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RELATIONSHIP OF BIOCHEMICAL DRUG EFFECTS TO THEIR ANTITUMOR ACTIVITY—I

DIACRIDINES AND THE CELL MEMBRANE

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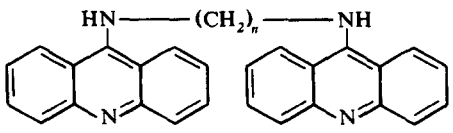
Abstract—Based on prior and current experimental results, a hypothesis is presented, that the most effective antineoplastic agents are those agents that modify directly or indirectly the interaction between cells. We also propose that the *in vitro* assays for antitumor agents in use today may select for unnecessarily toxic agents. Previous results from this laboratory indicated that the antitumor activity of the diacridines correlates best with their effect on the plasma membrane, despite the fact that the prominent biochemical site of action of the diacridines is the inhibition of nucleic acid metabolism. A method has been developed that permits the sensitive determination of the permeability of cells in culture, as well as changes in the intracellular acid-soluble and acid-insoluble nucleic acid pools. The evidence indicates that P-388 cells normally extrude acid-soluble components into the medium, that the diacridines attach to the plasma membrane, alter the degree of extrusion of acid-soluble components of the cells, and modify the intracellular levels of newly synthesized nucleic acids. The present study, performed with four selected diacridines, shows that the diacridines affect these parameters to varying degrees.

Many antineoplastic agents have multiple sites of subcellular association and inhibit a number of biochemical reactions. Some of these have been studied in detail; others remain to be discovered. It is difficult, therefore, to be certain which, if any, of the prominent biochemical actions of a compound are primarily responsible for its antineoplastic effects. It is even conceivable that the antineoplastic activity of a compound may not be causally related to any of its known biochemical inhibitory actions.

This concept, the appreciation that the prominent biochemical sites of action of an antineoplastic agent may not be causally related to its antineoplastic properties, but may even antagonize its antineoplastic properties, constitutes the basis of our current research. To this purpose we have synthesized a large series of diacridines. We use these as a model system to identify their prominent biochemical activities and to relate them to those parameters that are most directly related to their antineoplastic activities.



This approach recognizes that many of the biochemical effects of an antineoplastic agent may be chemotherapeutically undesirable and should be minimized. With the use of the diacridines as a model system, this approach is intended to: (1) identify those cellular sites of action with which the diacridines associate and to define that association which correlates best with their antitumor activity; (2) differentiate those irrelevant or undesirable biochemical sites of action from those effects that are essential for their antineoplastic activity; and (3) synthesize analogs in which the desirable antineoplastic properties are emphasized.

It is anticipated that this information will not only lead to the limited goal of optimization of the antineoplastic activity of the diacridines but that it will provide a general method that defines those biochemical parameters that are most crucial for the growth of the cancer cells and toward which chemotherapeutic agents should be specifically directed.

The diacridines were synthesized in order to enhance the natural intercalative ability of the acridine ring [1–3]. Attainment of this primary goal was borne out fully by the fact that some of the diacridines can raise the T_m of DNA by more than 50–60° under these same conditions [1]. At this temperature, the DNA does not disengage itself from the diacridine; rather the diacridine decomposes to acridone which has a nominal effect on the T_m and frees the DNA. The maximal effect on the melting of DNA occurs with C_{10} [1]; it has been shown that the intercalation of both acridine rings of a diacridine into DNA requires a connecting chain with at least five methylene groups or better with six methylene groups, i.e. a diamino pentane or diaminohexane [4, 5]. In effect, optimal antitumor activity against P-388 ascites tumors was obtained when the value for n in the formula was 6 or 8 [1, 6, 7].

The diacridines are inhibitors of the growth of the leukemic P-388 cells and L-1210 cells, as well as of HeLa cells in culture. Their I_{50} in P-388 cells in culture is in the range of 0.2–0.4 × 10^{-6} M. Their primary site of inhibition is the synthesis of RNA, whereas the

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inhibitions of the synthesis of DNA and of protein appear to be secondary sites [2, 3]. They inhibit specifically the synthesis of 45S RNA [8], as well as its processing to 28S and 18S RNA [8]; this latter effect differentiates them from actinomycin D, which does not inhibit the processing of 45S RNA [8]. The diacridines were tested on an *in vitro* enzyme system which synthesizes well-defined mRNAs, the T7 DNA-dependent RNA polymerase transcribing the cistrons of T7 DNA, and were shown to inhibit the initiation of RNA synthesis but not the elongation of RNA [9]. This was in obvious contrast to the action of actinomycin D, which specifically inhibited the elongation of RNA but not the initiation of RNA synthesis [10]. It was shown furthermore that all the diacridines are concentrated by the cells, some of them as much as 800-fold over their concentration in the medium, and that their distribution between the nucleus and the cytoplasm varies with the individual diacridine [10].

These results indicated that the major biochemical site of action of the diacridines was related to their ability to intercalate with nucleic acids and to the metabolic consequences of the intercalation. However, these results did not prove that there was a necessary causal correlation between these major biochemical activities and the antitumor effects of the diacridines. In fact, statistical correlation of these various results and the antineoplastic activity of the diacridines indicated the membrane as the site that was most closely related to the antineoplastic action of the diacridines [6].

In order to determine some of the biochemical effects of the diacridines on the plasma membrane, we have selected four diacridines, two from each extreme of the curve expressing the per cent increase in life span (% ILS) vs rate of agglutination curve (Fig. 1). Of these, the 4-methoxy C6 and the 4-methoxy C8 diacridines have a relatively high and the highest % ILS (62 and 89 per cent, respectively), with low rates of agglutination; at the other extreme, the 4-butoxy C6 and the 4-butoxy C8 diacridines have negligible if any antineoplastic activity, while they exhibit high rates of cell agglutination.

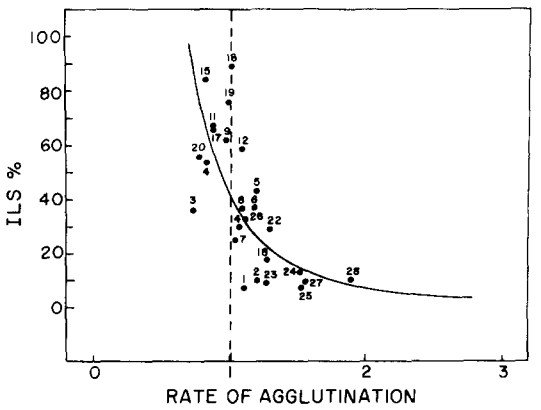

Fig. 1. Relationship of the rate of agglutination of P-388 cells to the per cent increase in life span (% ILS) of mice bearing P-388 ascites tumors. This figure is modified from Fig. 2 of Ref. 6. The vertical line represents the control agglutination value of P-388 cells that were not treated with diacridines; values to the left and right of this curve represent decreases and increases, respectively, in the rate of agglutination. Each number represents a different diacridine [6]. Key: 9 = 4-methoxy C_6, 16 = 4-methoxy C_8, 25 = 4-butoxy C_8. 4-Butoxy C_6 = 10 is not represented because it lyses P-388 cells under these conditions of assay. When used with Sarcoma-180 cells, its agglutination rate approximates that of 4-butoxy C_8.

In this manuscript, a method is presented that permits the determination of normal cellular permeability, as well as changes in the intracellular acid-soluble and acid-insoluble nucleic acid pools. The results show that cells normally extrude intracellular acid-soluble components into the medium, that the diacridines attach to the plasma membrane to various degrees, and that they alter the basal cellular excretion pattern as well as the levels of newly synthesized intracellular nucleic acids. A hypothesis is presented in the discussion which suggests that the most effective antineoplastic agents are those that modify the interaction "between" cells. We also propose that the *in vitro* assays for antitumor agents in use today may select for unnecessarily toxic agents.

MATERIALS AND METHODS

Determination of the permeability of the cells

Logarithmically growing P-388 cells (3×10^5 cells/ml) [1] were incubated at 37° for 30 min with [^{3}H]uridine (10 μCi/ml, 20 Ci/m-mole). The cells were washed with 37° growth medium plus 10% horse serum and incubated at 37° for 1 hr in growth medium containing non-radioactive uridine (25 μg/ml). They were centrifuged and resuspended in 37° growth medium, with 1×10^{-5} M diacridine, and incubated at 37°. Samples were removed at 0, 20 and 40 min, and centrifuged immediately (5 min at 1000 *g*); the supernatant fluid was designated "extracellular medium". The cells were suspended in 5% trichloroacetic acid, cooled in ice, and the radioactivity in the acid-soluble fraction and the precipitate was determined. The radioactivity in the three fractions, extracellular medium, acid-soluble, and acid-insoluble, of cells was calculated on the basis of the total counts/min/10^6 cells.

Fluorescence studies

With P-388 cells. P-388 cells were washed in Fischer's medium [1] and suspended in phosphate buffered saline to yield 7×10^5 cells/ml. They were treated with an equal volume of 2×10^{-5} M diacridine in phosphate buffered saline to yield 3.5×10^5 cells/ml and 1×10^{-5} M diacridines. Final values include corrections for samples of cells alone and diacridines alone.

With cell membranes. Red blood cell membranes [11] and P-388 cell membranes [12] were treated similarly with diacridines. The final concentration of membranes was approximately 300 μg/ml. Fluorescence values were adjusted for values as above.

All other methods, as well as materials used have been described in our previous publications [1–3].

RESULTS

Effect of the diacridines on the agglutination of tumor cells

Figure 1 presents the correlation between the antitumor activity of the diacridines in mice bearing P-388

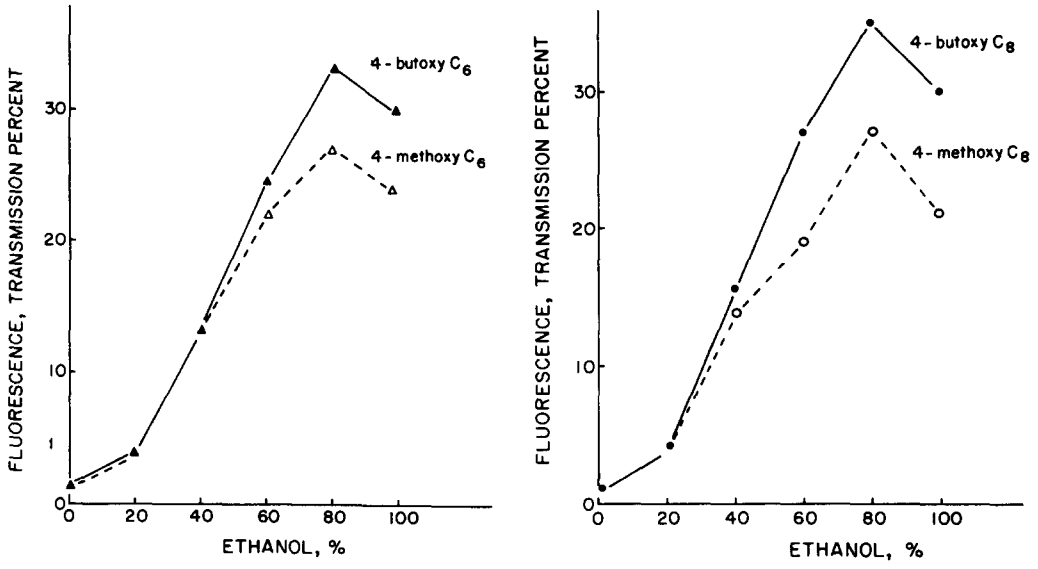

Fig. 2. Per cent increase in fluorescence transmission of diacridines dissolved in various ethanol solutions in water containing ethanol of different concentrations. Key: (●——●) 4-butoxy C_8; (▲——▲) 4-butoxy C_6; (○ – – ○) 4-methoxy C_8; and (△ – – △) 4-methoxy C_6.

ascites tumors (indicated as "per cent increase in life span", % ILS) and the ability of the diacridines to affect the rate of agglutination of the P-388 cells. This figure is modified from that presented previously [6], and emphasizes that some of the diacridines decrease the rate of agglutination, others greatly enhance it, while others have no effect. The correlation coefficient between these two variables is 0.8; this is particularly high when we consider that all other correlation coefficients obtained for a number of biochemical effects of these same diacridines and either the % ILS or the toxicity of the compounds (LD_{10}) is of the order of 5×10^{-5} to 0.3 [7]. This result, as well as the comparable effect of the diacridines on Sarcoma-180 cells [6], has been interpreted to indicate that the diacridines affect the cell membrane and that the antitumor activity of the diacridines is in some manner related to an effect of the diacridines on the plasma membrane.

Effect of ethanol on the fluorescence of diacridines

Excitation of the diacridines at 410 nm elicits a fluorescence maximum at 490 nm. The effect of ethanol, a non-polar lipophilic solvent, on this fluorescence is shown in Fig. 2. It can be seen that increasing concentrations of ethanol enhance the fluorescence of these compounds and that the degree of enhancement of fluorescence is approximately the same for the four diacridines; maximum enhancement occurs at 80% ethanol, while 100% ethanol gives reproducibly a lower enhancement of fluorescence. The values obtained within the short time required for mixing are stable and do not change with time.

Fluorescence of diacridines in the presence of intact P-388 cells

An increase in fluorescence is also observed when these diacridines are allowed to interact with P-388 cells. These results are presented in Fig. 3A and certain characteristics can be discerned. The increase in fluorescence occurs slowly and the maximum fluorescence enhancement occurs at different times for each diacridine. Furthermore, the maximum fluorescence enhancement attained in the presence of P-388 cells is different; it is characteristic of each diacridine.

These changes in fluorescence suggest that a slow interaction is occurring between the diacridines and a lipophilic area of the cell in which the diacridines are being dissolved. Furthermore, the 4-butoxy analogues can be differentiated from the 4-methoxy analogues by the much greater increase in fluorescence that they exhibit.

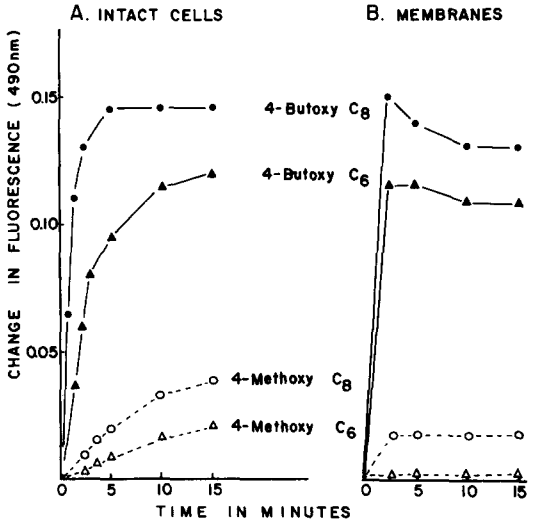

Fig. 3. Change in the fluorescence transmission with time of diacridines exposed to P-388 cells (A) and to red blood cell membranes (B). The changes in fluorescence transmission are in arbitrary units, normalized to the maximum value for 4-butoxy C_6. Key: (●——●) 4-butoxy C_8; (▲——▲) 4-butoxy C_6; (○ – – ○) 4-methoxy C_8; and (△ – – △) 4-methoxy C_6.

Fluorescence of diacridines in the presence of plasma membranes

In order to determine the type of interaction that occurs between the plasma membranes and the diacridines, washed red blood cell ghosts were isolated and reacted with the diacridines. A similar experiment was performed with highly purified plasma membranes of P-388 cells and very similar results were obtained, but not presented. The results with the purified red blood cell ghosts are presented in Fig. 3B and show that the changes in fluorescence that occur differentiate these four diacridines into the same two groups, i.e. the 4-butoxy analogs show a much greater enhancement in fluorescence than do the 4-methoxy analogues. Furthermore, it is apparent that maximum fluorescence is attained much more rapidly than with intact cells. These results reproduce very closely those presented in Fig. 3A with intact P-388 cells. A proportion of this fluorescence enhancement of the diacridines may be due, therefore, to their interaction with the plasma membrane; the use of the red blood cell ghosts also emphasizes that this fluorescence enhancement is not necessarily characteristic of the plasma membrane of the P-388 cells alone.

Relationship between fluorescence enhancement and uptake of diacridines by cells and plasma membranes

The differences observed in fluorescence enhancement between these diacridines could reflect the differences in uptake of diacridines by the cells or the membranes. Table 1 shows the relative uptake of diacridines by cells and membranes and the extent of fluorescence enhancement of the diacridines by these fractions. The results show an extensive uptake of 4-methoxy C_8 by cells. However, the 4-butoxy analogs are taken up more extensively by the membranes and the fluorescence enhancement seems to be related more to the uptake by the membranes.

Effect of the diacridines on the permeability of P-388 cells

The effect of diacridines on the permeability of the plasma membrane was determined; this was used as an index of a disruptive effect on the plasma membrane.

A common method of determination of altered permeability is the exposure of cells to trypan blue and determination of the per cent of trypan blue positive cells. We have developed a new assay which avoids the uncertainties inherent in the trypan blue method. This consists of growing cells for a short period of time in radioactive uridine. After suitable preparation, the cells are exposed to the experimental variables; the radioactivity present in the medium, as well as the acid-soluble and acid-insoluble radioactivity present in the cells, is determined. Changes in these components can then be interpreted both in terms of the ability of the cell to retain its acid-soluble pools as well as its RNA. Figure 4 shows the loss of radioactivity, from uridine-labeled P-388 cells, to the medium (Fig. 4A), and the concurrent changes in the radioactivity of the cellular acid-soluble compartment (Fig. 4B) and of the cellular acid-insoluble compartment (Fig. 4C).

The following can be discerned. The control P-388 cells show a progressive loss of acid-soluble intracellular radioactivity and this loss can be detected in the medium. The nature of this radioactive label is being determined. The intracellular acid-insoluble label, however, remains relatively stable. The 4-methoxy C_6 diacridine has a nominal effect, if any, on these control parameters. Although the uptake of 4-methoxy C_6 is low compared to that of the other diacridines, it is, nevertheless, concentrated 26-fold from the medium by the P-388 cells under these conditions. It is not taken up very much by the membranes and it shows little, if any, fluorescence enhancement with P-388 cells and with red blood cell membranes; yet it is as inhibitory to RNA synthesis as are 4-butoxy C_6 and 4-butoxy C_8 [6].

On the other hand, the 4-methoxy C_8 and the 4-butoxy C_8 diacridines, which show very different fluo-

Table 1. Summary of selected parameters of four diacridines*

		4-Methoxy C_6 (9)	4-Methoxy C_8 (16)	4-Butoxy C_6 (10)	4-Butoxy C_8 (25)
(A)	Uptake				
	P-388 cells	1.0	6.2	9.0	4.6
	Membranes of P-388 cells	1.0	2.3	2.6	4.7
	RBC membranes	1.0	5.2	12.8	27.0
(B)	Fluorescence enhancement				
	P-388 cells	1.0	2.0	12.0	14.5
	P-388 membranes	1.0	2.0		13.3
	RBC membranes		2.0	12.7	15.2
(C)	Rate of agglutination				
	P-388 cells	1.0	1.02		1.6
	Sarcoma-180 cells	1.0	1.05	2.1	2.2
(D)	% ILS	62	89	<10	<10

* Numbers in parentheses are from Fig. 1. Values in A and C are relative to 4-methoxy $C_6 = 1.0$; values in B are relative to 4-methoxy $C_8 = 2$. Uptake by P-388 cells: values were taken from Ref. 6; uptake by membranes: membranes were exposed to 1×10^{-5} M diacridines for 15 min. Fluorescence enhancement: values were obtained from Fig. 3. Rate of agglutination and % ILS values were obtained from Figs. 1 and 2 of Ref. 6.

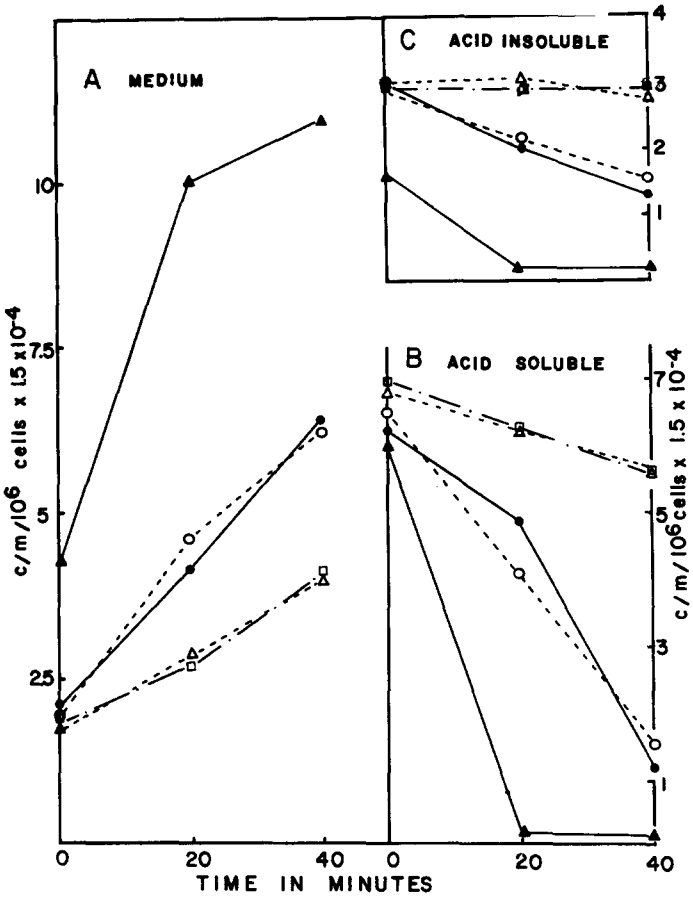

Fig. 4. Changes of the radioactivity pattern of P-388 cells with time of exposure to diacridines. P-388 cells, prelabeled with [^{3}H]uridine, were exposed to 1×10^{-5} M diacridines and the release of radioactivity into the medium (A), as well as the changes in the acid-soluble radioactivity of the cells (B) and of the acid-insoluble radioactivity (C) of the cells, was monitored. All values are expressed on the basis of 10^6 cells. Key: (□——□) control; (●——●) 4-butoxy C_8; (▲——▲) 4-butoxy C_6; (○ – – ○) 4-methoxy C_8; and (△ – – △) 4-methoxy C_6.

rescence enhancement with P-388 cells and with membranes and are taken up to very different extents by the membranes, seem to exert very similar effects on these parameters; the cells become equally permeable, and they lose their intracellular acid-soluble radioactivity as well as their acid-insoluble RNA into the medium at the same rate.

The 4-butoxy C_6 was selected because of certain unique characteristics. The 4-butoxy C_6, under conditions of this assay, lyses the P-388 cells [6]. This lysis is illustrated very well by the results presented in Fig. 4 (panels A, B and C). The acid-soluble radioactivity in the media has already increased by zero time, while the acid-insoluble RNA starts falling. By 20 min most of the cellular radioactivity can be accounted for in the medium.

Table 1 summarizes the various biological parameters that have been determined to date for these four compounds. The values obtained from the agglutination of S-180 cells have been included to accommodate a relative value for 4-butoxy C_6 in cells which are not lysed by this compound.

DISCUSSION

In this study, four diacridines were selected from the two extremes of the "Rate of Agglutination vs % ILS" curve which had suggested that the antitumor effectiveness of the diacridines is related primarily to reactions associated with the plasma membrane. The present results emphasize that these diacridines interact with the plasma membrane and modify the permeability of the cells.

The variable fluorescence enhancement that occurs in the presence of P-388 cells or of P-388 membranes or of red blood cell ghosts, with the diacridines that we have tested, seems to be related most closely to uptake of diacridines by the membranes. However, this limited study indicates that the modification of cellular permeability is not proportional to the uptake of the diacridines by the membrane. The permeability of cells treated with 4-butoxy C_8 or 4-methoxy C_8 is affected very similarly, yet these compounds are taken up to different extents by the membranes and the cells. It is possible that each diacridine enters a different area of lipophilicity of the cell plasma membrane or that they enter the same area

but that structural constraints enforce a different reaction with the neighboring molecules. Our projected synthesis of diacridines that will link covalently with adjacent groups on the membrane will permit us to define the molecular interaction that occurs with the nearest neighbors of the plasma membrane to which the diacridines are exposed.

Our results with this limited sample of four diacridines indicate that there is no obvious correlation between the extent of alteration of cellular permeability and antitumor activity. This is indicated very clearly in the summary presented in Table 1. 4-Methoxy C_6 (% ILS = 62) does not affect cellular permeability, while 4-methoxy C_8 (% ILS = 89) and 4-butoxy C_8 (% ILS < 10) have similar effects on the permeability of the P-388 cells. However, we do not know what information will be derived from a larger series.

The common denominator of the agglutination test and of the % ILS is that they are both expressions of the effect of the diacridines on cell to cell interaction. All the other tests we have used, and which are normally used, measure the effect of a drug on individual cells; these do not show a high correlation coefficient.

We suggest that attention should be paid to developing better *in vitro* assays aimed at determining the effects of agents on the interaction between cells. Such *in vitro* assays may correlate better with % ILS values.

In general, we propose that the most effective antineoplastic agents are those agents that directly or indirectly modify the interaction between cells and that the *in vitro* assays for antitumor agents in use today may select for unnecessarily toxic agents.

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