

Cellular Accumulation and Disposition of Aclacinomycin A

Merrill J. Egorin,¹ Ronald E. Clawson, Louis A. Ross, Norman M. Schlossberger, and Nicholas R. Bachur

Laboratory of Clinical Biochemistry, Baltimore Cancer Research Program, Division of Cancer Treatment, National Cancer Institute, NIH, Baltimore, Maryland 21201

ABSTRACT

The cellular accumulation and disposition of the anthracycline antitumor antibiotic aclacinomycin A (ACM) were compared to those of daunorubicin. Although both drugs were avidly accumulated by cells, intracellular concentrations of ACM were two to three times those of daunorubicin. Whereas lowered temperature (0°) reduced intracellular accumulation of both drugs, 10 mM sodium azide had no effect on accumulation of either ACM or daunorubicin. Both drugs exited from cells placed in drug-free medium, a process that was reduced at 0° but not altered by 10 mM sodium azide. Unlike whole cells, isolated nuclei accumulated more daunorubicin than ACM. This process was not altered at 0°. Both drugs were lost from nuclei placed in drug-free buffer, a process that was reduced at 0°. Unlike daunorubicin, which localized in cell nuclei, ACM localized in the cytoplasm with no detectable nuclear fluorescence. Although both drugs produced dose-dependent inhibitions of [³H]thymidine and [³H]uridine incorporation by L1210 and P388 cells, ACM inhibited both processes at lower concentrations than did daunorubicin. While daunorubicin inhibited [³H]thymidine incorporation more effectively than [³H]uridine incorporation, the reverse was observed with ACM.

INTRODUCTION

ACM² is an anthracycline antibiotic isolated from *Streptomyces galileus* (16). Previous studies have shown that ACM possesses antitumor activity against a number of animal (11, 15) and human (10) tumors, but differs from the clinically used anthracyclines such as Adriamycin in its effects on tumor cell nucleic acid synthesis. ACM has been proposed as a member of a class of anthracyclines that inhibit RNA synthesis preferentially to DNA synthesis (5, 15). Whereas the whole-animal pharmacology of ACM and the effects of ACM on tumor cell macromolecular biosynthesis have been reported, the cellular pharmacodynamics of this compound have not been reported. This paper reports our studies comparing ACM and daunorubicin with regard to various aspects of cellular drug accumulation and disposition.

MATERIALS AND METHODS

Cell Lines. L1210 murine leukemia cells were maintained *in vitro* by serial culture in Roswell Park Memorial Institute Tissue Culture Medium 1630 (NIH Media Unit, Bethesda, Md.) containing 50 units penicillin per ml, 50 µg streptomycin per ml, and 2 µmol L-glutamine per ml (Flow Laboratories, Rockville,

Md.) and 15% FBS (Flow Laboratories; Medium A). Under these conditions, cells had a population doubling time of 14 to 18 hr and achieved a maximum cell density of 1.5 to 2.0 × 10⁶ cells/ml.

Two strains of P388 murine leukemia, one sensitive and one resistant to daunorubicin and Adriamycin were obtained from Dr. Randall Johnson, National Cancer Institute, Bethesda, Md., and maintained *in vitro* by serial culture in a 1:1 mixture of Roswell Park Memorial Institute Tissue Culture Medium 1630 and Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 0.01 mM mercaptoethanol, 15% FBS and penicillin, streptomycin, and L-glutamine as above (Medium B). Under these conditions both the P388/S and P388/ADR cells had population doubling times of 12 to 14 hr and achieved maximum cell densities of 2.0 to 2.6 × 10⁶ cells/ml. Except where noted, all experiments using L1210 cells were performed in Medium A and all experiments using P388 cells were performed in Medium B.

Mouse fibroblasts, obtained from the American Type Culture Collection, Rockville, Md., were maintained *in vitro* by serial culture as previously described (14).

Isolation of Human Neutrophils. Peripheral venous blood was collected on the day of the experiment in a syringe containing heparin (10 units/ml) and centrifuged at 100 × g for 5 min. The platelet-rich plasma supernatant was discarded, and the remaining cells were sedimented by centrifugation at 3400 × g for 5 min. The cells were resuspended in an equal volume of hydroxyethyl starch solution [0.154 M NaCl (6 g/100 ml); Volex, McGaw Laboratories, Milledgeville, Ga.] and allowed to sediment at 1 × g for 60 min. The resulting supernatant was collected and mixed with 2 volumes of 0.15 M NH₄Cl at 4° to lyse un sedimented erythrocytes. Leukocytes, sedimented by centrifugation at 175 × g for 8 min at 4°, were resuspended in 36 ml 0.154 M NaCl, layered on a Ficoll (Sigma Chemical Co., St. Louis, Mo.)-Hypaque (Winthrop Laboratories, New York, N. Y.) gradient (2), and centrifuged at 400 × g for 40 min at room temperature. The final sediment, consisting of greater than 95% neutrophils, was resuspended in Roswell Park Memorial Institute Tissue Culture Medium 1640 and used in drug uptake studies.

Drugs. Daunorubicin hydrochloride (NSC 82151) and ACM (NSC 208734) were obtained from the Developmental Therapeutics Program, NCI, Bethesda, Md. Purity of this material was confirmed by thin-layer chromatography (4) and high performance liquid chromatography by a modification of the method of Eksborg *et al.* (8).

Fluorescence Spectra. Fluorescence excitation and emission spectra of solutions of drugs in 0.154 M NaCl and 0.3 N HCl in 50% ethanol were obtained with an absolute spectrofluorometer (Model SPF 1000CS; American Instrument Co., Silver Spring, Md.).

Incubation Conditions. Tumor cells were washed twice with 0.154 M NaCl, resuspended in fresh medium, and 10⁶ cells were preincubated for 60 min prior to addition of drugs. The

¹ To whom requests for reprints should be addressed, at National Cancer Institute, Baltimore Cancer Research Program, 655 West Baltimore Street, Baltimore, Md. 21201.

² The abbreviations used are: ACM, aclacinomycin A; FBS, fetal bovine serum; P388/S, anthracycline-sensitive P388 murine leukemia; P388/ADR, Adriamycin- and daunorubicin-resistant subline of P388 murine leukemia.

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final incubation volume was 1 ml and, unless indicated, all incubations were performed at 37°, 5% CO₂, and 95% humidity.

Assay of Drug Content. After incubation in drug containing medium, 10 ml of iced 0.154 M NaCl was added to the incubation mixture, and the cells were centrifuged at 2500 × g for 5 min at 4°. The resulting cell pellets were washed with another 10 ml of iced 0.154 M NaCl before being resuspended in 2 ml of 0.3 N HCl in 50% ethanol. Fluorescence was determined with excitation of 470 nm and emission of 585 nm for daunorubicin and excitation of 450 nm and emission of 585 nm for ACM. Drug content was calculated by comparison with drug-free control cultures and simultaneously performed daunorubicin and ACM standards (1).

Efflux Experiments. After incubation with 10 μM daunorubicin or ACM for 120 min, cells were washed twice with 10 ml of iced 0.154 M NaCl and resuspended in 10 ml of drug-free medium at either 0 or 37° and containing 0 or 10 mM sodium azide. After varying periods of incubation, cells were sedimented and assayed for drug content.

Drug Uptake and Efflux from Isolated Nuclei. Nuclei were isolated by homogenization of cells after swelling in hypotonic buffer (6). Studies of ACM and daunorubicin uptake and export by nuclei were performed as described above for whole cells except a buffer (50 mM Tris, 92 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, and 4 mM CaCl₂, pH 7.4) was used in place of medium and 0.154 M NaCl.

Fluorescence Microscopy. Cells incubated with daunorubicin or ACM for varying periods were washed once or twice with 0.154 M NaCl and were resuspended in a small volume of 0.154 M NaCl. Cell suspensions were examined immediately on an American Optical Model 10 microscope fitted with an HBO 50 mercury arc lamp and FITC interference filter for incident illumination and 500 and 515 nm secondary filters. Photomicrographs were made on Kodak Ektachrome film (ET 135, ASA 160).

Incorporation of Radioactive Macromolecular Precursors. After incubation for 120 min with ACM or daunorubicin, cells were pulsed with 0.1 ml of medium containing either 1 μCi [*methyl*-³H]thymidine (2.0 Ci/mmol, New England Nuclear, Boston, Mass.) or 2 μCi [*G*-³H]uridine (8 Ci/mmol, New England Nuclear). After incubation for 60 min with radiolabeled nucleosides, cells were assayed for incorporation of ³H into trichloroacetic acid-precipitable material as previously described (9).

RESULTS

Fluorescence Spectra. As previously described (1), daunorubicin has a fluorescence spectrum with an activation maximum at 470 nm and 2 emission maxima at 554 and 585 nm. ACM has a fluorescence spectrum with an activation maximum at 450 nm and 2 emission maxima at 546 and 585 nm.

Drug Uptake by Whole Cells. The uptake of both ACM and daunorubicin was dose dependent (Chart 1), but at any given drug concentration ACM accumulation was approximately twice that of daunorubicin. Although all cell types studied accumulated more ACM than daunorubicin, P388/ADR cells accumulated less of both ACM and daunorubicin than did P388/S cells (Chart 1). Both daunorubicin and ACM uptake were temperature dependent; cellular accumulation at 0° being much less than at 37°. Sodium azide (10 mM) had no effect on

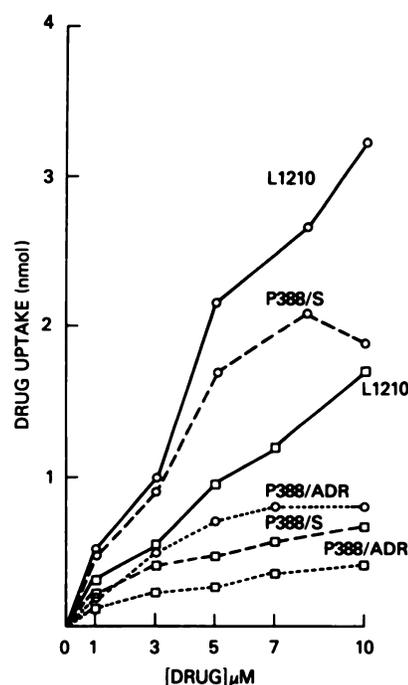


Chart 1. Accumulation of ACM and daunorubicin by tumor cells. L1210, P388/S, and P388/ADR cells were incubated at 37° for 120 min with various concentrations of ACM (○) or daunorubicin (□) and assayed for drug accumulation as described in "Materials and Methods." Chart depicts the means of a representative experiment done in duplicate.

the intracellular accumulation of either drug.

ACM accumulated intracellularly in L1210 cells more rapidly than daunorubicin, equilibrium being achieved by 60 to 90 min for ACM as compared to 120 to 180 min for daunorubicin (Chart 2). Although we have found FBS enhanced accumulation of some anthracyclines,³ the presence of 15% FBS in the incubation medium did not affect cellular accumulation of either daunorubicin or ACM (data not shown).

Both daunorubicin and ACM exited the cells by a process that was temperature sensitive (Chart 2). Sodium azide (10 mM) did not alter the efflux of either anthracycline from cells (data not shown).

Fluorescence microscopy allowed evaluation of the intracellular distribution of daunorubicin and ACM. Daunorubicin, as previously described (7, 17), was concentrated in cell nuclei with minor cytoplasmic localization (Fig. 1A). Unlike daunorubicin, ACM showed pronounced cytoplasmic accumulation with little if any drug fluorescence observed in cell nuclei (Fig. 1B). These distribution patterns were apparent after 5 min of incubation of cells with drugs, and the pattern did not change during incubation of up to 120 min. As little as 0.05 nmol of drug could be detected. These characteristic intracellular drug localization patterns were most easily defined in human neutrophils, but were also observed in L1210, P388/S, and P388/ADR leukemic cells, and L929 fibroblasts.

It was possible to saturate cellular uptake of daunorubicin. If L1210 cells were incubated for 2 hr with a saturating concentration of daunorubicin (50 nmol/ml), and 10 nmol ACM per ml was then added, there was no change in intracellular fluorescence. This could be explained by failure of ACM to enter the

³ M. J. Egorin, unpublished data.

cells or replacement of daunorubicin by ACM with no change in total intracellular fluorescence. Sedimented cells incubated with daunorubicin only were red, those incubated with ACM only were yellow, while those incubated with both drugs were orange. This implies the presence of both drugs within the cells. When the cells were observed with fluorescence microscopy, they demonstrated both bright nuclear and cytoplasmic fluorescence, again implying the intracellular presence of both daunorubicin and ACM.

Drug Uptake by Isolated Nuclei. Unlike intact L1210 cells, isolated L1210 cell nuclei accumulated more daunorubicin than ACM (Chart 3). Whereas the uptake of daunorubicin by

10^6 nuclei was somewhat greater than the uptake of this drug by an identical number of whole cells, 10^6 nuclei accumulated much less ACM than did the equal number of whole cells (Chart 3). Reduced temperature did not alter the nuclear uptake of daunorubicin or ACM. At equilibrium, nuclei incubated at 0° contained the same amount of daunorubicin or ACM as did nuclei incubated at 37° (Chart 3). Both ACM and daunorubicin were lost from drug-loaded nuclei placed in drug-free buffer, and this process was reduced by lowering the temperature to 0° (Chart 3).

Inhibition of Cellular Nucleic Acid Synthesis. Both daunorubicin and ACM produced dose-dependent inhibition of [3 H]-thymidine and [3 H]uridine incorporation by L1210 and P388

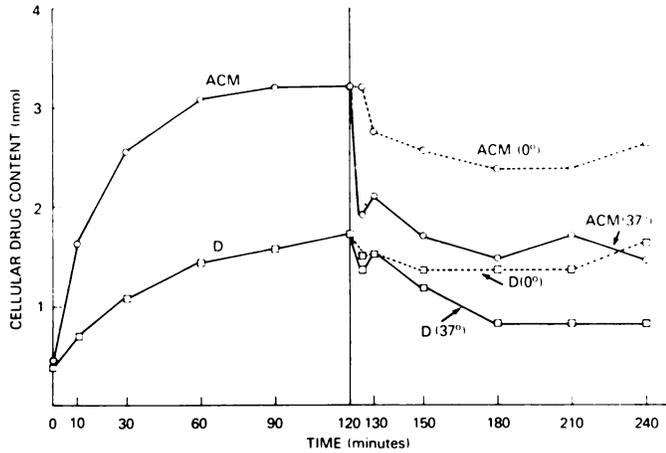


Chart 2. Time course of accumulation and effect of temperature on the efflux of daunorubicin (D) and aclacinomycin (ACM) from L1210 cells. Cells (10^6) were incubated at 37° with $10 \mu\text{M}$ daunorubicin (\square — \square) or ACM (\circ — \circ), and after various times were assayed for drug accumulation as described in "Materials and Methods." After 120 min, all remaining cultures were washed twice with 10 ml iced 0.154 M NaCl, resuspended in 10 ml Roswell Park Memorial Institute Tissue Culture Medium 1630 at 0° (\square — \square , \circ — \circ) or 37° (\square — \square , \circ — \circ), and assayed for remaining drug content after various times of incubation. Chart depicts the means of a representative duplicate experiment.

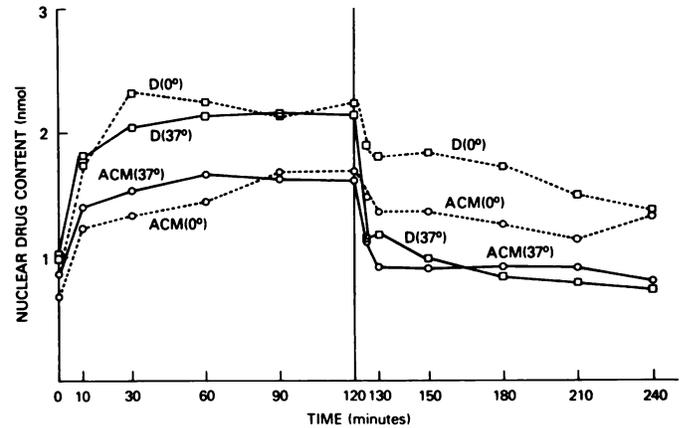
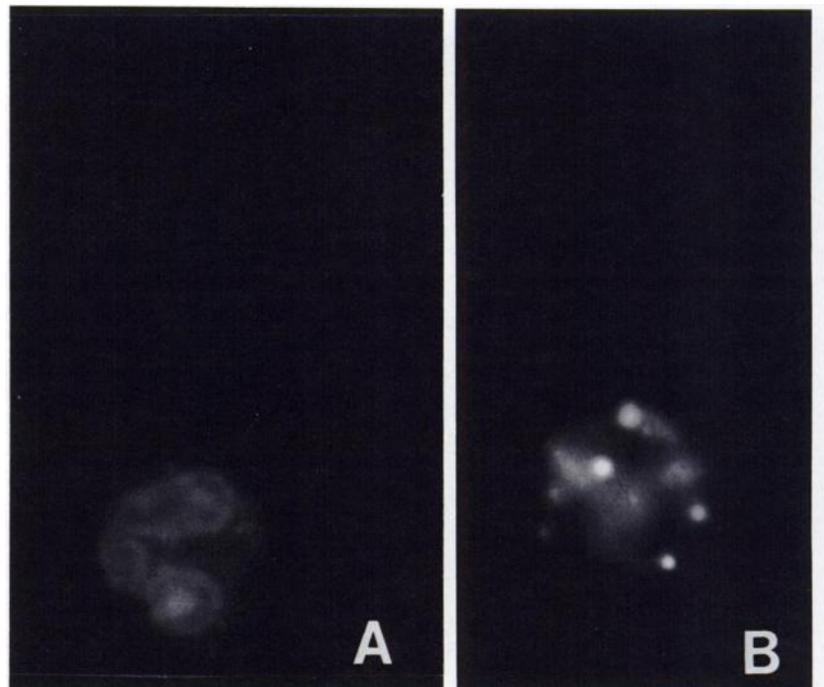


Chart 3. Effect of temperature on the accumulation and efflux of daunorubicin (D) and aclacinomycin (ACM) in L1210 cell nuclei. L1210 cell nuclei were isolated as described in "Materials and Methods." Nuclei (10^6) were incubated with $10 \mu\text{M}$ ACM (\circ) or daunorubicin (\square) at 0° (\circ — \circ , \square — \square) or 37° (\circ — \circ , \square — \square), and assayed after various times for drug content as described in "Materials and Methods." At 120 min, all remaining cultures were washed twice with 10 ml iced buffer, resuspended in 10 ml buffer at 0° or 37° , and assayed for remaining drug content after various times of incubation. Chart depicts the means of 2 to 5 duplicate experiments.

Fig. 1. Fluorescence microscopy of human neutrophils incubated with (A) daunorubicin or (B) ACM. Human neutrophils were isolated as described in "Materials and Methods" and incubated with $10 \mu\text{M}$ daunorubicin or ACM. After 60 min, cells were washed once with iced 0.154 M NaCl and examined under fluorescent microscopy as described in "Materials and Methods." $\times 500$.



cells (Chart 4). For both processes, ACM proved a more potent inhibitor than did daunorubicin (Chart 4; Table 1). This difference in antimetabolic effect could not be explained simply by the greater intracellular concentration of ACM, since at equivalent intracellular drug concentrations ACM was still a more effective inhibitor (Chart 5). P388/ADR cells were far more sensitive to ACM than to daunorubicin (Chart 4). On the other hand, at equal intracellular ACM concentrations, the inhibition of [³H]thymidine incorporation by P388/ADR cells was still less than the inhibition of P388/S cells. As previously reported (2, 4), ACM was a more effective inhibitor of [³H]uridine incorporation than [³H]thymidine incorporation while the reverse held true for daunorubicin (Table 1).

DISCUSSION

ACM represents a group of anthracycline antitumor antibiotics that share common structural and cytotoxic features (5,

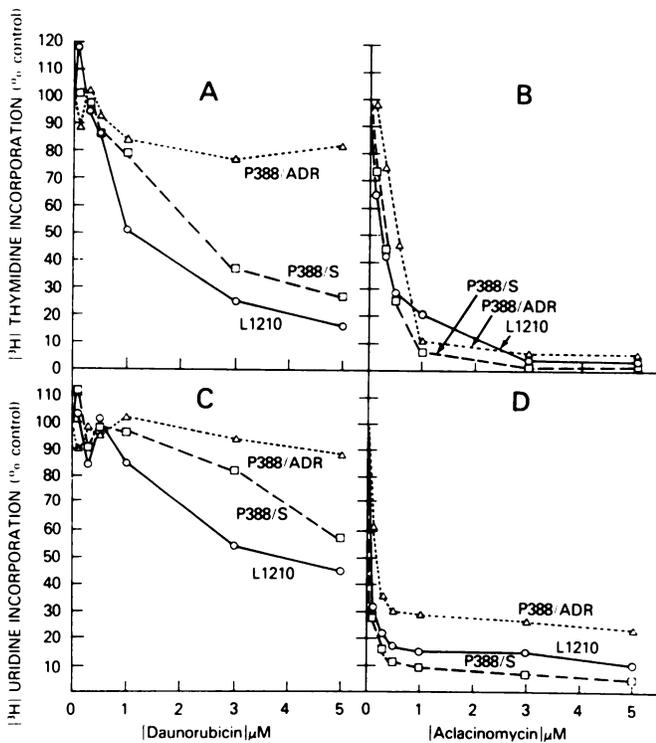


Chart 4. Effect of daunorubicin and ACM on [³H]thymidine and [³H]uridine incorporation by tumor cells. O, 10⁶ L1210; □, P388/S; or Δ, P388/ADR cells were incubated for 2 hr with varying concentrations of (A and C) daunorubicin or (B and D) ACM, and incorporation of [³H]thymidine (A and B) or [³H]uridine (C and D) into trichloroacetic acid precipitable material was then assessed as described in "Materials and Methods." Chart depicts the means of 2 experiments, each done in triplicate.

Table 1

Inhibitory concentration values of daunorubicin and ACM for [³H]thymidine and [³H]uridine incorporation by tumor cells

Cell type	50% inhibitory concentration of drug (μM)			
	[³ H]Thymidine		[³ H]Uridine	
	Daunorubicin	ACM	Daunorubicin	ACM
L1210	1.2 ± 0.2 ^a	0.32 ± 0.1	3.2 ± 0.7	0.1 ± 0.05
P388/S	2.3 ± 0.2	0.2 ± 0.06	3.5 ± 0.7	0.1 ± 0.05
P388/ADR	7.5 ± 0.4	0.35 ± 0.01	7.2 ± 0.9	0.2 ± 0.1

^a Mean ± S.E. of 3 to 11 experiments.

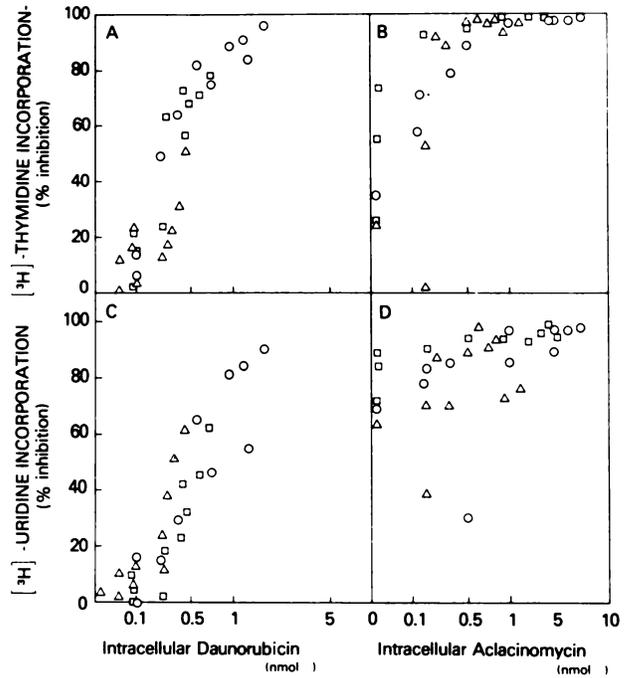


Chart 5. Effect of intracellular drug concentration on [³H]thymidine and [³H]uridine incorporation by tumor cells. O, 10⁶ L1210; □, P388/S; or Δ, P388/ADR cells were incubated with various concentrations of (A and C) daunorubicin or (B and D) ACM, and drug uptake and incorporation of [³H]thymidine (A and B) and [³H]uridine (C and D) into trichloroacetic acid precipitable material were determined as described in "Materials and Methods." Chart represents the summation of the means of 3 experiments, each done in triplicate.

15). Although previous reports have described the effects of these drugs on cellular macromolecular synthesis and tumor growth in experimental animals, the cellular accumulation and disposition of these compounds remained undefined. Our studies demonstrate a number of similarities and several significant differences in the cellular accumulation and localization of ACM and daunorubicin.

The cellular mechanism for uptake and efflux for both drugs may be similar since both of these processes were reduced at lowered temperature and not altered by sodium azide. Moreover, P388/ADR cells accumulated less of both drugs than did P388/S cells. For daunorubicin, this reduced intracellular drug concentration has been attributed to enhanced efflux from resistant cells (12, 18) and presumably ACM shares this property. On the other hand, all cell types studied accumulated ACM more rapidly and to a greater degree than daunorubicin. This implies some difference in handling of the 2 drugs, such as a more rapid influx of ACM or tighter intracellular binding of ACM. Our studies demonstrate no difference in the efflux of daunorubicin or ACM from cells and therefore argue against reduced export of ACM or tighter binding causing the difference in cellular accumulation of these 2 drugs.

There are major differences in the intracellular disposition of these 2 anthracyclines. Fluorescence microscopy showed ACM located primarily in the cytoplasm, whereas daunorubicin was found almost exclusively in the nucleus. This observation is reinforced by the fact that isolated nuclei accumulate more daunorubicin than ACM.

Our observations on the abilities of daunorubicin and ACM to inhibit [³H]thymidine and [³H]uridine incorporation by L1210 and anthracycline-sensitive P388/S cells agree with previous

reports (5, 15). The relative sensitivity to ACM of P388/ADR cells has not been previously described. This increased sensitivity to ACM is particularly interesting in view of the ability of these cells to accumulate less ACM than do P388/S cells. On the other hand, previous *in vivo* studies have shown P388/ADR to be resistant to ACM (13). Also, other studies with the P388/ADR tumor line have demonstrated *in vivo* resistance to emetine and the *N,N*-dimethyl analogs of daunorubicin and Adriamycin⁴ despite apparent *in vitro* sensitivity to these agents (3). The basis for these disparate results is at present unknown.

Another question raised by our studies is how a cytoplasmically located drug inhibits DNA synthesis and in fact does so at lower intracellular concentrations than does the nuclearly situated daunorubicin. It may be that the amounts of nuclear ACM required to cause reduced radionucleoside incorporation are too small to be detected with fluorescence microscopy. Alternatively, a nonfluorescent form of ACM may be responsible for its inhibition of nucleic acid synthesis. This could be a truly nonfluorescent metabolite or severely quenched ACM complexed with DNA or chromatin. On the other hand, alteration of cytoplasmic nucleic acid precursor metabolism may explain this phenomenon. We are currently attempting to clarify these issues.

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