R_m^2 - 0.89\ (\pm 0.54)\ I_{NH} + 0.98\ (\pm 0.45)\ \log Q_{0.1} + 0.34\ (\pm 0.35)\ \log \alpha - 1.39 \quad (8)$$

$$n = 48 \qquad r = 0.91 \qquad s = 0.34 \qquad F_{6,41} = 34.8$$

DISCUSSION

The factors that affect the activity of *m*-AMSA derivatives in *in vivo* antitumor assays can be divided into three main groups: (a) factors influencing the drug disposition and metabolism in the animal, and ultimately the level and duration of drug exposure of the leukemia cells; (b) factors influencing the proportion of extracellular drug which associates with the target of cytotoxic action, and the time of this association; and (c) factors influencing the intrinsic activity of the drug when it is bound to the target of cytotoxic action.

The *in vitro* results reported here are based on a growth-inhibition assay under conditions of continuous exposure to drug (subject to the rate of drug degradation under conditions of culture). These conditions differ from those *in vitro*, where the time of exposure to drug is much shorter (14, 15). A clonogenic assay following pulse exposure *in vitro* would be more appropriate, but is very laborious to perform. However, the high correlation between the *in vitro* and *in vivo* data presented here ($r \equiv 0.81$) shows that, at least in this series, growth inhibition assays can predict quite well the expected potency *in vivo*.

The results also show unexpectedly that the lipophilicity of the drug is the most important parameter relating *in vitro* and *in vivo* activity (Eq. 1). This can be rationalized in terms of a model where drugs diffuse from the injection site to target cells by passage through a series of membranes (16). Drugs which are either too lipophilic or too hydrophilic do not partition optimally between lipid and aqueous phases.

Equation 1 provides an excellent method for predicting antitumor dose potency *in vivo* for the *m*-AMSA series. With the elucidation of such relationships, it is now possible to utilize cell cultures as an effective tool for analogue development in the 9-anilinoacridine series.

m-AMSA and its analogues react with simple thiols and with protein sulfhydryl groups (13). Thiolytic half-

[2] L. R. Ferguson, Cancer Research Laboratory, Auckland University School of Medicine, personal communication.

[3] B. C. Baguley, unpublished results.

life is a significant term in the description of *in vitro* activity (Eq. 8) and supports the observation that *m*-AMSA breaks down in culture medium (15). However, *in vivo* dose potency contains no such term (8). Calculation of the corresponding regression equation with the additional parameters expressing fluorescence quenching ($Q_{0.1}$) and percentage ionized form (α) produces the following equation:

$$\log (1/D_{40}) = 0.70\ (\pm 0.46) \log K - 3.49\ (\pm 1.12)\ R_m^2 + 0.62\ (\pm 0.36) \log \alpha + 0.62\ (\pm 0.51)\ log\ Q_{0.1} + 0.76 \quad (9)$$

$$n = 48 \qquad r = 0.86 \qquad s = 0.38 \qquad F_{4,43} = 28.3$$

It is possible that biological half-life *in vivo* is determined more by the rate of clearance from blood (for instance, by liver) or by enzymatic breakdown (17) than by thiolysis in blood or tissues. The dependence of dose potency on log α (Eq. 9) might reflect an uptake mechanism which responds to the charged form of the drug, although other explanations are possible.

Uptake of drugs by cells requires partition of the drug across the plasma membrane, and it is interesting that *in vitro* biological activity is related to a parabolic term in lipophilicity (Eqs. 4, 5, and 8). The optimal lipophilic-hydrophilic balance occurs at the same point ($R_m = 0$) as that for equations linking *in vivo* and *in vitro* activity (Eqs. 1 and 2) and equations linking either *in vivo* or *in vitro* activity to other physicochemical parameters. It should be noted that R_m values were deliberately defined so that optimal antitumor activity was associated with $R_m = 0$ (18). It is therefore not surprising that a linear term for R_m was not significant.

If DNA is the target of cytotoxic action of derivatives of *m*-AMSA, the DNA association constants of these derivatives would be expected to play an important role in their *in vitro* activity. The DNA association constant is the dominant term in the regression equations (Table 2), supporting the above hypothesis. Our current model for the mode of action of *m*-AMSA and its derivatives is that drug intercalation induces enzymatically caused breaks in the DNA, which in turn lead to chromatid breaks, faulty chromosome segregation at mitosis and cell division, and cell death. The DNA association constant may determine to a large extent the proportion of added drug which is bound at target site(s) of the DNA.

The association constants for drug binding to poly(dA-dT) have been used in the regression equations. These data were used because the data for this polymer are comparatively easy to derive (10) and because they were used in a previous paper (8). Data for calf thymus DNA have been collected for a number of *m*-AMSA analogues, and are highly collinear with data for poly(dA-dT) (cf. ref. 10). Data for poly(dG-dC) have also been collected for 24 of the derivatives listed in Table 1 and also show a high correlation coefficient ($r = 0.9$) with the data for poly(dA-dT). In order to detect a relationship between DNA site selectivity and biological activity, a data set of agents spanning a greater range of DNA binding selectivity must be chosen. Experiments along these lines are in progress.

A previous study (11) has underlined the importance of the substituents on the anilino-ring for *in vitro* activity and has indicated that activity cannot be predicted by DNA binding alone. It has been suggested that a DNA-drug-protein complex is involved in the action of *m*-AMSA, with the anilino-ring substituents making productive contacts with the putative target protein (7, 8, 11). The position of the anilino-ring substituents will in turn be influenced by the position of the intercalated acridine, so that acridine substitution could determine the binding geometry of the anilino function, as well as affecting the over-all DNA binding constant.

In a closely related series of agents, in which the 3′-methoxy group of *m*-AMSA is absent, helical unwinding angles have now been determined for a number of congeners using closed-circular duplex DNA.[4] Strongly binding compounds, including the 2-amino, 3-amino, and *N*-monosubstituted 3-amino derivatives, have consistently lower unwinding angles than other strongly binding congeners, which have unwinding angles close to that previously determined (4) for *m*-AMSA. This subgroup of derivatives is also virtually inactive in frame-shift mutagenesis assays, whereas other strongly binding AMSA derivatives are strongly mutagenic in the *Salmonella typhimurium* TA 1537 tester strain (12). It is therefore possible that the indicator variable used in Eq. 5 reflects changes in the geometry of drug-DNA binding.

The physical basis for the drug-induced quenching of DNA-bound ethidium is as yet unknown (8). The degree of induced quenching varies considerably with substitution (Table 1), and the inclusion of a significant term for fluorescence quenching in Eqs. 3, 4, and 9 is suggestive evidence that quenching reflects biologically significant changes in DNA binding. Quenching is low for bulky substituents (e.g., Compounds 11, 22, 34, 35 and 44 in Table 1), and the investigation of Compound 44 revealed an unwinding angle of 8°, as compared with 20° for *m*-AMSA (data not shown). Steric distortion of intercalation, or the favoring of nonintercalative versus intercalative DNA binding, could give rise both to a decrease in the induced quenching and a decrease in biological activity.

Although conclusions from regression equations should be made with caution, the equations developed on the basis of the data in Table 1 provide suggestions for the direction of future research in this area. Compounds with a high therapeutic index should be hydrophilic and have high *in vitro* activity. In general, high *in vitro* activity should be associated in turn with high DNA binding, high induced quenching of ethidium, the absence of a substituted or unsubstituted amino group, high pK_a, and correct lipophilic-hydrophilic balance (R_m close to 0). Unfortunately, the compounds satisfying the first three criteria (particularly the 3-chloro, 3-bromo, and 3-iodo derivatives) have lowered pK_a values and are too lipophilic. Current research is being directed toward the synthesis of drugs which fulfill all of the criteria specified by the regression equation.

[4] B. C. Baguley, R. Nash, and H. E. D. Lane, in preparation.

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Send reprint requests to: Dr. Bruce C. Baguley, Cancer Research Laboratory, Auckland University School of Medicine, Private Bag, Auckland, New Zealand.