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EFFECTS OF ACRONYCINE ON CELL-SURFACE PROPERTIES OF MURINE LEUKEMIA CELLS

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Abstract—The alkaloid acronycine interacts with cell-surface components of the murine leukemia cells resulting in inhibition of nucleoside transport. Other consequences of this interaction are altered binding of fluorescent probes, and altered cell behavior in a two-phase aqueous polymer system. Amino acid transport was not affected by acronycine, but high levels of the drug inhibited synthesis of protein.

The drug acronycine (Fig. 1) has antitumor activity in several experimental systems [1]. Drug-induced inhibition of growth of murine leukemia L5178Y cells in culture has been reported [2]. The apparent inhibition of incorporation of exogenously-supplied uridine into RNA [2] was not caused by drug-induced inhibition of intracellular uridine biotransformations, suggesting [3] inhibition of uridine transport by acronycine. In this report, we show that the interaction between acronycine and L1210 and L5178Y murine leukemia cells has several demonstrable consequences, including inhibition of nucleoside transport across the cell membrane.

MATERIALS AND METHODS

Uridine and thymidine, both labeled with ^{14}C in the 2 position of the pyrimidine ring (10–25 mCi/mmole), [*carboxyl*-^{14}C]cycloleucine (30 Ci/mole) and L-leucine-1-[^{14}C] (15 Ci/mole) were purchased from New England Nuclear Corp., Boston, MA. These were diluted with carrier to obtain stock solutions of 0.1–10 μM, containing approx. 5,000 cpm/μl of solution as measured by liquid scintillation counting.

Acronycine (NSC 403169) was provided by the Division of Cancer Treatment, National Cancer Institute, NIH. The drug was dissolved in *N,N*-dimethylformamide at a level of 10 mg/ml. No more than 3 μl of DMF* was added per ml of cell culture; an equivalent amount of DMF was added to control tubes in all experiments shown here.

Dansyl cadaverine was purchased from Vega-Fox Biochemicals, Tucson, Arizona; solutions of 10 mM were prepared in DMF. ANS (1-anilino-8-naphthalenesulfonate) was purchased from Pierce Chemical Co., Rockford, IL; aqueous solutions of 10 mg/ml were employed. The Mg salt of ANS was converted to the sodium salt by treatment with Dowex 50.

Data relating to the effect of acronycine on survival of tumor-bearing animals was provided by Mr. I. Wodinsky, Arthur D. Little Corp., Cambridge, MA, and by the Division of Cancer Treatment, NCI, NIH.

L5178Y cells were obtained from Dr. H. B. Bosmann, University of Rochester Medical Center, Rochester, NY, and were grown in sealed flasks using Fisher's medium + 10% fetal serum. L1210 cells were obtained from the Arthur D. Little Corp., Cambridge, MA, and were grown in sealed flasks with MEM-Eagle's medium (spinner modification) + 10% fetal calf serum. All biologicals were purchased from Grand Island Biological Corp., Grand Island, NY.

Studies were generally carried out using 7×10^6 cells/ml suspended in growth media with an equimolar amount of HEPES (pH 7.4) replacing $NaHCO_3$ to minimize pH drift. Cell suspensions (1 ml portions) were incubated together with specified levels of acronycine, for 5–10 min at 10° or at 37°. If washing was specified, the cells were then collected by centrifugation (30 sec at 1000 *g*) and quickly suspended in 0.9% NaCl. After an additional centrifugation, cells were resuspended in HEPES-buffered growth medium.

Nucleoside transport was measured at 10° to minimize subsequent nucleoside incorporation into nucleic acid [4]. After drug treatment, cell suspensions were chilled at 10°, and incubated with 0.2–100 μM levels of labeled nucleosides. The level of radioactivity was maintained at approx. 50,000 cpm/ml of cell suspension by addition of carrier nucleoside. After 3 min at 10°, the cells were collected by centrifugation (30 sec.) and washed once in 0.9% NaCl containing 100 μM persantin to prevent loss of nucleoside pools (5–7). The packed cells were then dispersed in 500 μl

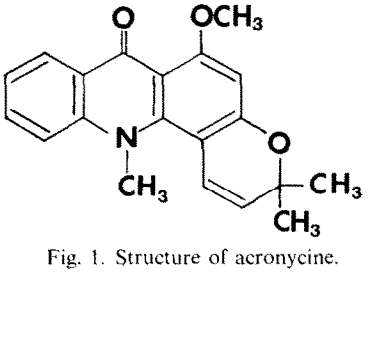

Fig. 1. Structure of acronycine.

*Abbreviations: DMF: *N,N'*-dimethylformamide; HEPES: *N*-2-hydroxyethylpiperzaine-*N*-2'-ethanesulfonic acid; ANS: 1-Anilino-8-naphthelenesulfonic acid; DCV: Dansyl cadaverine; PEG: Polyethylene glycol.

of 0.9% NaCl and intracellular radioactivity was measured by liquid scintillation counting. The appearance of label in intracellular nucleosides, nucleotides and nucleic acid was measured as described in Ref. 8.

The effect of acronycine on nucleoside exodus from cells was measured by pre-loading cells (10 min, 10°) in medium containing 25 μM levels of labeled uridine or thymidine. These cells were collected by centrifugation and suspended in fresh medium at 10° containing 0–300 μM acronycine. At intervals, aliquots of the suspension were removed, the cells collected, and intracellular radioactivity was measured as described above.

Drug effects on protein synthesis were measured at 37° by monitoring incorporation of labeled leucine into the acid-insoluble material. To measure amino acid uptake, the leucine analog cycloleucine [8] was employed. Drug-treated cells were incubated for 10 min at 37° in medium containing 0.1 mM [^{14}C]-cycleucine; the cells were collected by centrifugation, washed twice with 0.9% NaCl at 4°, and radioactivity in the cell pellets was measured.

Drugs effects on incorporation of radioactive nucleosides into nucleic acid were measured as described before [9].

Electrophoretic mobility of cells was measured [10, 11] at 25° in a buffer composed of 14.5 mM NaCl, 0.4 mM $NaHCO_3$ and 4.5% sorbitol at pH 7.2, using a Rank Bros., Mark II apparatus (Rank Bros., Cambridge, England). For each determination, 10 cells were measured with change of polarity between each measurement.

The partition coefficient [12, 13] of cells was measured in the two-phase aqueous polymer systems described in Table 4. To 9.9 ml of the complete system was added a 0.1 ml suspension of 8×10^5 cells in 150 mM NaCl. The cells had previously been treated (10 min, 37°) with 0–300 μM acronycine at 37°, then washed once with 0.9% NaCl. After gentle mixing, an aliquot of 0.5 ml was removed from the tube and diluted with 9.5 ml of Isoton (Coulter Electronics, Inc) for determination of cell number using a Coulter Z_F counter. Meanwhile, the polymer phases were then allowed to separate at 0° or at 10° as specified. The volume and number of cells in the top phase was measured. The *partition coefficient* was the number of cells in the top phase expressed as percent of the total number of cells present.

The fluorescence of cells after treatment with dansyl cadaverine [14] was measured in an Aminco recording fluorometer at room temperature. One ml portions of cell suspensions in HEPES-buffered growth medium were treated with 0–300 μM acronycine for 5 min at 37°, washed once in 0.9% NaCl, and resuspended in fresh medium. Dansyl cadaverine was added (final concentration = 0.1 mM) and the incubation was continued for an additional 30 min at 37°. The cells were collected by centrifugation, washed once with 0.9% NaCl and suspended in 2 ml of 0.9% NaCl for fluorescence measurements. An excitation wavelength of 340 nm was used, with light emission measured at 515 nm.

Studies employing the fluorescent dye ANS were carried out by a different procedure. Cells were suspended in a buffered-salts medium (TES-E, described in Ref. 9) at a concentration of 7×10^6 cells/ml. One ml portions were incubated with 0–300 μM acronycine for 5 min at 37°, washed once, and suspended in TES- E buffer at 10°. An aqueous solution of ANS (final level = 10 μM) was then added, in incubation was continued for 90 min, and fluorescence measured (excitation wavelength was 375 nm, emission measured at 480 nm).

Control experiments were carried out in which the fluorescent dyes were omitted, to permit correction for inherent fluorescence of cells and of acronycine.

RESULTS

Drug-induced inhibition of nucleoside transport. During 10 min incubations at 10°, extracellular uridine was readily accumulated by L5178Y or L1210 cells (approx. distribution ratio = 1). After incubations, we found less than 5% of intracellular radioactivity was incorporated into acid-soluble material (RNA). The intracellular acid-soluble radioactivity was composed of 75% uridine and 25% uridine nucleotides.

Table 1. Effects of acronycine on uridine uptake (10°) and incorporation into RNA (37°)

Uridine level μM	Cell line	Uptake 0*	Uptake 20*	Uptake 100*	RNA synthesis 0†	RNA synthesis 20†	RNA synthesis 100†
0.2	L5178Y	0.20	0.086	0.030	0.135	0.061	0.023
10		6.02	3.09	1.18	4.06	2.35	0.93
30		8.99	6.34	3.34	7.98	5.04	2.55
100		11.0	9.95	5.23	11.6	9.3	5.2
300		12.7	12.3	7.7	13.1	12.1	7.3
0.2	L1210	0.2	0.094	0.052	0.073	0.059	0.044
10		5.18	2.45	1.29	2.43	2.13	1.58
30		8.36	3.8	2.05	5.3	4.5	3.1
100		10.2	4.8	2.4	9.0	7.6	5.4
300		11.1	5.4	2.9	11.0	9.8	5.9

Data are in terms of μmoles of uridine accumulated/l of cell water, in a typical experiment.

* Level of acronycine (μmoles/liter) present during 10 min incubations at 10° together with specified concentrations of [^{14}C]uridine.

† Drug and [^{14}C]uridine were present at 37° during a 10 min incubation; incorporation of label into RNA was measured as described in the text.

When acronycine was present during these incubations at 10°, we found the drug (at levels of 20 μM) to be a competitive inhibitor of uridine uptake by L5178Y cells (Table 1). Results shown in Table 1 represent intracellular nucleoside levels achieved after 10 min incubations, and indicate an apparent K_m of 25 μM for the uridine uptake process in L5178Y cells. The competitive inhibition of such uptake by acronycine had an apparent K_i = approx. 20 μM. Acronycine was a less effective inhibitor of uridine uptake in L1210 cells; uptake K_m = 35 μM, apparent K_i = 75–100 μM. In the L1210 cell line, drug-induced inhibition of uridine uptake showed kinetics of non-competitive inhibition; at a 20 μM acronycine level, inhibition was not reversed by increased uridine concentration. These results were not altered when radioactive thymidine replaced uridine. In another series of experiments, we found that a 10 μg/ml level of cycloheximide failed to affect uptake of uridine or thymidine, although protein synthesis was inhibited by 95 per cent in either cell line.

Effects of acronycine on nucleoside uptake at 37° can be inferred only indirectly, since incorporation of nucleoside into nucleotides and RNA rapidly occurs at this temperature. The data of Table 1 indicate that the degree of inhibition by acronycine was essentially the same whether uridine uptake at 10° or incorporation of uridine into RNA at 37° was being measured. In light of data indicating a lack of effect of acronycine on metabolism of intracellular uridine [3], we interpret the data of Table 1 to indicate that acronycine inhibits nucleoside transport equally well at 10° and at 37°.

Nucleoside exodus. L5178Y and L1210 cells were pre-loaded with radioactive uridine (extracellular nucleoside level = 25 μM) at 10°, then suspended in fresh medium at the same temperature. Nucleoside loss was rapid, with a half-time of 0.3 min as previously described [6, 7]. In the presence of acronycine, this exodus was markedly slowed (Table 2). In these experiments a 30 μM acronycine level was as effective as 100 μM persantin as an inhibitor of nucleoside exodus.

Amino acid uptake and protein synthesis. Uptake of the leucine analog cycloleucine [8] was unaffected by 300 μM acronycine in L5178Y or L1210 cells. The amino acid was concentrated 2-fold from an extracellular 0.1 mM level. But 100 μM acronycine inhibited the incorporation of [^{14}C]leucine into protein by 50 per cent in both cell lines. In another experiment, we found that 10 μg/ml cycloheximide inhibited protein synthesis by 95 per cent without affecting uptake of cycloleucine. These data indicate inhibition of protein synthesis, but not of amino acid transport, at high acronycine levels.

Table 2. Inhibition of uridine exodus by acronycine

Drug level μM	Time min	L1210 cpm/10^7 cells	L5178Y cpm/10^7 cells
None	0	3895	4980
	3	195	925
	10	25	25
10	3	2920	4915
	10	1745	4305
30	3	3890	5050
	10	3865	4735
Persantin 100 μM	10	3730	4895

Cells were incubated for 10 min at 10° in medium containing 25 μM of [^{14}C]uridine, then suspended in fresh medium for specified times in the presence of 0, 10, 30 μM acronycine, or 100 μM Persantin. Intracellular radioactivity was measured at specified intervals after the suspension in fresh medium.

Table 3. Effects of acronycine on the partitioning of cells in a two-phase polymer system

	L5178Y	L5178Y	L1210	L1210
Components:				
Dextran (w/v)	5%	5%	5%	5%
PEG (w/v)	4%	3.08%	4%	3.08%
NaCl (mM)	0	70	0	140
Sucrose (mM)	0	70	0	0
NaPhosphate (mM)	120	10	120	10
Temperature (mM)	0°	10°	0°	10°
Acronycine level (μM):	Partition coefficient			
0	45	30	24	39
10	41	26	21	32
30	33	21	16	29
100	25	15	14	26

The two-phase system contained specified levels of the components listed above. Partition coefficient represents the % of the total number of cells found in the upper phase after 60 min at the specified temperature. These numbers are reproducible to ± 10% for a given cell batch.

Whole-cell microelectrophoresis. The electrophoretic mobility of L5178Y cells across a 60 V potential gradient was −1.56 ± 0.14 in units of μm/V/cm/sec. The corresponding value L1210 cells was −2.01 ± 0.09 μm/V/cm/sec. These values were not significantly altered by incubation of cells for 10 min at 10° or at 37° in medium containing 100 μM acronycine.

Two-phase partition studies. These studies were carried out in systems described in Table 3. The precise composition of these mixtures was based on the requirement that at least 20 per cent of the cell population partition into the upper phase of control tubes. Systems permitting observation of the partition coefficient in solutions containing 10 mM and in 120 mM phosphate buffer were utilized. Under these conditions, we found that the partition coefficient of both L5178Y and L1210 cells was markedly reduced following treatment with acronycine. Under conditions employed in these experiments, cycloheximide (10 μg/ml) did not alter the partition coefficient of either cell line.

Studies involving fluorescent membrane probes: dansyl cadaverine. Binding of dansyl cadaverine [14] was substantially enhanced by prior treatment with acronycine of L5178Y cells (Table 4). Similar results were obtained employing the L1210 cell line. In contrast, exposure of cells to high acronycine levels caused a decrease in total fluorescence, perhaps suggesting a chaotropic effect of this agent at a high concentration.

Fluorescence studies involving ANS. Prior treatment of L5178Y cells with acronycine markedly decreased

Table 4. Effect of acronycine on the fluorogenic interaction between L5178Y cells and membrane probes

Acronycine level μM	Fluorescence units DCV*	ANS†
0	183	71
10	240	68
30	270	47
100	283	42
300	90	10

* Cells were incubated with acronycine at 37°, then washed and treated with dansyl cadaverine at 37° as described in the text. These results were reproducible with an accuracy of ± 5% in replicate experiments.

† Cells were treated with acronycine at 37°, then washed incubated with ANS at 10° as described in the text. Data shown here were reproducible to within ± 5% in replicate experiments.

the fluorescent interaction between ANS and L5178Y cells (Table 4). Similar results were also obtained with the L1210 cell line. Since components of the growth medium contributed to the blank, cells were suspended in a buffered-salts medium for this study.

Antitumor effect of acronycine in vivo. Although inhibition of growth of L5178Y cells *in vitro* by acronycine has been shown [2], the agent was found to be ineffective against this tumor *in vivo*.* The drug was also essentially ineffective *in vivo* against the L1210 murine leukemia.†

DISCUSSION

Under conditions described here, we found that the drug acronycine interacts with L5178Y and L1210 murine leukemia cells to produce several measurable effects.

1. Inhibition of nucleoside transport both inward and outward.
2. Decreased partition of cells in a two-phase aqueous polymer system.
3. A decrease in the fluorogenic interaction between cells and the membrane probe ANS, a dye known [20] to become strongly fluorescent in a hydrophobic environment.
4. An increased fluorogenic interaction between cells and the dye dansyl cadaverine.

We interpret these data to indicate that the drug acronycine produces an alteration in the cell surface of the murine leukemia cell and that this alteration results in impaired transport of one class of biological material: the nucleosides.

Dunn [3] had provided data strongly suggesting inhibition of nucleoside transport by acronycine; the drug impaired incorporation of extracellular uridine into RNA but did not affect nucleotide formation or incorporation of intracellular nucleotides into RNA. We have confirmed this suggestion, using methods described previously [6, 7].

At levels which strongly inhibited nucleoside transport, acronycine had no effect on uptake of a neutral amino acid, cycloleucine. At higher drug levels, inhibition of incorporation of leucine into protein was found, however. A more substantial inhibitor of protein synthesis, cycloheximide, did not inhibit nucleoside transport, nor did it alter any cell-surface property measured here. The interference with protein synthesis produced by acronycine might, however, be ultimately responsible for the drug-induced inhibition of cell growth in culture [2].

In order to more fully characterize the acronycine-cell interaction, we have employed several biophysical methods previously used to characterize cell-surface properties.

The distribution of a cell population between two layers of a phosphate-containing dextran-PEG mixture provides a sensitive measurement of the cell-surface electronegativity to a depth of 60A [12, 13, 15–19]. In systems containing substantial (120 mM) levels of phosphate ion, the unequal distribution of phosphate between the phases results in a preferential partitioning of electronegative cells into the top layer; the partition coefficient of different cell preparations is thus lowered if the electronegativity of the cell is decreased. In this study, we found that the partition coefficient was decreased by acronycine treatment, even in the presence of only 10 mM phosphate. Under these conditions, the relative affinity of the cell-surface for the two different polymer-rich layers becomes the major determinant of partitioning behavior [12, 19]. We found no evidence for any alteration of electrophoretic mobility of whole cells upon treatment with acronycine. Walter [19] concluded that the composition of cell-surface fatty acids was one determinant of partition ratio of cells in a system containing low levels of phosphate. Other such determinants are suggested by our studies involving the fluorescent membrane probes.

The fluorescent interaction between ANS and the cell-surface has been characterized as a result of binding of the dye to hydrophobic surface regions [20–24], although the process may be more complex. In the present study, we found that prior treatment with acronycine markedly reduced the fluorescent interaction between murine leukemia cells and ANS. This finding suggests that acronycine-treated cells might have a less 'hydrophobic' cell surface than untreated cells, and that the reduced partition ratio of treated cells reflects the alteration.

The L5178Y and L1210 cell lines appear to be relatively insensitive to antitumor effects of acronycine *in vivo*, although the former exhibited sensitivity to the drug *in vitro* [2, 3]. In these cell lines, we could readily delineate several effects of acronycine on partitioning behavior, and impaired transport of nucleosides. The biophysical techniques examined here appear to provide sensitive means for assessing drug effects at the cell surface [25].

At the present state of our knowledge on correlations between cell-surface phenomenon and drug action *in vivo*, we can only speculate on the consequences of the effects being measured here. The rapidity with which acronycine causes cell-surface alter-

* Data provided by Mr. Wodinsky, Arthur D. Little Corp., Cambridge, MA. Drug was administered i.p. (140 mg/kg) for 9 successive days to animals which had been inoculated i.p. with 10^6 tumor cells on day 1 without significantly prolonging survival.

† Data provided by Division of Cancer Treatment, NCI. A drug dose of 50 mg/kg was employed; conditions were otherwise as specified in footnote * (above).

ations suggests that these are primary effects, rather than secondary results of drug-induced inhibition of macromolecular biosynthesis.

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