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Uptake, Efflux, and Hydrolysis of Aclacinomycin A in Friend Leukemia Cells[1]

Alain Fourcade, Jean-Jacques Farhi, Myriam Bennoun, and Haim Tapiero[2]

Département de Pharmacologie Cellulaire et Moléculaire et de Pharmacocinétique, Unité Simone et Cino del Duca de Pharmacologie Humaine des Cancers, Institut de Cancérologie et d'Immunogénétique (INSERM U-50) 94804 Villejuif, France

ABSTRACT

The kinetics of uptake by cells and nuclear incorporation of aclacinomycin A was studied in Friend leukemia cells. It was shown that uptake is a very rapid process. The intracellular concentration is maximum in 10 min and mainly (about 75%) localized in the nucleus. Most of the incorporated drug will disappear from the cell by a two-step mechanism: (*a*) efflux from the nucleus to the cytoplasm; and (*b*) deglycosidation at C-7 to the alkavinone form in the cytoplasmic fraction. The cellular uptake was temperature dependent but was not prevented by sodium azide treatment. We assumed, therefore, that it is related to the composition and to the dynamic structure of the cell surface membrane. Nuclear outward transport and deglycosidation were inhibited by sodium azide and low temperatures; this suggests that they are regulated by an active transport process and by an enzymatic activity, respectively.

INTRODUCTION

ACM[3] is one of the new antitumor antibiotics isolated from *Streptomyces galileus* MA 144-MI (13). Like ADM and DNR, ACM belongs to the anthracycline group, and it shares the same general basic structure. It is composed of bifunctional molecules, with hydrophobic and hydrophilic regions (Chart 1). The hydrophobic region is made up of a resonating ring system, AKV, which introduces an intrinsic fluorescence property to the molecule. By comparison with ADM and DNR, the structure of ACM is characterized by an ethyl group at C-9 instead of the acetyl or the hydroxyacetyl group, the methoxycarbamyl group at C-10, and the hexopyranoses (rhodosamine, 2-deoxyfucose, and cinerulose) attached via glycosidic linkage at C-7.

ACM was classified by some authors as nonnucleophilic (1, 4). This classification will not be supported by the present study. Recently, we have shown that FLC variants resistant to ADM,ADM-resistant FLC and to DNR,DNR-resistant FLC were cross-resistant to ADM and to DNR but not to ACM. The uptake of ACM across the plasma membrane and the incorporation into the nuclei of ADM-resistant FLC and DNR-resistant FLC were the same as in sensitive cells (16). Moreover, the nuclear incorporation of ACM was also found in various human cell lines (data not shown). Since it was assumed that ACM like other anthracyclines intercalates in DNA and alters the DNA-dependent DNA polymerase and RNA polymerase activities (11), the cellular uptake, nuclear distribution, efflux, and qualitative stability of ACM will constitute decisive factors for the cytotoxic effect and consequently for the therapeutic effectiveness.

In the following studies, these different parameters have been analyzed in FLC.

MATERIALS AND METHODS

Cell Culture. FLC were derived from a clone of Friend virus-transformed 745A cells. Cells were grown in a modified Eagle's spinner medium lacking calcium and containing 10 mM sodium phosphate and nonessential amino acids (Gibco Bio-Cult, Ltd., Paisley, Scotland). The medium was supplemented with 10% fetal calf serum (Lot K 3862015; Gibco Bio-Cult) and antibiotics. All cell cultures were incubated at 37° in a CO_2 incubator. Cell densities were determined by repeated cell counts using a hemacytometer, and cell viability was measured by counting the cells excluding 0.1% trypan blue. To maintain continued exponential growth, cells were seeded at 0.1×10^6/ml and passaged every 2 to 3 days. Cultures were replaced every 3 months from frozen stocks, and tests for *Mycoplasma* were consistently negative.

Drugs. All chemicals were of analytical grade. ACM and related metabolites were kindly provided by Roger Bellon Laboratory (Paris, France).

Condition for Drug Exposure and Determination of Intracellular Drug Concentration. Unless otherwise mentioned, 5×10^6 cells were exposed to drug in their respective fresh growth medium. For each drug concentration tested, at least 2 separate cultures were used.

After incubation with drugs, cells grown in suspension were washed with Hanks' balanced salt solution (Institut Pasteur, Paris, France). ACM that was incorporated in total cells was extracted by resuspending the cell pellet in 1 ml Hanks' solution containing 0.1% Nonidet P-40. After addition of 6 ml ethyl acetate (Merck, Darmstadt, Germany) and shaking for 5 min at room temperature, the organic phase was evaporated, and the dried pellet was resuspended in the mobile phase, chloroform:methanol:acetic acid:water:triethylamine (68:20:10:2:0.01, v/v).

The distribution of ACM and its related compound was determined in the nuclear and cytoplasmic fractions by resuspending cells in ice-cold Hanks' balanced salt solution containing 0.1% Nonidet P-40. After 5 min incubation in ice, cells were centrifuged, and the resulting supernatant was separated from the nuclear pellet. Extraction of ACM from these 2 fractions was carried out as described above. High-pressure liquid chromatography analysis was carried out according to the method of Ogasawara *et al.* (10) in a Waters model (Waters Associates, Milford, Maine) liquid chromatography equipped with a WISP injector 710 B, dual Model 6000 A pumps. A Schoeffel FS 970 fluorometer and Schoeffel M 450 UV absorbance detector (Schoeffel Instrument Corporation, Kratos, Inc.) were connected to the system to allow the fluorescence and the absorbance to be monitored at 435 and 254 nm, respectively. The fluorescence emission over 500 nm was obtained with a cut-on filter. A Merck Lichrosorb SI 60 [40 mm (inner diameter) x 25 cm] was used in these analyses. Elution was performed with chloroform:methanol:acetic acid:water:triethylamine (68:20:10:2:0.01, v/v) at a flow rate of 1 ml/min and an ambient room temperature of 18°. Reagents were prepared daily and filtered through a 0.5-μm Millipore FH filter (Millipore Corporation, Bedford, Mass.). The chart speed of the recorded data was 1 cm/min.

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: ACM, aclacinomycin A; ADM, Adriamycin; DNR, daunorubicin; AKV, aklavinone; FLC, Friend leukemia cells.

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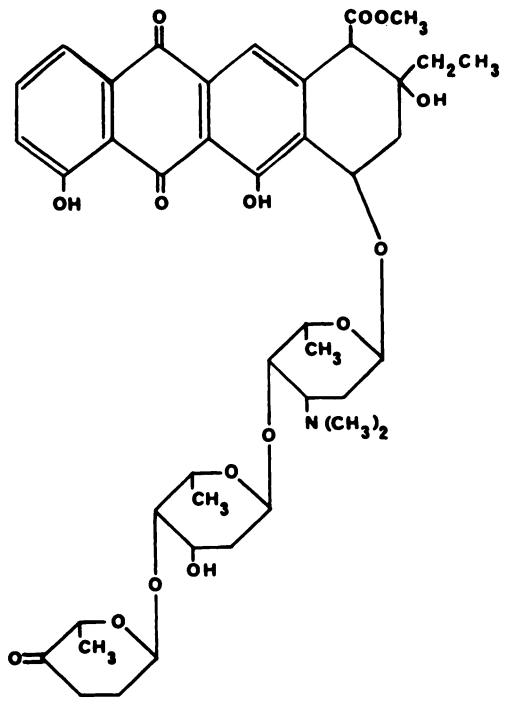

Chart 1. Formula of ACM.

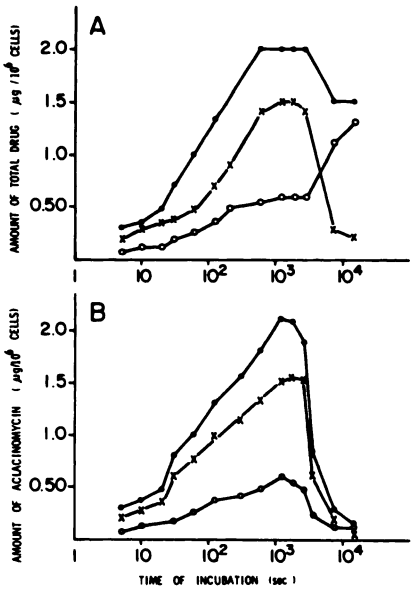

Chart 2. Time-dependent incorporation of ACM into FLC. Samples of 5 × 10^6 cells were resuspended in 1 ml medium supplemented with 10% fetal calf serum and containing 20 μg ACM. Cells were incubated at 37° for different time periods after which cells were washed twice with ice-cold Hanks' balanced salt solution. The incorporated drug extracted from the total cells (●), nuclear (×), and cytoplasmic fraction (○) was subjected to high-pressure liquid chromatographic analysis as described in "Materials and Methods." The amount of extracted ACM plus the related metabolites (*A*) and exclusively ACM (*B*) were determined.

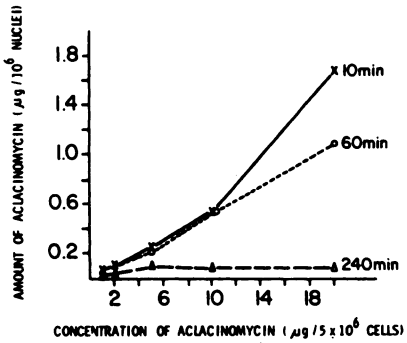

Chart 3. Relationship between cell exposure to various concentrations of ACM and the amount that remained in the nuclear fraction. Samples of 5 × 10^6 cells were resuspended in 1 ml medium supplemented with 10% fetal calf serum and containing various concentrations of ACM. Cells were incubated at 37° for 10, 60, and 240 min and washed twice with ice-cold Hanks' balanced salt solution. The incorporated ACM extracted from nuclei was subjected to high-pressure liquid chromatographic analysis as described in "Materials and Methods."

RESULTS

Kinetics of Uptake and Efflux of ACM in Sensitive FLC. The kinetics of uptake by sensitive FLC and its distribution observed in the cytoplasmic and the nuclear fractions were studied by incubating 5 × 10^6 cells in the presence of ACM (20 μg/ml) at 37°. Uptake is a rapid process; more than 20% of the maximum uptake was in 5 sec and was found mainly in the nuclei. Maximum incorporation was obtained in 10 to 30 min and was followed by a decrease observed in nuclear fraction only. The decrease was observed in both nuclear and cytoplasmic fractions when exclusively ACM was determined (Chart 2). Nuclear distribution of ACM was related, as expected, to the initial concentration in the incubation medium. However, after 4 hr incubation, the maximum amount that remained in the nuclear fraction (about 80 ng/10^6 nuclei) was the same whether the initial concentration was 5 or 20 μg ACM per ml (Chart 3). In order to correlate the amount that remained in the nuclear fraction with the cytotoxic effect, samples of 5 × 10^6 cells were exposed to 1, 2, 5, 10, and 20 μg ACM per ml. After 4 hr incubation at 37°, the cells were washed, seeded at 0.1 × 10^6 cells/ml in fresh medium, and incubated at 37° in a CO_2 incubation for 72 hr. Under these conditions, cell growth was inhibited only when the amount of ACM that remained in the nuclear fraction was at its maximum level (cells exposed to up to 5 μg ACM per ml).

Although the mechanism of cellular uptake is not known, it is assumed to be related to the dynamic structure of the cell membrane rather than to an active transport. This assumption was supported by the nonsignificant effect of the sodium azide in concentrations as high as 5 × 10^{-2} M. However, when cells were incubated for 5 min at different temperatures, maximum uptake was reached at 37°. At lower temperatures, uptake was reduced to almost null at 0° (Chart 4). Nuclear incorporation of ACM was not affected by low temperatures, and the percentage of influxed drug, which was localized nuclearly, was fixed. Furthermore, uptake of ACM by isolated nuclei was about the same at 4° and at 37°. Nuclear efflux, on the contrary, was not only temperature dependent (Chart 4), but it was also inhibited by sodium azide (Table 1). Cells incubated for 5 min at 37° in the presence of ACM (20 μg/ml) were washed, transferred in fresh medium containing different concentrations of sodium azide, and incubated again for 4 hr at 37°. The amount of ACM remaining in the nuclei was related to the sodium azide concentration. From these results, we conclude that nuclear incorporation is a passive process, whereas efflux is probably an active one.

Metabolism of ACM. When nuclear efflux occurred, ACM that had accumulated in the cytoplasmic fraction was degly-

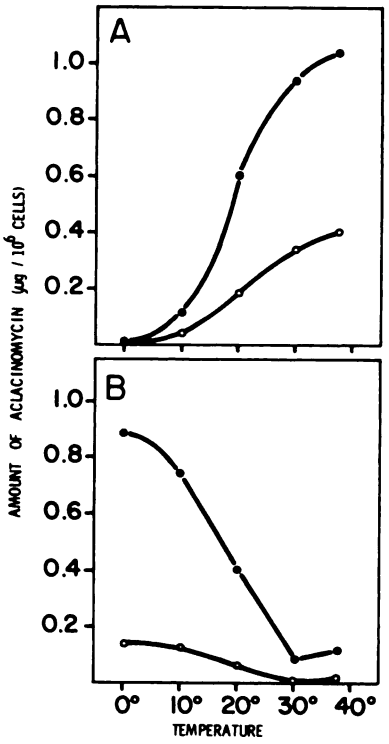

Chart 4. Temperature-dependent incorporation and efflux of ACM in FLC. Samples of 5 × 10^6 cells were resuspended in 1 ml medium supplemented with 10% fetal calf serum and containing 20 μg ACM. *A*, cells incubated for 5 min at different temperatures; *B*, cells, which were incubated 5 min at 37°, centrifuged, resuspended in 1 ml fresh medium without ACM, and incubated for 4 hr at different temperatures. The incorporated drug in the nuclei (●) and in the cytoplasm (○) was extracted and subjected to high-pressure liquid chromatographic analysis as described in "Materials and Methods."

Table 1

Inhibition of efflux and hydrolysis of ACM by sodium azide-treated FLC

An amount of 5 × 10^6 cells was incubated in 1 ml medium containing 20 μg ACM. The incubation was carried out for 5 min at 37°, and cells were then centrifuged, resuspended in 1 ml medium containing different concentrations of sodium azide, and incubated for 4 hr at 37°. Cells were then washed, nuclei and cytoplasm were separated, and drug was extracted and subjected to high-pressure liquid chromatography analysis as described in "Materials and Methods."

	Nuclear fraction			Cytoplasmic fraction		
Treatment	ACM (μg)	AKV (μg)	AKV (%)	ACM (μg)	AKV (μg)	AKV (%)
Control (5 min)	5.1	0	0	2.1	0	0
Untreated	0.36	0.18	33.3	0.15	2.7	94.7
Sodium azide 1 × 10^{-2}M	2.0	0	0	0.32	0.9	73.8
Sodium azide 2 × 10^{-2}M	2.2	0	0	0.57	0.45	44.1
Sodium azide 5 × 10^{-2}M	3.5	0	0	0.90	0.08	8.2

cosidated at C-7 to give the AKV metabolite identified by its earlier elution from the high-pressure liquid chromatography column. Cytoplasmic accumulation of this metabolite was related to the time when the cells were incubated (Chart 5). After 2 hr, more than 95% of the detected drug was in the AKV form. The deglycosidation, which is probably due to an enzymatic activity, was inhibited when cells were incubated in the presence of sodium azide. However, the amount accumulated was related to the sodium azide concentration (Table 1). ACM hydrolysis was related also to the temperature of incubation.

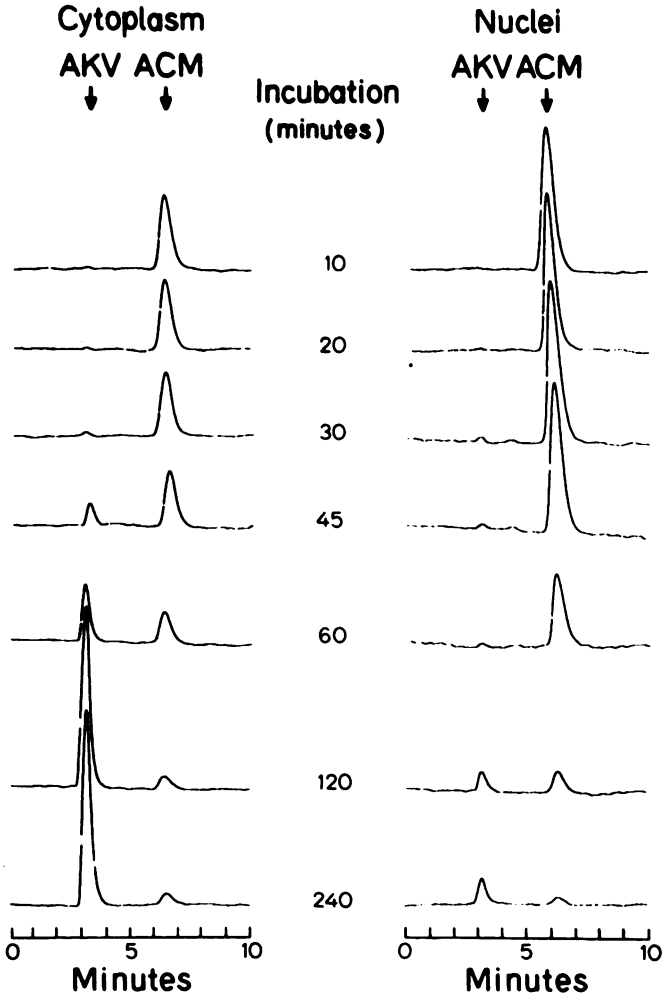

Chart 5. Time-dependent hydrolysis of ACM in FLC. Samples of 5 × 10^6 cells were resuspended in 1 ml medium supplemented with 10% fetal calf serum and containing 20 μg ACM. Cells were incubated at 37° for different time periods and washed twice with Hanks' balanced salt solution buffer. The incorporated drug was extracted from nuclei (*right*) and cytoplasm (*left*) and subjected to high-pressure liquid chromatographic analysis as described in "Materials and Methods."

Cells incubated for 5 min at 37° in the presence of ACM (20 μg/ml) were washed, transferred in fresh medium, and incubated for 4 hr at different temperatures. Under those conditions, maximum hydrolysis was observed when cells were incubated at 37°. At lower temperatures, hydrolysis was inhibited to be null below 10° (Chart 6). In the nuclear fraction, AKV was detected after only 2 hr incubation at 37°, time corresponding to the maximum accumulation in the cytoplasm. The amount accumulated in nuclei never exceeded 10% of the cellular AKV. This indicates that deglycosidation does not occur in the nuclei. It is assumed that AKV detected in the nuclear fraction is due to a nuclear membrane binding rather than to an incorporation through the nuclear membrane. This assumption was supported by incubating 5 × 10^6 cells at 37° in the presence of 5 μg AKV for 10 min to 2 hr. The results showed that AKV uptake was related to time of exposure. All the incorporated AKV was found only in the cytoplasmic fraction. We conclude, therefore, that the decrease in the amount of incorporated ACM is a consequence of efflux and hydrolysis,

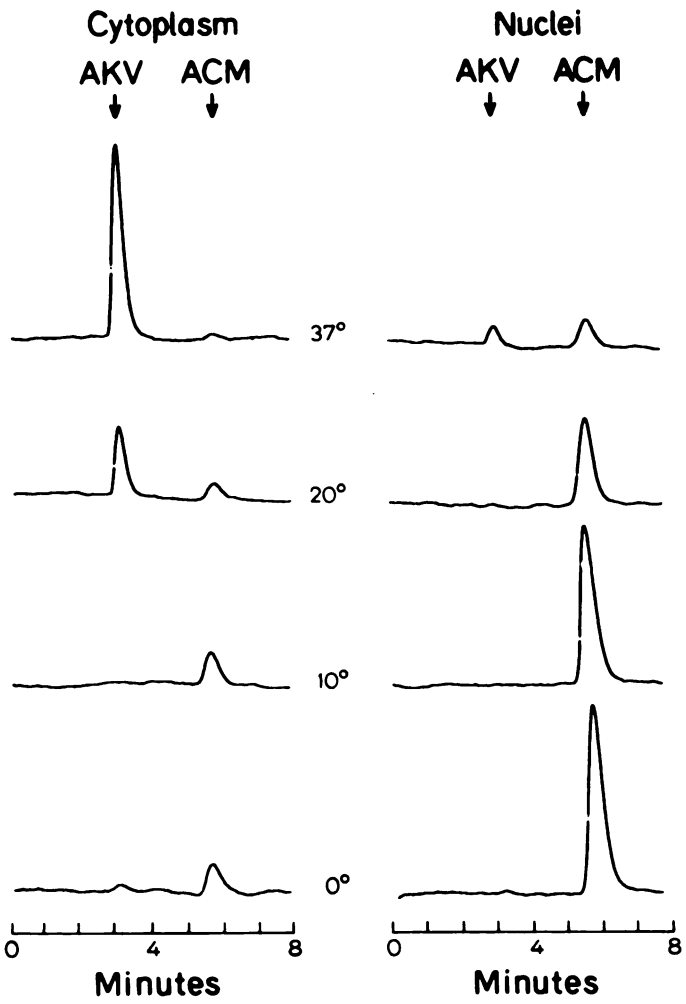

Chart 6. The effect of temperature on hydrolysis of ACM in FLC. Samples of 5×10^6 cells were resuspended in 1 ml medium supplemented with 10% fetal calf serum and containing 20 μg ACM. Cells incubated for 5 min at 37° were centrifuged, resuspended in fresh medium without ACM, and incubated for 4 hr at different temperatures. Drug extracted from nuclei (*right*) and cytoplasm (*left*) was subjected to high-pressure liquid chromatographic analysis as described in "Materials and Methods."

2 different mechanisms which occur in nuclear and cytoplasmic fractions, respectively.

DISCUSSION

ACM was selected for clinical trial on the basis of its wide spectrum of activity against experimental tumors (7, 11) and low cardiotoxicity in animal models (3, 7, 19). Furthermore, ACM was reported to be nonmutagenic as compared to DNR and ADM (18). The nature of the interaction of anthracyclins with a given cell population is still not known. The initial event seems to be a multistep process leading to the cytotoxic activity of the drug (2, 9, 14, 15, 20). From the physical and chemical points of view, ACM, like other anthracyclines, is a bifunctional molecule containing on one end a hydrophobic region and on the other end a hydrophilic region. In the present studies, we attempted to analyze the incorporation and the subcellular distribution of ACM in FLC. Our results have shown that incorporation of ACM is time dependent and temperature sensitive. It may represent a passive transport process across the cell membrane since it is not inhibited by sodium azide at 37°. The possibility that the composition and the dynamic structural organization of the cell surface might be associated with the rate of drug incorporation cannot be excluded. Changes in membrane dynamic and membrane composition were shown to occur during cell growth and cell differentiation (5, 6, 8, 17, 21). These changes differed according to the cell line studied. Since one possible approach for increasing the effectiveness of antitumor drugs is to increase their local concentration at the specific cell sites, studies on the affinity to various cells are currently under investigation in our laboratory. The intracellular drug concentrations of the anthracycline derivatives are determined not only by the uptake capacity and the affinity for the binding sites but also by the active efflux and the intracellular stability of the drug. The present results show that an active outward transport is responsible for the decreased ACM level in nuclei. This was evidenced by a blockade with sodium azide and by incubating cells at low temperatures. In these studies, cell fractionation, extraction, and analysis of ACM have shown that the drug is rapidly deglycosidated at C-7 in the cytoplasm and that, in 2 hr, more than 95% of the incorporated drug is in the AKV form. This intracellular hydrolysis was, as expected, temperature dependent and inhibited by NaN_3, which indicates that it is under enzymatic control. Since the NADPH: cytochrome *c* reductase has been reported to be involved in the reductive 7-deglycosidation of DNR and ADM, it was suggested that the same enzyme is also involved in the deglycosidation of ACM (12).

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