Diacridines, Bifunctional Intercalators. Chemistry and Antitumor Activity[1]

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The synthesis and the characterization of a number of diacridines connected through the 9-amino position of the acridine rings by alkyl chains of varying lengths and with various substituents on the acridine ring are described. An interesting chemical property has been noted; whereas the 3-amino monoacridines cannot form stable dihydrochloride salts, the corresponding diacridines can form stable trihydrochloride salts. The biological activity of the diacridines encompasses a broad spectrum of action. Their antitumor activity (% ILS) and their toxicity have been correlated with their biological actions. The % ILS, as measured by inhibition of growth of P-388 ascites cells in BDF/1 mice, shows no significant correlation with their ability to inhibit the growth of P-388 cells in culture (I_{50}). The toxicity of the diacridines does not correlate with the inhibition of DNA or of RNA synthesis, the uptake of the diacridines by P-388 cells, or with % ILS. The only significant correlation that has been found to date between the antitumor effectiveness of the diacridines and their effects on intact cells occurs between % ILS and cell agglutination. These results emphasize that caution should be used in attributing the "antitumor activity" of a single compound or of a small number of congeners of a given chemical structure to a particular site of biological inhibition. Furthermore, the results suggest that effective antitumor drugs are those that affect the host–tumor interaction and that the toxicity of the drugs may not be essential to their antitumor properties.

In considering the design of new antitumor agents, one of the important target areas has been the interference either with the synthesis or with the function of nucleic acids. Taking advantage of the known ability of acridines to interact with both DNA and RNA, we have synthesized a series of diacridines, connected at the 9-amino position of the acridine ring by alkyl chains of varying length and with various substitutions on the acridine ring (see Table I). The rationale that such compounds should bind strongly to nucleic acids has been presented.[2a] Previously we have shown that diacridines bind to DNA and RNA more strongly than the parent compound, 9-aminoacridine;[2a] a similar conclusion has been reached by LePecq et al.[2b] A recent application of these principles to bis(quinaldines) has been published.[21]

Our initial studies emphasized the critical length of $n = 6$ and $n = 8$ for antitumor activity.[2a] Consequently, all later modifications relied heavily on this length of connecting chain, and structural changes were confined to the acridine rings. Subsequent in vitro studies with supercoiled PM2 DNA provided a possible interpretation of this in vivo result. These results indicated that in order that both acridine rings be intercalated into supercoiled PM2 DNA, the value of n (see formula, Table I) has to be 6 or higher;[3] when $n \leq 4$ only one of the two rings is intercalated into PM2 DNA. The diacridine with $n = 5$ assumes an intermediate position.

We have reported that the diacridines are potent inhibitors of the growth of P-388 and L1210 cells and of HeLa cells[4] in culture, as well as of the synthesis of RNA and of DNA and protein in these cells,[5] and that they specifically inhibit the synthesis of 45S RNA as well as its processing to 28S and 18S RNA.[6,7] At higher concentrations they inhibit the methylation of tRNA.[7] From an in vitro enzyme system which synthesizes well-defined mRNA's (T7 DNA dependent RNA polymerase transcribing the late cistrons of T7 DNA), it has been shown that the diacridines inhibit the initiation of RNA synthesis and not the elongation of RNA,[8] the latter being a characteristic of actinomycin D in this biological system.[8]

Considering the broad spectrum of in vitro and in vivo inhibitory effects of these compounds, it was anticipated that the overall inhibition of cell growth would also reflect the summation of these various inhibitions. We therefore synthesized a large series of diacridines and related the inhibition of cell growth (I_{50}) of P-388 cells to the % ILS for P-388 ascites cells in mice. In this fashion it was anticipated that a correlation between particularly interrelated phenomena could be detected.

A study of Figure 1(a) indicates that the two monofunctional diacridines **1** and **2**, which can intercalate only one of their acridine rings into DNA, can be distinguished from all the others; in these, the connecting alkyl chain ($n = 2$ and 4) is too short to permit synchronous intercalation of both rings.[3] These are much less inhibitory to the growth of P-388 cells in culture (ca. $^1/_6$–$^1/_{20}$) and also have very poor antitumor activity in mice as defined by the % ILS. From our other published data,[4-8] these two compounds also have less of an effect on the inhibition of RNA synthesis and on the processing of 45S RNA.

Such diacridines that act as monofunctional intercalators can only represent a small sample; nevertheless, this small sample suggests that the longer connecting alkyl chains that correspond to the double intercalating structures are required for antitumor activity. It is possible that the bifunctional diacridine structure is required to differentiate between a general effect on host tissues and a more specific effect on the tumor; however, a number of other plausible explanations can be forwarded by the available data.

All the other diacridines represented in Figure 1(a) have connecting chains which permit them to act as bifunctional intercalators. Whereas these show a great deal of variation in antitumor activity, the molar concentrations of the diacridines required to inhibit the growth of P-388 cells in cultures are very similar. This narrow range of I_{50} values emphasizes that the % ILS can vary from maximum to minimum with almost identical I_{50} values, thus emphasizing a lack of correlation between these two parameters [correlation coefficient (r) = 0.11].

We have also related the in vivo toxicity of the diacridines to their antitumor activity; Figure 1(b) shows the graph of the LD_{10} of the 47 diacridines to their % ILS. No obvious correlation can be discerned and the computer derived correlation coefficient $r = 0.01$. Moreover, if this graph is divided into four equal quadrants, it is seen that there are approximately as many compounds of low % ILS within the high as within the low LD_{10} quadrants, just as there are approximately as many compounds of high % ILS within the corresponding high and low LD_{10} quadrants. This suggests that the more toxic congeners have no advantage as antitumor agents; this is in keeping with the low correlation coefficient that was derived.

In another publication[19] we have indicated that the % ILS of the diacridines does not correlate with a number

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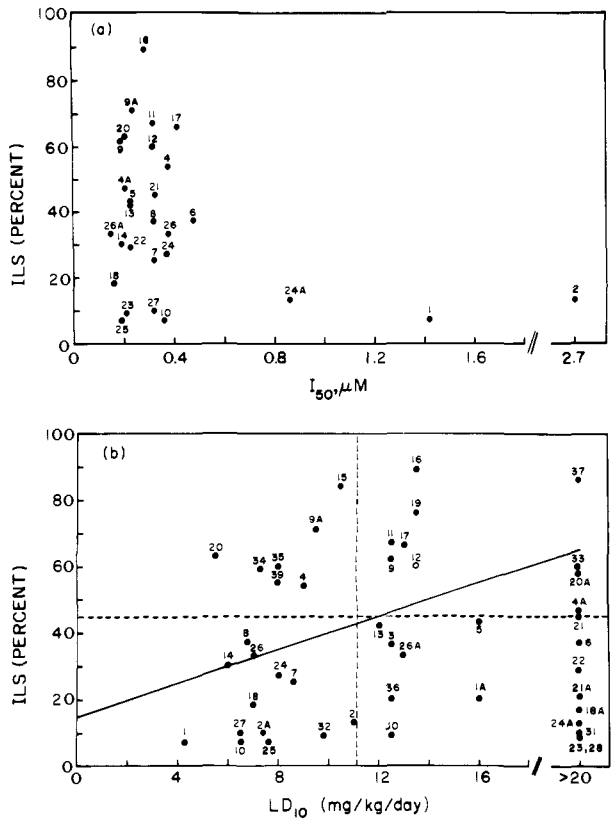


Figure 1. Correlation of (a) % ILS and I_{50} as defined in the Experimental Section and (b) % ILS and LD_{10} of individual diacridines. The dotted lines divide the graph into four quadrants, valued at LD_{10} = 11 and % ILS = 45. Equations and correlation coefficients (r) for the two graphs: (a) $y = 32.50 + 28.14x$, r = 0.11 (excluding compounds **1**, **2**, and **24a**); (b) $y = 14.43 + 2.52x$, r = 0.01.

of other metabolic effects of the diacridines. In Figure 2 we have plotted the LD_{10} of the diacridines against these same metabolic effects, i.e., inhibition of RNA synthesis [Figure 2(a)] and of DNA synthesis [Figure 2(b)] and against the uptake of diacridines by P-388 cells [Figure 2(c)] as well as against the rate of agglutination of wheat germ agglutinin treated P-388 cells as this is affected by the diacridines [Figure 2(d)]. None of these appears to show any good correlation [correlation coefficients (r) = 0.013, 0.031, 0.0014, and 0.28, respectively]. Consequently, the only significant correlation that can be made so far is the inverse correlation between % ILS and the rate of agglutination of lectin-treated cells that we have reported (correlation coefficent of 0.80).[19]

To our knowledge this is the first time that a correlation of the antitumor effectiveness to the inhibition of growth of the same tumor cells in culture, by a large homologous series of drugs, has been made. These results emphasize the lack of correlation that exists between these two parameters. However, they also emphasize that it is difficult to attribute a cause–effect relationship between (a) the inhibition of a particular biochemical reaction, (b) the inhibition of growth of cells in culture, and (c) their antitumor activity, based upon data obtained with a single compound or with a small number of congeners of a given structure.

It should be noted that whereas the % ILS correlates with the rate of agglutination of lectin-treated cells (r = 0.8), the toxicity of these same drugs does not show such correlation with this rate of agglutination (r = 0.28). Consequently, it would appear that the interaction of these compounds with membrane-associated sites relates best to their antitumor activity while the toxicity of these compounds is not related to membrane-associated sites.

These results suggest to us that the antitumor activity of the diacridines is not the result of the inhibition of any one of these various biochemical or growth parameters of the tumor cells. Rather, the sum total of the results presented in these two papers suggests that the antitumor activity of the diacridines is related to membrane-associated reactions and perhaps may be an expression of a differential effect exerted on the membranes of host and tumor cells or that the effective diacridines modify the interaction between host and tumor cells.

The biological significance of the inverse relationship that exists between the % ILS and the rate of agglutination is best understood if we take into account (a) the high correlation coefficient for this relationship, (b) the lack of a correlation for all the other parameters tested, and (c) that all the diacridines interact with the membranes of P-388 cells.

It is our interpretation that this inverse relationship measures the extent to which the diacridines can interfere with the interaction between the host and the tumor cells. The hypothesis, which is presently subjected to experimental proof in our laboratory, is that those diacridines that enhance the rate of agglutination also permit the association of tumor cells to host tissues and thereby facilitate the growth of the tumor cells; on the other hand, those diacridines that decrease the rate of agglutination, or at least do not enhance it, interfere with or minimize the association of tumor cels with host tissues. As a result of their inability to associate with and support themselves within the environment of the host cell, the growth of the tumor cells is inhibited. If viewed in this way, what appears to be an inverse relationship immediately becomes a direct relationship with a high correlation coefficient; i.e., an enhancement of agglutination correlates with growth of tumor cells within host tissues. This interpretation emphasizes that effective antitumor drugs are those that affect the host–tumor interaction and not necessarily the toxic compounds that interfere with cell growth.

We furthermore suggest that the toxicity is unrelated to these phenomena and that it results in the inhibition of both host and tumor cells and introduces a detracting factor to the antitumor properties of these compounds.

Many other interpretations are, of course, possible; however, this hypothesis constitutes the basis of our projected research in isolating the relevant membrane-associated structures.

A number of tentative conclusions can be made concerning the relationship between % ILS, the toxicity, and the structure of the diacridines.

(a) Most effective antitumor compounds are those in which the connecting hydrocarbon chain has 6–8 methylenes (n = 6–8, Table I).

(b) Alkoxy substitutions in the 4 position give the highest % ILS values; however, such substitutions tend to enhance the toxicity of the unmodified diacridine ring. As the substituent increases in size, the enhanced toxicity appears to counteract the increase in % ILS. Consequently, the optimal alkoxy substituent is 4-methoxy. The toxicity of this compound can be decreased by a 6-NO_2 substituent **37** without affecting the % ILS. In contrast, a 1-methyl substituent **36** decreases the % ILS of the 4-methoxy compound and also slightly enhances its toxicity.

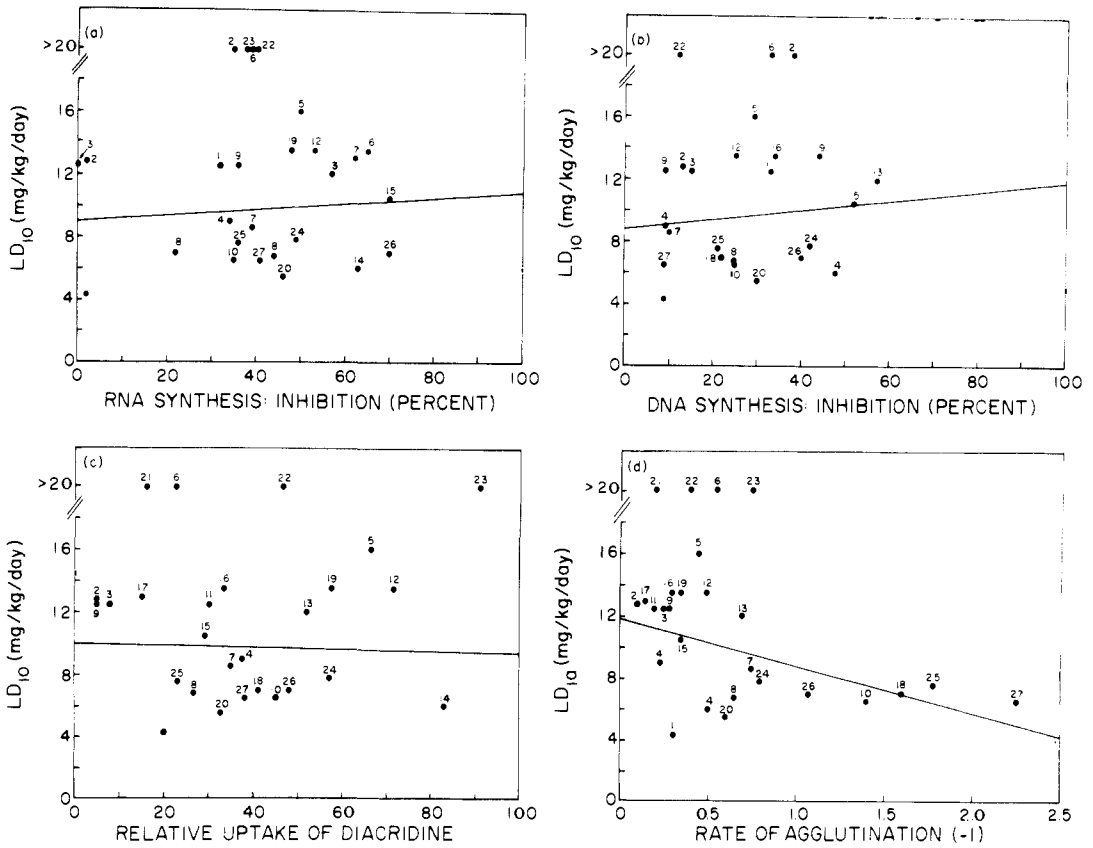


Figure 2. Least-squares regression analysis of (a) LD_{10} and inhibition of RNA synthesis in P-388 cells, (b) LD_{10} and inhibition of DNA synthesis in P-388 cells, (c) LD_{10} and uptake of diacridines by P-388 cells, and (d) LD_{10} and rate of agglutination of P-388 cells. Statistics: equations used to obtain correlation coefficients (r) and appropriate curves are (a) $y = 9.01 + 0.018x$, $r = 0.013$; (b) $y = 8.8 + 0.031x$, $r = 0.031$; (c) $y = 10 - 0.031x$, $r = 0.0014$; and (d) $y = 11.86 - 3.08x$, $r = 0.28$. Those compounds with LD_{10} larger than 20 mg/kg/day were excluded from the regression analyses.

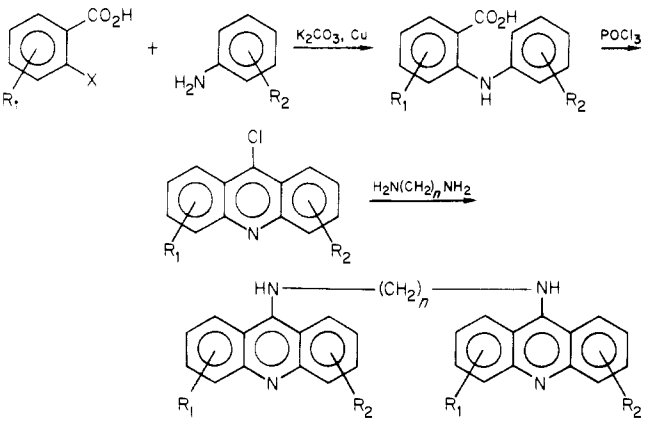
(c) Alkoxy substitutions in the 3 position are comparable in their effect to similar substitutions in the 4 position. The major difference is that the greater toxicity of the bulkier 3-propoxy substituent **20** is not counteracted by a fall in the % ILS as it is in the 4-alkoxy series. The 6-NO_2 substituent **21a** decreases the toxicity of the 3-methoxy substituent as it decreases the toxicity of the 4-methoxy substituent; however, it largely eliminates the antitumor effectiveness of the 3-methoxy group.

(d) The 2-methoxy substituents, **7** and **14**, are toxic and show a lower % ILS. A comparison of the 3- and the 4-methylthio substituents **24** and **24a** also emphasizes the increase in toxicity that occurs with such groups as one compares positions 4 to 3 to 2.

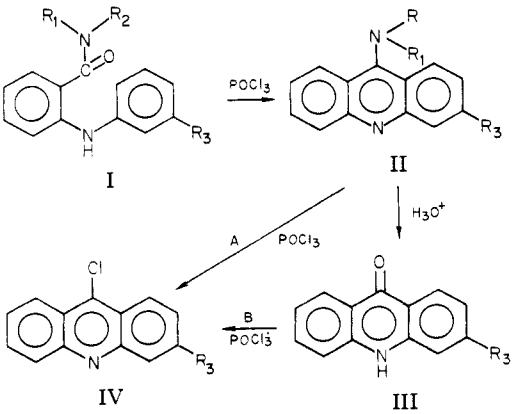
Chemistry. Most of the diacridines listed in Table I were prepared by the reaction of appropriate 9-chloroacridines with α,ω-diaminoalkanes (2 to 1 molar ratio) in phenol.[2a] The required 2- and 4-substituted 9-chloroacridines were synthesized by cyclization of 2-phenylaminobenzoic acids which, in turn, were prepared by an Ullmann synthesis[9] (Scheme I).

Three routes for the preparation of 3-substituted 9-chloroacridines were employed. Synthesis of 3-nitro and 3-chloro derivatives by unequivocal synthesis from the condensation of the readily available 4-nitro-2-chlorobenzoic acid and 2,4-dichlorobenzoic acid with anilines is a straightforward process.[9] However, when meta-substituted anilines (commercially available) were used in the acridine synthesis, a mixture of 1- and 3-substituted 9-chloroacridines was obtained. Only in cases with *m*-alkoxyanilines were reasonable yields of 3-substituted (3-methoxy and 3-propoxy) 9-chloroacridines obtained. These could be separated from the 1 isomers by simple

Scheme I



Scheme II



fractional crystallization. Ackerman et al.[10] reported that cyclization of alkylamides of 2-(3-substituted phenylamino)benzoic acid gave 3-substituted acridines exclusively. This procedure was utilized to cyclize the corresponding *N,N*-dialkylamide (I, $R_1 = R_2 = CH_3$, Scheme II) to the 3-substituted 9-(*N,N*-dialkylamino)acridines (II, $R_1 = R_2 = CH_3$), which could be readily hydrolyzed to 3-substituted acridones (III, Scheme II).[11,12] Treatment of these acridones with $SOCl_2$–DMF[12-16] yielded the desired 3-substituted 9-chloroacridines (IV). The 9-chloroacridines, bearing 3-MeS, 3-MeO-7-Cl, 3-Me, 3-MeO-6-Cl, and 3-MeO-6-NO_2 substituents, were prepared in this fashion in good yields. It was also possible, due to the steric lability of the 9-*N,N*-dialkylamino group of an acridine, to transform 9-*N,N*-dialkylaminoacridines directly to the corresponding 9-chloroacridines (IV, Scheme II). This could be accomplished if the cyclization of *N,N*-dimethylamides of 2-phenylaminobenzoic acids (I, $R_1 = R_2 = CH_3$) was performed either in refluxing $POCl_3$ or in $POCl_3$ in toluene or in $POCl_3$ in benzene. However, when it was refluxed in $POCl_3$ in methylene chloride, 9-*N,N*-dimethylaminoacridine was the only product observed by NMR.

Catalytic hydrogenation (Raney nickel) or reduction ($Fe-H^+$)[17] of the 3-nitro compound (**6** and **21**) yielded the corresponding 3-amino derivatives. The latter procedure proved to be the easier of the two methods and also resulted in better yields. The 3-amino derivatives (**4a** and **20a**, Table I) could be obtained either as the dihydrochloride or as the trihydrochloride salts. The isolation of the trihydrochloride salts by crystallization from hot ethanol was unexpected since the second pK_a of 3-aminoacridine or of 3,9-diaminoacridine is extremely low and cannot be detected except in strongly acidic solutions.[18] It would therefore be expected that the trihydrochloride salts, if formed under our conditions, would not have sufficient stability to survive the conditions of purification. However, we have been able to isolate the trihydrochloride salt of **4a** and we have corroborated this finding by converting the dihydrochloride salt of **20a** to the corresponding trihydrochloride salt (as determined by C, H, N, and Cl analysis); this emphasizes that the trihydrochloride salts of these diacridines are stable. In a previous report[2a] it was noted that a diacridine prefers the conformation with the two acridine rings on top of each other, providing sufficient overlap of the π-electron cloud for maximum stability. Our present analytical result could be interpreted to mean that the protonation of one of the 3-amino groups is stabilized by intramolecular hydrogen bonding with the other 3-amino group of the diacridine.

Experimental Section

Cell Culture Conditions, Assays of Metabolic Effects, and Antitumor Testing. P-388 cells were maintained as a suspension culture as previously described,[4] as well as in ascites form in DBA/2 mice.[2a] For the antitumor studies, 1×10^6 cells from the ascites tumor were administered intraperitoneally to BDF/1 mice on day 0. Drugs were administered starting on day 1 and continued for 9 consecutive days. Each drug was administered to groups of six mice starting at the highest dose of 20 mg/kg/day and at half doses thereafter up to 0.625 mg/kg/day (i.e., 20, 10, 5, 2.5, 1.25, and 0.625 mg/kg/day). The day of death was determined and the ratios of the life spans of the treated vs. those of the control animals were calculated. These are expressed as % ILS [(treated × 100)/(control − 100)].[2a] The % ILS that corresponds to each dose was recorded. In Table I the dose that corresponds to the optimal % ILS is provided. The effect on the growth rate of cells was determined by incubating P-388 cells in the presence of varying amounts of diacridines and determining the amount required to inhibit the rate of growth by 50% = I_{50}. The conditions for assays of inhibition of RNA and DNA synthesis, relative uptake of diacridines, and rate of agglutination of P-388 cells have been described.[19]

Statistics. Equations and correlation coefficients (r) were obtained from least-squares regression analysis; these are Figure 1(a), $y = 32.50 + 28.14x$, $r = 0.11$ (compounds **1**, **2**, and **24** were excluded in this regression analysis); Figure 1(b), $y = 14.43 + 2.52x$, $r = 0.01$; Figure 2(a), $y = 9.01 + 0.018x$, $r = 0.013$; Figure 2(b), $y = 8.80 + 0.031x$, $r = 0.031$; Figure 2(c), $y = 10.0 - 0.031x$, $r = 0.0014$; Figure 2(d), $y = 11.86 - 3.08x$, $r = 0.28$. Those compounds with LD_{10} larger than 20 mg/kg/day were excluded in the regression analyses. The correlation coefficient for % ILS vs. agglutination of cells in the presence of the diacridines is 0.80.[19]

Toxicity. Each assay for the antitumor activity of the diacridines was performed on groups of six mice at 20 mg/kg/day and at half-dose intervals up to 0.625 mg/kg/day (i.e., 20, 10, 5, 2.5, 1.25, and 0.625 mg/kg/day). The LD_{10} values were obtained from plotting dosage-mortality data at day 5.[20]

Synthesis. Elemental analyses were performed by Baron Consulting Co., Orange, Conn. All the compounds reported in Table I had analytical data of C, H, and N which were within 0.4% of the calculated values. Melting points (uncorrected) were taken in open capillary tubes in a Thomas-Hoover capillary melting point apparatus. For high melting compounds (**4a** and **20a**), a Melt-Temp (metal block) melting point apparatus was used. For each type of compound or intermediate, a representative synthetic procedure is described below.

1,8-Di(3-methoxyacridin-9-ylamino)octane (15). A solution of 2.503 g (10.3 mmol) of 3-methoxy-9-chloroacridine and 0.84 g (5 mmol) of 1,8-diaminooctane in 20 mL of phenol was heated at 100 °C for 2 h. The reaction mixture was then added dropwise into a stirring mixture of 200 mL of ether and 100 mL of ethyl acetate. The crude precipitated product was collected (by filtration), washed with ethyl acetate, and triturated with chloroform. Recrystallization from ethanol–methanol (twice) provided 1.96 g of pure product (60%), mp 217–220 °C. Anal. ($C_{36}H_{38}N_4O_2 \cdot 2HCl \cdot 0.5H_2O$) C, H, N. The free base was obtained by the following procedure.

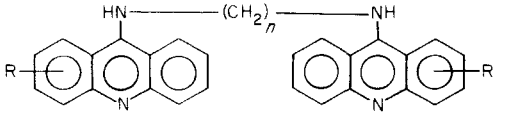
A methanol solution of the above dihydrochloride salts was added slowly with stirring to a cold 3% NaOH solution (350 mL). The precipitate was collected, washed, and crystallized from MeOH–$CHCl_3$ (75%): mp 182.5–184 °C. Anal. ($C_{36}H_{38}N_4O_2 \cdot 0.5H_2O$) C, H, N.

4-Butoxy-9-chloroacridine. Method C. A solution of 2-(2-butoxyphenylamino)benzoic acid (10 g) in 50 mL of $POCl_3$ was heated at 120–125 °C for 1.5 h. The excess $POCl_3$ was removed by evaporation under vacuum. The chloroform solution of the residue was poured slowly into 6% NH_4OH solution in ice–water. The chloroform phase was washed (ice–water), dried (Na_2SO_4), and evaporated to dryness. Crystallization from cyclohexane gave 8.4 g of pure product (84%), mp 114.5–115.5 °C. Anal. ($C_{17}H_{16}ClNO$) C, H, N.

3-Methoxy-7,9-dichloroacridine. Method A. A suspension of 4-chloro-2-(3-methoxyphenylamino)benzoic acid (6.05 g, 21.8 mmol) and 5.45 g of PCl_5 (24.3 mmol) in 150 mL of dry petroleum ether was refluxed for 45 min. The reaction mixture was filtered hot (charcoal) and the residue was washed with boiling petroleum ether. The combined filtrates were concentrated to a small volume until crystallization commenced. The acid chloride (5.8 g, mp 86–88 °C) was collected and then dissolved in 180 mL of dry toluene. To the toluene solution, 4 g each of powdered dimethylamine hydrochloride and $NaHCO_3$ were added with vigorous stirring and the mixture was then refluxed for 1 h. The cooled toluene solution was washed with water and dried over anhydrous Na_2SO_4. A small portion of the toluene solution was evaporated to dryness and the residue recrystallized from cyclohexane to give the corresponding *N,N*-dimethylamide, mp 93.5–95.5 °C. Anal. ($C_{16}H_{17}ClN_2O_2$) C, H, N.

To the major portion of the toluene solution, 40 mL of $POCl_3$ was added and the mixture heated to reflux for 15 h. The reaction mixture was evaporated to dryness under vacuum; the nonvolatile residue was dissolved in chloroform and added to a stirring solution of 3% NH_4OH in ice–water (450 mL). The organic layer was washed (ice–water) and dried (Na_2SO_4) and the $CHCl_3$ removed under vacuum. Crystallization from cyclohexane gave 4.4 g (73%) of 3-methoxy-4,9-dichloroacridine, mp 194–195 °C (lit.[2b] mp 195–196 °C).

Table I



compd no.	n	R	mp, °C	formula	method[a]	optimal dose, mg/kg	% ILS	I_{50}, μM	LD_{10} at 5th day,[d] mg/kg/day
1	2	H	*b*		C	5.0	7	1.42	4.3
1a	2	4-ethyl	144–146	$C_{32}H_{30}N_4$	C	5.0	20	*c*	16.0
2	4	H	*b*		C	20.0	13	2.71	11.0
2a	4	4-ethyl	145–146	$C_{34}H_{34}N_4$	C	10.0	10	*c*	7.4
3	5	H	190–191.5	$C_{31}H_{28}N_4 \cdot 0.5H_2O$	C	7.5	36	*c*	12.5
4	6	H	*b*		C	2.5	54	0.38	9.0
4a	6	3-NH_2	400	$C_{32}H_{32}N_6 \cdot 3HCl \cdot 0.5H_2O$	D	10.0	47	0.21	>20.0
5	6	4-ethyl	155–156	$C_{36}H_{38}N_4$	C	5.0	43	0.23	16.0
6	6	3-nitro	212.5–213	$C_{32}H_{28}N_6O_4$	C	1.25	37	0.48	>20.0
7	6	2-methoxy	122–123	$C_{34}H_{34}N_4O_2$	C	2.5	25	0.32	8.6[e]
8	6	3-propoxy	175–176	$C_{39}H_{44}N_4O_2 \cdot 0.5H_2O$	C	1.25	37	0.32	6.8
9	6	4-methoxy	197.5–198.5	$C_{34}H_{34}N_4O_2$	C	5.0	62	0.19	12.5
9a	6	3-methoxy	167–168.5	$C_{34}H_{34}N_4O_2$	A,C	1.25	71	0.24	9.5
10	6	4-butoxy	147.5–148.5	$C_{40}H_{36}N_4O_2$	C	2.5	7	0.36	6.5
11	7	H	171–173	$C_{33}H_{33}N_4 \cdot 0.5H_2O$	C	5.0	67	0.32	12.5
12	8	H	*b*		C	2.5	60	0.32	13.5
13	8	4-ethyl	111–118	$C_{38}H_{42}N_4$	C	10.0	42	0.23	12.0
14	8	2-methoxy	186–187.5	$C_{36}H_{38}N_4O_2 \cdot 0.5H_2O$	C	7.5	30	0.19	6.0[e]
15	8	3-methoxy	182.5–184	$C_{36}H_{38}N_4O_2 \cdot 0.5H_2O$	C	2.5	84	*c*	10.5
16	8	4-methoxy	182.5–184	$C_{36}H_{38}N_4O_2$	C	2.5	89	0.29	13.5
17	8	3,5-dimethoxy	199–201	$C_{38}H_{42}N_4O_2$	C	1.25	66	0.42	13.0
18	8	3-methoxy-7-chloro	165–166.5	$C_{36}H_{36}N_4O_2Cl_2$	A,B	0.63	18	0.16	7.0
18a	8	3-methoxy-6-chloro	273 dec	$C_{36}H_{36}N_4O_2Cl_2 \cdot 2HCl \cdot 0.5H_2O$	A	5.0	17	*c*	>20.0
19	8	4-ethoxy	191–192	$C_{38}H_{42}N_4O_2 \cdot H_2O$	C	3.75	76	*c*	13.5[e]
20	8	3-propoxy	146–148	$C_{40}H_{46}N_4O_2$	C	1.25	63	0.21	5.5[e]
20a	8	3-NH_2	338 dec	$C_{34}H_{38}N_6 \cdot 2HCl \cdot 0.5H_2O$	D,E	1.25	58	*c*	>20.0
21	8	3-nitro	188–189	$C_{34}H_{34}N_6O_4 \cdot H_2O$	C	5.0	45	0.33	>20.0
21a	8	3-methoxy-6-nitro	207.5–209.5	$C_{36}H_{36}N_6O_6 \cdot H_2O$	B	10.0	21	*c*	>20.0
22	8	3-chloro	167.5–168.5	$C_{34}H_{32}N_4Cl_2$	C	20.0	29	0.23	>20.0[e]
23	8	3-bromo	151–153.5	$C_{34}H_{32}N_4Br_2 \cdot H_2O$	B	2.5	09	0.21	>20.0
24	8	3-methylthio	268 dec	$C_{36}H_{38}N_4S_2 \cdot 2HCl$	B	5.0	27	0.37	8.0
24a	8	4-methylthio	164–173	$C_{36}H_{38}N_4S_2$	C	5.0	13	0.86	>20.0
25	8	4-butoxy	140–142	$C_{42}H_{50}N_4O_2$	C	5.0	07	0.19	7.6
26	10	H	*b*		C	10.0	33	0.38	7.0
26a	10	4-ethyl	105–108.5	$C_{40}H_{46}N_4$	C	2.5	33	0.15	13.0
26b	10	4-aza	150–160	$C_{34}H_{36}N_6 \cdot 0.5H_2O$	C	10.0	22	*c*	

27	12	H	148.5–163	$C_{36}H_{40}N_6 \cdot 0.5H_2O$	C	10.0	10	0.32	6.5
28	12	4-aza	276–279	$C_{30}H_{26}N_4O_2 \cdot 2HCl \cdot 3H_2O$	C	20.0	10	c	
29	2	3-methoxy	178–180	$C_{36}H_{38}N_4O_2 \cdot 0.5H_2O$	C	5	0	c	7.2
30	2	4-butoxy	267–269	$C_{32}H_{32}N_4O_2 \cdot 2HCl \cdot 2.5H_2O$	C	1.25	9	c	12.5
31	4	3-methoxy	105–109	$C_{36}H_{38}N_4O_2 \cdot 1.5H_2O$	C	20	10	c	> 20.0
32	4	4-propoxy	245 dec	$C_{34}H_{36}N_6 \cdot 2HCl \cdot H_2O$	C	1.25	9	d	9.8
33	8	2-amino	252 dec	$C_{36}H_{38}N_4 \cdot 2HCl \cdot H_2O$	C	2.5	60	c	> 20.0
34	8	3-methyl	190–193	$C_{38}H_{42}N_4 \cdot 2HCl$	B	2.5	59	c	7.3
35	8	3,4-dimethyl	245 dec	$C_{38}H_{42}N_4 \cdot 2HCl \cdot 0.5H_2O$	C	2.5	60	c	8.0
36	8	4-methoxy-1-methyl	244 dec	$C_{36}H_{36}N_6O_6 \cdot 2HCl \cdot 0.5H_2O$	C	5	20	c	12.5
37	8	4-methoxy-6-nitro	166.5–167.5	$C_{36}H_{36}N_4O_2Cl_2$	C	10	86	c	> 20.0
38	8	2-methoxy-6-chloro	173–177	$C_{38}H_{42}N_4O_2 \cdot 2HCl \cdot H_2O$	C	20	9	c	> 20.0
39	10	3-methoxy			C	5	55	c	8.0

[a] Method A,B indicates that the precursor (the corresponding 9-chloroacridine) was prepared according to Scheme II, route A or B, respectively; method C, prepared by the procedure outlined in Scheme I; and methods D and E, prepared by reduction of the corresponding 3-nitro analogue. [b] Previously reported in ref 2a. [c] Not measured. [d] The total number of animals started on most tests is six, unless specified. [e] The total number of animals started on test is four.

Method B. When the toluene solution of the *N,N*-dimethylamide in method A was evaporated to dryness and treated with $POCl_3$ in benzene (refluxed) for 8 h and worked up, the TLC and NMR analysis showed that the crude product was a mixture of 9-chloro and 9-*N,N*-dimethylamino derivatives in a 2 to 3 ratio. Refluxing in ethanol–3% HCl for 2 h gave 80% of 3-methoxy-7-chloroacridone. Treatment of the acridone with $SOCl_2$–DMF in chloroform (route B, Scheme II) gave the desired product in 84% yield, mp 194–196 °C.

1,8-Di(3-aminoacridin-9-ylamino)octane Dihydrochloride (20a) and Its Trichloride. (a) Dihydrochloride. Method E. The 3-nitro derivative **21**·2HCl (3.3 g) and iron powder (40 mesh, 15 g) were suspended in 250 mL of aqueous ethanol (60%). To this was added 2 mL of $FeCl_3$ solution (32.5 g of $FeCl_3$ dissolved in 100 mL of water).[17] The vigorously stirring mixture was heated to boiling on a water bath and refluxed for 30 min. The boiling reaction mixture was filtered and the insoluble solid washed with 60% EtOH (boiling). The combined filtrate was concentrated to 70 mL, resulting in precipitation of the product. Crystallization from 95% EtOH (twice) gave 2.0 g of pure product, mp 348 °C (darkens before decomposition). Anal. Calcd for $C_{34}H_{36}N_6 \cdot 2HCl$: C, 67.88; H, 6.37; N, 13.97; Cl, 11.80. Found: C, 67.96; H, 6.48; N, 14.18; Cl, 12.22.

Method D. This compound could also be prepared by the catalytic hydrogenation (Raney nickel) of compound **21**.

(b) Trihydrochloride. To the above dihydrochloride (0.3 g) dissolved in ethanol, 0.5 mL of concentrated HCl was added. The solution was evaporated to dryness under vacuum below 40 °C and the residue crystallized from 95% EtOH (twice) to give 0.25 g of the trihydrochloride salt, mp 345 °C dec. Anal. Calcd for $C_{34}H_{36}N_6 \cdot 3HCl \cdot 2H_2O$: C, 60.58; H, 6.38; N, 12.47; Cl, 15.81. Found: C, 60.75; H, 6.14; N, 12.38; Cl, 15.91.

1,6-Di(3-aminoacridin-9-ylamino)hexane Trihydrochloride (4a). Method D. 1,6-Di(3-nitroacridin-9-ylamino)hexane (**6**, 1 g), 0.7 g of Raney nickel, 5 mL of acetic acid, and 1 mL of concentrated HCl in 100 mL of ethanol were shaken in a Parr hydrogenator at 40 psi for 40 h. Fresh catalyst (0.5 g) was added at 20 h and once more at 30 h. TLC showed that the reaction was complete. The reaction mixture was filtered and the greenish filtrate discarded. The insoluble solid was extracted with a large volume of boiling ethanol. The crude product was obtained by evaporation and recrystallized (twice) from 95% ethanol to give 0.31 g of product, mp 400 °C. Anal. Calcd for $C_{32}H_{32}N_6 \cdot 3HCl \cdot 0.5H_2O$: C, 62.09; H, 5.86; N, 13.58. Found: C, 62.0; H, 6.05; N, 13.49.

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Pyridine Derivatives as Potent Inducers of Erythroid Differentiation in Friend Leukemia Cells

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Friend erythroleukemia cells in culture can be induced to differentiate along the erythroid pathway by dimethyl sulfoxide and a variety of organic polar compounds. Since this system appears to be a reasonable model to uncover agents with the potential to decrease the malignant phenotype of certain cancer cells through maturation, we have instituted a search for new and effective inducers of differentiation. To this end, we wish to report on the capacity of a series of pyridine derivatives to induce erythroid differentiation, which is monitored by the attainment of specialized function (i.e., the ability to synthesize hemoglobin detected as benzidine-stained cells). Three types of derivatives, acetamidopyridines, acetamidopyridine *N*-oxides, and acetamido-1-methyl-2-pyridones, were prepared and characterized. All but one of the compounds in these classes induced an accumulation of hemoglobin by Friend cells, although pyridine alone was not an effective inducer of differentiation. 2-Acetamidopyridine, 1-methyl-2-pyridone, and 1-methyl-5-acetamido-2-pyridone caused induction of maturation equivalent to dimethyl sulfoxide at concentrations approximately 100-fold lower than the polar solvent. It is interesting to note that all of the compounds tested which caused differentiation in this cell system contained the sequence $-C(=O)-N(R)-$ (R = H or CH_3) either attached to the heterocyclic ring or as part of the ring structure itself.

Friend and her co-workers[1] have reported that murine virus transformed leukemia cells grown in culture can be induced to differentiate along the erythroid pathway by treatment with dimethyl sulfoxide (Me_2SO). The process of differentiation is characterized by the synthesis and accumulation of globin mRNA[2] and hemoglobin,[3] the appearance of erythroid antigens on the cell surface,[4] the induction of spectrin,[5] a large variety of morphological alterations similar to those associated with the differentiation of normal proerythroblasts to orthochromatic normoblasts,[6] and loss of cell proliferative capacity.[7-9] *N*,*N*-Dimethylformamide and a wide variety of other organic polar compounds,[10,11] as well as some polymethylene bisacetamides, such as hexamethylene bisacetamide,[12] have been shown to be potent inducers of differentiation in this system. These compounds have in common a hydrophilic or polar group covalently joined to a hydrophobic portion of the molecule. Other structurally unrelated molecules, including butyric acid,[13] hypoxanthine and some purine analogues,[14] and ouabain,[15] can also stimulate erythroid differentiation, suggesting that there may be more than one mechanism involved in this process.

Furthermore, there is evidence[1] that Me_2SO-treated cells, which have attained specialized function, possess lower malignant potential as compared to untreated cells. Thus, Friend erythroleukemia cells in culture provide a valuable system to study the design of agents which are capable of inducing cell differentiation and decreasing the malignant potential of a tumor. A continued search for new potent inducers and a study of the relationship between structure and the capacity to induce differentiation would appear to be useful to gain an understanding of the mechanisms of erythroid differentiation in the erythroleukemia cells. It is a primary objective of this laboratory to develop new inducing agents which (a) function at low concentrations and (b) induce a high percentage of cells to differentiate. Since certain amide-containing agents and pyridine *N*-oxide have been reported[10-12] to be effective inducers of the differentiation of Friend leukemia cells, we have synthesized and studied the biological activities of amides of three classes of pyridine derivatives, including pyridines, pyridine *N*-oxides, and 2-pyridones. The biological findings demonstrate that many derivatives of pyridine, an inactive agent in this system, are capable of inducing differentiation of the Friend erythroleukemia. 2-Acetamidopyridine, 1-methyl-2-pyridone, and 1-methyl-5-acetamido-2-pyridone were among the most potent inducers studied, being effective at more than $^1/_{100}$th the concentration of Me_2SO required for equivalent induction.

Chemistry. Various acetamidopyridine derivatives were prepared by treatment of corresponding aminopyridines with pyridine and acetic anhydride; their *N*-oxides were obtained by reaction with *m*-chloroperbenzoic acid in chloroform.[16] Using the Chichibabin reaction,[17] a mixture of 4-phenylpyridine and sodium amide in dimethylaniline was heated at 110 °C to give 2-amino-4-phenylpyridine (**3**) as the major product. Following the procedure of Caldwell and Kornfeld,[18] β-acetamidopyridine derivatives were obtained from corresponding β-nitropyridines. Acetylation of 2-hydroxy-6-aminopyridine and partial hydrolysis of the resulting diacetate product afforded 2-hydroxy-6-acetamidopyridine[19] which exists predominantly in the oxo form, 6-acetamido-2(1*H*)-pyridone (**11**).[20] N-Methylation of 2-hydroxy-5-nitropyridine with dimethyl sulfate gave 1-methyl-5-nitro-2-pyridone (**13**).[20,21] 1-Methyl-6-acet-

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