Cancer Chemother Pharmacol (1982) 8:243–249

Cancer Chemotherapy and Pharmacology

Cellular Pharmacokinetics of Aclacinomycin A in Cultured L1210 Cells

Comparison with Daunorubicin and Doxorubicin

A. Zenebergh, R. Baurain, and A. Trouet

Université of Louvain and International Institute of Cellular and Molecular Pathology, Avenue Hippocrate, 75, B-1200 Bruxelles, Belgium

Summary. *Aclacinomycin, which is more lipophilic than daunorubicin and doxorubicin, is taken up and released more rapidly and extensively by L1210 cells. After 5 h of incubation 91% of aclacinomycin is found in the nuclei of L1210 cells and the drug present in the post-nuclear fraction is distributed between the lysosomes and the cytosol. After an incubation of 5 h aclacinomycin decreases the density of the lysosomes. This effect is not observed either with doxorubicin or daunorubicin or when the cells are incubated with aclacinomycin for only 30 min.*

Introduction

The anthracycline antitumor antibiotics can be divided into two categories on the basis of their molecular site of action; the first one includes drugs like daunorubicin (DNR), doxorubicin (DOX) and rubidazone, which inhibit the synthesis of DNA, RNA and nucleolar RNA, with a preferential effect on DNA polymerase α [15]. The second class includes drugs like aclacinomycin A (ACM), marcellomycin and musettamycin, which preferentially inhibit the synthesis of nucleolar RNA [7, 13, 15].

ACM is an interesting compound which, like DNR and DOX, is used in cancer chemotherapy [12, 14], and it is characterized by a decreased cardiac toxicity in golden hamsters [5] and mutagenicity [23].

Conflicting results have been published with regard to the subcellular localization of ACM. Recently Egorin et al. [8] reported the localization of ACM in the cytoplasm of human neutrophils with no detectable drug fluorescence in the nucleus, while Seeber et al. [21] found the drug concentrated mainly in the nucleus of Ehrlich ascites cells.

Reprint requests should be addressed to A. Zenebergh

In this paper we compare the cellular pharmacokinetics of ACM, DOX and DNR in cultured L1210 cells with the aid of cell fractionation techniques and drug determination by high-pressure liquid chromatography (HPLC) and fluorometry.

Materials and Methods

DNR and DOX were obtained, respectively, from Rhône-Poulenc, SA (Paris, France) and from Farmitalia-Benelux (Brussels, Belgium). ACM and aklavinone were kindly provided by Dr. De Jager from the Institut de Cancérologie et d'Immunogénétique (Paris, France). RPMI 1640 culture medium and fetal and newborn calf sera were obtained from Gibco Biocult (Paisley, UK).

Cell Cultures. L1210 ascitic cells obtained from leukemic DBA_2 mice were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum [26].

Accumulation and Release Experiments. L1210 cells were incubated in 5-ml Falcon tubes in the presence of anthracyclines at a concentration of 10 µg/ml in the culture medium (4 ml cell suspension; 0.4–0.6 mg cell protein). They were then washed twice with PBS at 4° C, centrifuged for 5 min at 1,500 rpm (IEC centrifuge, rotor 253), and resuspended in 1 ml distilled water.

For the release experiments the washed cells were reincubated in 4 ml drug-free fresh medium.

To allow us to study the effect of metabolic inhibitors on drug accumulation and release, iodoacetate (1 m*M*) and antimycin A (2 µ*M*) were added to the culture medium [17].

Cell Fractionation Experiments. After incubation, the cells were washed twice with 0.15 *M* NaCl and resuspended in 0.25 *M* sucrose, 3 m*M* imidazole at pH 7.0. The homogenization, the preparation of the post-nuclear supernatant (PNS), and the assays of marker enzymes, proteins, and nucleic acids were performed as described previously [26].

Drugs Analysis by HPLC. Anthracyclines were extracted from biological samples by a chloroform-methanol (4 is : 1, v/v) mixture, as described previously [2]. Aliquots were injected into a Hewlett-Packard liquid chromatograph fitted with a 250 × 4.6 mm column prepacked with silica particles (Lichrosorb Si-60-7 u, Merck, Darmstadt, FRG). Elution was performed with a mixture

0344-5704/82/0008/0243/$ 01.40

of chloroform-methanol-acetic acid and $MgCl_2$ 0.3 m*M* (145 : 45 : 8 : 7 by volume) at a flow rate of 1.2 ml/min. A Gilson Spectro-glo flow fluorometer (Gilson, Middleton, USA) was used as a detector, with excitation and emission filters of 480 and 560 nm for ACM [16] and 520 and 600 nm for DOX and DNR [2].

Determination of the Partition Coefficients. The octanol/PBS partition coefficients (K) of ACM, DNR, and DOX were determined by measuring the optical density at 435 nm (ACM) and 475 nm (DNR and DOX) of the drugs in solution at 50 µg/ml in PBS before and after extraction with an equal volume of octanol.

Determination of the pKa of ACM. The pKa of ACM was determined by titrating a solution of 20 mg ACM in 25 ml distilled water with NaOH 1 m*M* and measuring the pH after each addition of 0.5 ml of NaOH.

Determination of the Binding Parameters of the Drugs to DNA. To determine the binding parameters of ACM, DNR, and DOX to herring sperm DNA (Type VII, Sigma Chemicals Co., St Louis, MO, USA) we used a spectral titration method based on the progressive hypochromic and bathochromic shifts induced by DNA in the visible absorption spectra of the drug [19].

Incorporation of Radioactive Precursors. After incubation for various periods of time with the anthracyclines, cells were washed and reincubated in 1.0 ml medium containing either 1 µCi [methyl-^{3}H]thymidine 18–25 Ci/mmol or 1 µCi [5-^{3}H]uridine 25–30 Ci/mmol (New England Nuclear, UK) for 15 min. The cells were then washed twice with the culture medium supplemented with 10 m*M* cold nucleosides and precipitated with ice-cold TCA (5% final concentration). The precipitate was washed twice with TCA and resuspended in NaOH 1 *N*. The radioactivity was measured in a Packard model 2405 scintillator (Packard Instrument Co. Inc., Downers Grove, IL, USA).

Results

The partition coefficient between octanol and phosphate-buffered saline for ACM, DNR, and DOX are 21.8, 3.3, and 0.5, respectively, while pKa values of 7.3 and 8.2 were determined for ACM and DNR, respectively.

As indicated in Table 1, the affinity constants for the in vitro binding of the three anthracyclines to herring sperm DNA at 22° C and pH 7.4 are of the same order of magnitude.

Table 1. Apparent binding parameters of the anthracyline drugs to herring sperm DNA at 25° C in PBS buffer pH 7.4

Compound	DNA binding parameters	
	Ka. $10^{-6}M^{-1}$	nmax.
Aclacinomycin	0.23	0.12
Daunorubicin	0.31	0.16
Doxorubicin	0.54	0.15

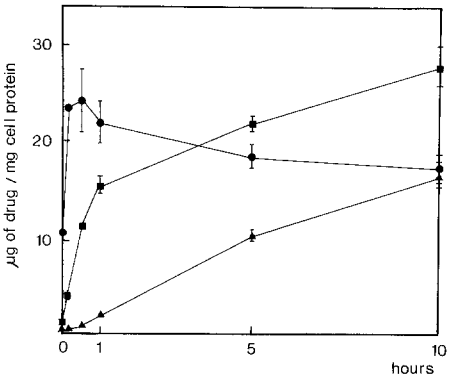

Fig. 1. Time course of accumulation of anthracyclines by L1210 cells. Cells were incubated for various times at 37° C in the presence of DNR (■), DOX (▲) or ACM (●) each at a concentration of 10 µg/ml. Drug accumulation was determined as described in *Materials and Methods*. Results are expressed as µg drug/mg cell protein. Mean ± SD of three experiments

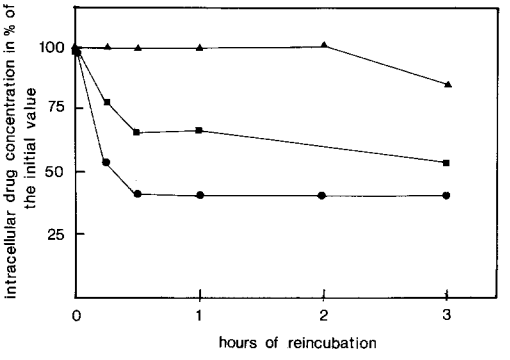

Fig. 2. Release of anthracyclines by L1210 cells. Cells were first incubated for 5 h in the presence of 10 µg/ml DNR (■), DOX (▲) or ACM (●), and then reincubated in a fresh medium for various times. The remaining intracellular drug content is expressed as the percentage of the cellular concentration before reincubation

The uptake of ACM, DNR and DOX by cultured L1210 cells incubated in the presence of 10 µg drug/ml is illustrated in Fig. 1.

The uptake of ACM occurs at a greater speed than that of DNR and DOX, the maximal drug concentration being reached after 30 min of incubation. In addition to the parent compound two fluorescent metabolites were separated by HPLC. The first one, which elutes like the aglycone of ACM, increases rapidly up to 1 h, when it represents about 15% of the total drug. The second metabolite, as yet unidentified, increases steadily during the incubation, representing 20% of the intracellular fluorescence after 10 h. The uptake of DNR is biphasic, with an initial rapid phase followed by a slower one, while the intracellular DOX levels increased steadily during the assay.

Figure 2 illustrates the release of drug by cells preincubated for 5 h with the anthracyclines at 10

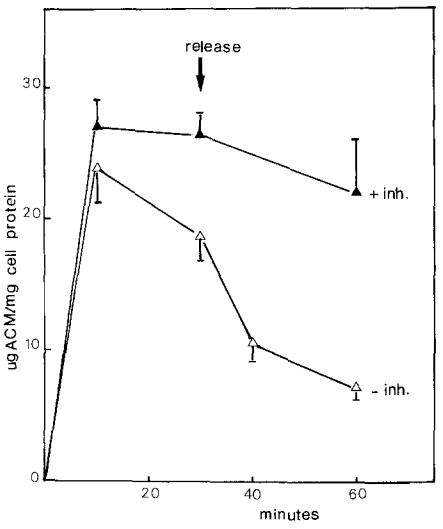

Fig. 3. Effect of metabolic inhibitors on the accumulation and release of ACM by L1210 cells. Cells were incubated in the presence of ACM at a concentration of 10 μg/ml in the presence (▲) or absence (△) of inhibitors (iodoacetate 1 m*M* + antimycin A 2 μ*M*). Results are expressed as μg drug/mg cell protein. Mean ± SD of three experiments

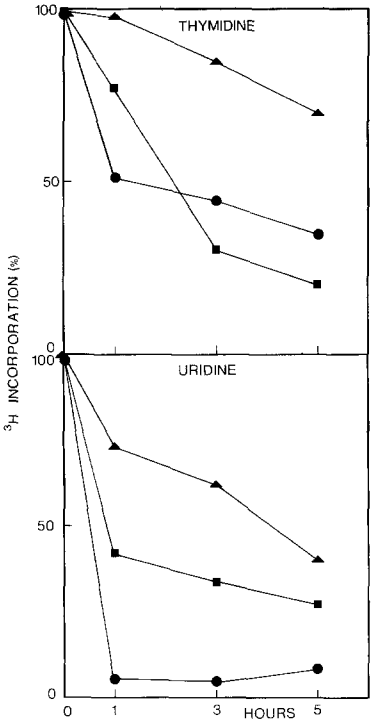

Fig. 4. Inhibition of ^{3}H-thymidine and ^{3}H-uridine incorporation by anthracyclines. L1210 cells were incubated in the presence of ACM (●), DOX (▲) or DNR (■). The results are expressed as percentages of the amount of ^{3}H-nucleotides incorporated in cells incubated without any drug

μg/ml and incubated subsequently in 4 ml drug-free medium. ACM is released to the highest extent, about 60% excaping from the cells in less than 30 min. Between 30% and 40% of DNR is released from the cells during the first 30 min, the intracellular levels remaining stable thereafter. DOX does not escape from L1210 cells during the first 2 h of reincubation. Similar results were obtained when cells were preincubated with the drugs for only 30 min.

The accumulation and release of ACM in the presence or absence of inhibitors is compared in Fig. 3. The inhibitors have no significant effect on the accumulation of ACM, but affect the release of the drug very significantly during incubation of the cells in a fresh medium.

The inhibition by the anthracyclines of the incorporation of radioactive nucleotides in L1210 cells is illustrated in Fig. 4. After a preincubation of 1 h with ACM, DNR, and DOX at 1 μg/ml, the incorporation of [^{3}H]thymidine is inhibited to 48%, 23%, and 2%, respectively. The inhibition of incorporation of [^{3}H]uridine is more rapid and extensive with ACM than with DNR or DOX.

Cells incubated with anthracyclines for 5 h were then fractionated. Despite an adequate homogenization of the cells incubated with ACM, up to 18% of phosphoglucomutase remains in the nuclear fraction, and in the case of cells incubated with DOX, 26% of DNA contaminates the PNS. Respectively, 91%, 71%, and 62% of ACM, DOX, and DNR was found in the nuclear fraction (Table 2). The PNS were fractionated by isopycnic centrifugation in a sucrose gradient. Figure 5 shows in the three columns A, B, and C the frequency distribution of the drugs and of the marker enzymes of lysosomes, plasma membrane, cytosol, and the DNA of cells incubated in the presence of ACM, DNR, and DOX, respectively.

Table 2. Percentage of drugs and marker enzymes found in the nuclear fraction of L1210 cells incubated with the anthracyclines at 10 μg/ml

Cells incubated in presence of	Percentage in nuclear fraction			
	30 min ACM	5 h ACM	5 h DNR	5 h DOX
Drug	91	91	62	71
DNA	99	98	96	74
RNA	38	35	21	26
Cathepsin B	30	25	14	22
N-Acetyl-β-glucosaminidase	35	27	22	28
Cytochrome C oxidase	26	14	12	15
Inosine diphosphatase	22	20	15	18
Phosphoglucomutase	27	18	10	18
Proteins	46	45	33	37

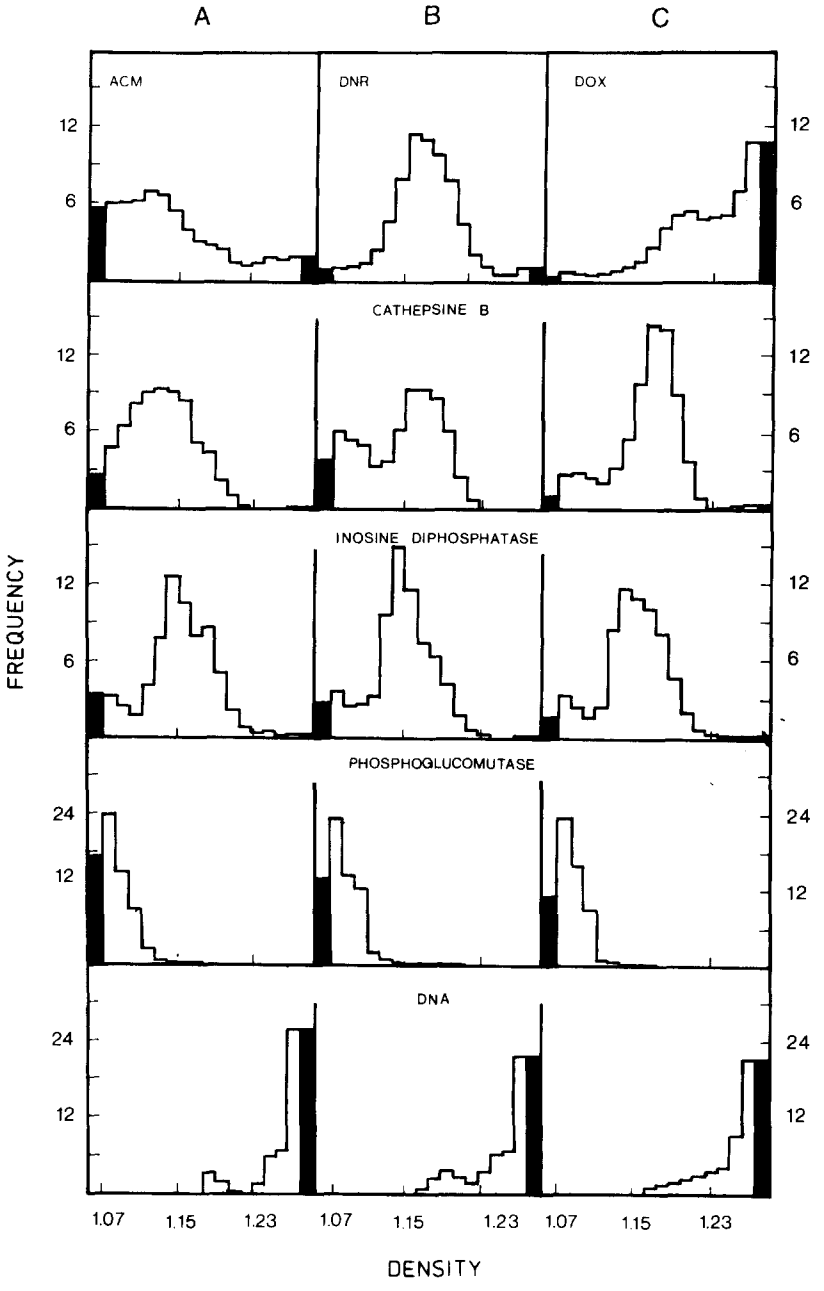

Fig. 5. Distribution patterns of anthracyclines and enzymes after isopycnic centrifugation in a sucrose gradient of the PNS from L1210 cells. The cells were incubated for 5 h with 10 μg anthracyclines/ml. Column A, cells incubated in presence of ACM; column B, cells incubated in presence of DNR; and column C, cells incubated in presence of DOX. Results are plotted in the form of normalized histograms [10]. On the *abscissa* is the density scale, divided into 15 equal sections of density increment $\Delta\varrho = 0.013$ over the span 1.070–1.270. The frequency, plotted on the *ordinate* is $\Delta Q/(\Sigma Q \cdot \Delta\varrho)$, where ΔQ is the amount of constituent present within the section and ΣQ the sum of the amounts found on all the subfractions. The surface area of each section of the diagram gives the fractional amount of constituent present within the section. *Solid blocks* on each side of the distribution profile represent material recovered below 1.07 and above 1.27; they are arbitrarily constructed over the density spans 1.05–1.07 and 1.27–1.30. The total area of each histogram is then equal to 1

Inosine diphosphatase was used as a marker enzyme for plasma membrane [26]. The distribution of DNR is similar to that of the major peak of cathepsin B, which equilibrates at a density of 1.17 g/cm^3, corresponding to intact lysosomes. The major part of DOX can be associated to the DNA contaminating the heavier fractions of the gradient. When L1210 cells are incubated with ACM, the distribution of cathepsin B is shifted towards lower densities.

Figure 6 shows that when L1210 cells were incubated for only 30 min with ACM the density of the lysosomal enzymes was not modified. ACM has a bimodal distribution, one mode corresponding to the lysosomal enzymes, with a peak density at 1.17 g/cm^3, while the other could correspond to the phosphoglucomutase, with a peak density of 1.09 g/cm^3.

In each fraction of the gradients, the percentage of drug that could be attributed to either cytosol (ACM) or DNA (DOX and DNR) and lysosomes has been calculated according to a previously published method [20]. Taking into account the results presented in Table 2, the following subcellular distributions have been calculated for the three anthracyclines and shown in Table 3. DNR and DOX are

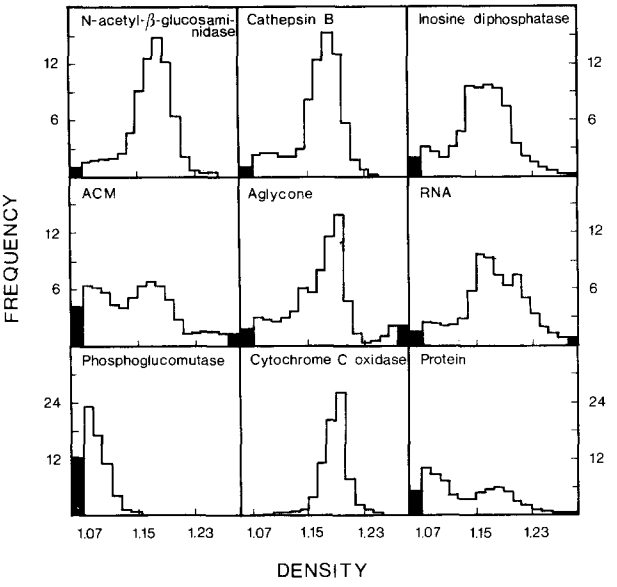

Fig. 6. Distribution patterns of ACM, its aglycone, enzymes, and constituents after isopycnic centrifugation in a sucrose gradient of the PNS from L1210 cells. The cells were incubated for 30 min with 10 μg/ml ACM. Results are presented as described in Fig. 5

Table 3. Subcellular distribution of ACM, DNR, and DOX accumulated by L1210 cells[a]

Drug	Percentage of drug associated to		
	Cytosol	Lysosomes	ADN
ACM[b]	2	6	91
DNR	0	36	64
DOX[b]	0	7	88

[a] L1210 cells were incubated for 5 h with the anthracyclines at 10 μg/ml culture medium

[b] Of the ACM in the extract, 1% could not be attributed to either the cytosol or lysosomes; and 5% of DOX could not be attributed to either the DNA or lysosomes

associated to DNA and lysosomes, while ACM is also present in small amounts in the cytosol. The aglycone of ACM, aklavinone, has a distribution profile similar to those of the lysosomal enzymes *N*-acetyl-β-glucosaminidase and cathepsin B.

Discussion

The retention of anthracyclines by various cell lines has been explained by a leak and pump mechanism in which the leak is the passive diffusion of the drugs into the cells and the pump is an active outward efflux [4, 9, 18, 22]. The speed of uptake of drugs by cells is therefore governed mainly by their lipophilic character, the more lipophilic drugs being taken up at a greater speed [1, 24]. Figures 1 and 2 show that the speeds at which ACM, DNR, and DOX are taken up and released by cultured L1210 cells are in parallel with their lipophilicity, expressed as their octanol/PBS partition coefficients.

The faster accumulation of ACM could explain the more rapid inhibition of RNA and DNA synthesis that has been observed (Fig. 3). We confirm in this study that RNA synthesis is more markedly affected than DNA synthesis by ACM [15, 25], while for DNR and DOX the two synthesis pathways are similarly inhibited.

The existence of an outward pump mechanism for ACM is supported by the results of uptake and release experiments in the presence of inhibitors of cellular energy production. For ACM the drug uptake is greater in presence of antimycin and iodoacetate and the efflux considerably slowed down, as shown in Fig. 3.

The subcellular localization of the anthracyclines will depend, on the other hand, on their affinity for the acidic lysosomal compartment, since they are weak bases, and on their binding affinity for DNA.

According to the lysosomotropic theory [6], ACM with a pKa of 7.3 should be trapped eight times more rapidly and at lower levels in the lysosomes than drugs like DNR and DOX, which have a pKa of 8.2 [3]. On the other hand, the in vitro affinity of ACM for DNA is slightly lower than that of DNR and DOX.

After incubation of L1210 cells for 5 h with ACM, 90% of the drug fluorescence is associated to the nuclei. Our data confirm those of Seeber et al. [21], who reported recently that 79% of ACM is found in the nuclei of Ehrlich ascitic cells. The results conflict, however, with those of Egorin et al. [8], who did not find detectable drug fluorescence in the nuclei of human neutrophils. But these latter data should be interpreted with reference to the fact that the fluorescence of ACM is extensively quenched in the presence of DNA [7, 11]. Dissociation of the anthracycline-DNA complexes and extraction of the free drugs are therefore prerequisites for the analysis of nuclear fractions.

The three anthracyclines studied display similar subcellular localizations, with some quantitative differences.

DNR enters the L1210 cells quite rapidly and is not very actively metabolized intracellularly [2]. After 5 h, the balance between the pKa and the DNA-binding constant Ka is such that 64% and 36% of the drug are found associated to the nuclei and to the lysosomes, respectively (Table 3). The cytosolic drug remains essentially as it is and is not extruded too rapidly. The drug present in the cytosol can

progress to fill the nuclei or/and the lysosomal compartments. Peterson and Trouet [17] have shown that in fibroblasts the nuclear compartment is filled more rapidly than the lysosomal compartment. DOX, being the least lipophilic drug, enters the L1210 cells more slowly than DNR and ACM. The drug is trapped preferentially in the nuclei, and this could result from its increased DNA affinity (greater Ka, same pKa as DNR). After 5 h the steady-state levels are not yet reached and the lysosomal reservoirs are only beginning to fill with DOX.

ACM is mainly associated to the nuclei, with a very small proportion of the drug being found in the cytosol (3%) and lysosomes (6%). This could partly be explained by the lower pKa of ACM and consequent lower affinity for the acid lysosomal compartment. Despite the low concentration of ACM in the lysosomes after 5 h of incubation, their equilibrium density is decreased after isopycnic centrifugation.

We have observed that ACM is metabolized partly by L1210 cells, to a compound that behaves like aklavinone and represents 15% of the intracellular fluorescence after 1 h. Another fluorescent metabolite increases steadily with time and represents 20% of the fluorescence after 10 h of incubation. The biological importance of this drug metabolism in the cellular pharmacology of ACM should be investigated.

It must be stressed that no appreciable anthracycline levels could be associated with either the mitochondria or the plasma membrane of cultured L1210 cells; this does not exclude, however, the possibility that very low amounts of drugs exert an effect at these sites.

It remains to be established whether the distribution properties of ACM are due only to its greater lipophilicity and its reduced waste in the lysosomal reservoir or its metabolism.

Acknowledgements. This work was supported by the Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite, Brussels (Belgium). We are indebted to Drs. G. De Jager and Dr. R. Hulhoven who provided us with aclacinomycin A and some of its metabolites, and to Mrs B. VandeVoorde-Aerts and Miss T. Aerts for their skillful technical assistance.

References

1. Bachur NR, Steele M, Meriwether WD, Hildebrand RC (1976) Cellular pharmacodynamics of several anthracycline antibiotics. J Med Chem 19: 651
2. Baurain R, Zenebergh A, Trouet A (1978) Cellular uptake and metabolism of daunorubicin as determined by high-pressure liquid chromatography: application to L1210 cells. J Chromatogr 157: 331
3. Calendi E, Di Marco A, Reggiani M, Scarpinato B, Valentini L (1965) On physico-chemical interactions between daunomycin and nucleic acids. Biochim Biophys Acta 103: 25
4. Danø K (1973) Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells. Biochim Biophys Acta 323: 466
5. Dantchev D, Tchouk S, Paintrand M, Hayat M, Bourut C, Mathé G (1979) Electron microscopic studies of the heart and light microscopic studies of the skin after treatment of golden hamsters with adriamycin, detorubicin, AD-32, and aclacinomycin. Cancer Treat Rep 63: 875
6. de Duve C, de Barsy T, Poole B, Trouet A, Tulkens P, Van Hoof F (1974) Lysosomotropic agents. Biochem Pharmacol 23: 2495
7. Du Vernay VH, Pachter JA, Crooke ST (1979) Deoxyribonucleic acid binding studies on several new anthracycline antitumor antibiotics. Sequence preference and structure-activity relationships of marcellomycin and its analogues as compared to adriamycin. Biochemistry 18: 4024
8. Egorin MJ, Clawson RE, Ross LA, Schlossberger NM, Bachur NR (1979) Cellular accumulation and disposition of aclacinomycin A. Cancer Res 39: 4396
9. Inaba M, Kobayashi H, Sakurai Y, Johnson RK (1979) Active efflux of daunorubicin and adriamycin in sensitive and resistant sublines of P388 leukemia. Cancer Res 39: 2200
10. Leighton F, Poole B, Beaufay H, Baudhuin P, Coffey JW, Fowler S, de Duve C (1968) The large-scale separation of peroxisomes, mitochondria and lysosomes from rat livers of rats injected with Triton WR-1339. J Cell Biol 37: 482
11. Masquelier M, Baurain R, Trouet A (1980) Amino acid and dipeptide derivatives of daunorubicin. 1. Synthesis, physicochemical properties, and lysosomal digestion. J Med Chem 23: 1166
12. Mathé G, Bayssas M, Gouveia J, Dantchev D, Ribaud P, Machover D, Misset JL, Schwarzenberg L, Jasmin C, Hayat M (1978) Preliminary results of a phase II trial of aclacinomycin in acute leukemia and lymphosarcoma. Cancer Chemother Parmacol 1: 259
13. Misumi M, Yamaki H, Akiyama T, Tanaka N (1979) Mechanism of action of aclacinomycin A. II. The interaction with DNA and with tubulin. J Antibiot (Tokyo) 32: 48
14. Ogawa M, Inagaki J, Horikushi N, Inoue K, Chinen T, Ueoka H, Nagura E (1979) Clinical study of aclacinomycin A. Cancer Treat Rep 63: 931
15. Oki T (1979) New anthracycline antibiotics. Jpn J Antibiot [Suppl] 30: 70
16. Peters JH, Murray JF (1979) Determination of adriamycin and aclacinomycin A in plasma by high-pressure liquid chromatography and spectrofluorometry. J Liquid Chromatogr 2: 45
17. Peterson C, Trouet A (1978) Transport and storage of daunorubicin and doxorubicin in cultured fibroblasts. Cancer Res 38: 4645
18. Peterson C, Baurain R, Trouet A (1980) The mechanism for cellular uptake, storage and release of daunorubicin. Studies on fibroblasts in culture. Biochem Pharmacol 29: 1687
19. Schneider Y-J, Baurain R, Zenebergh A, Trouet A (1979a) DNA-binding parameters of daunorubicin and doxorubicin in the conditions used for studying the interaction of anthracycline-DNA complexes with cells in vitro. Cancer Chemother Pharmacol 2: 7
20. Schneider Y-J, Tulkens P, de Duve C, Trouet A (1979b) Fate of plasma membrane during endocytosis. 1. J Cell Biol 82: 449
21. Seeber S, Loth H, Crooke ST (1980) Comparative nuclear and cellular incorporation of daunorubicin, doxorubicin, carminomycin, marcellomycin, aclacinomycin A and AD-32 in dau-

norubicin-sensitive and -resistant Ehrlich ascites in vitro. Cancer Res Clin Oncol 98: 109
22. Skovsgaard T (1980) Mechanism of resistance to daunorubicin in Ehrlich ascites tumor cells. Cancer Res 38: 1785
23. Umezawa K, Sawamura M, Matsushima T, Sugimura T (1978) Mutagenicity of aclacinomycin A and daunomycin derivatives. Cancer Res 38: 1782
24. Wheeler CS, Kessel D (1980) Influx of anthracyclines by murine leukemia cells as a function of drug lipophilicity and membrane fluidity. Pharmacologist 22: 291
25. Yamaki H, Suzuki H, Nishimura T, Tanaka N (1978) Mechanism of action of aclacinomycin A. I. The effect on macromolecular synthesis. J Antibiot (Tokyo) 31: 1149
26. Zenebergh A, Schneider Y-J, Loni MC, Trouet A (1981) Enzymatic characterization and analytical fractionation of L1210 cells. Biochimie 63: 411

Received Juli 27, 1981/Accepted January 25, 1982