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FATE OF ACLACINOMYCIN-A AND ITS METABOLITES EFFECT ON CELL GROWTH AND MACROMOLECULAR SYNTHESIS

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Abstract—The relationship between the structure and activity of aclacinomycin-A (ACM) metabolites was investigated *in vitro* in Friend leukaemia cells (FLC). The cytotoxic effect was related to the ease with which ACM and its metabolites accumulate in the nucleus. Cellular uptake and nuclear incorporation are influenced by the hexopyranoses linked to aklavinone (AKV) and by the two methyls linked to the L-rhodosamine amino groups. The effect of ACM and its metabolites on macromolecular synthesis depended on the drug concentrations and the exposure time. ACM was the most active in the inhibition of nucleic acid synthesis whereas it had no direct effect on protein synthesis even at high drug concentrations. When cells were treated for a short time with low drug concentrations (1 μM), RNA synthesis was inhibited to a greater extent than DNA synthesis. But when incubated for longer periods, inhibition of DNA synthesis increased further. RNA and DNA syntheses were both inhibited to about the same extent only when cells were exposed to the higher drug concentrations (10 μM). We conclude therefore that at low drug concentrations the effect on DNA synthesis is probably a consequence of RNA synthesis inhibition. The early DNA synthesis inhibition which occurs at higher drug concentrations may result from the direct action on the cellular genome.

Aclacinomycin-A (ACM) is a cancer chemotherapeutic agent currently under clinical investigation [1–3]. It has an anthracyclin glycoside structure with a hydrophobic region, aklavinone (CS-2), that has an intrinsic fluorescence property, and a hydrophilic region containing hexopyranoses (L-rhodosamine, 2-deoxyfucose and L-cinerulose) attached to it via a glycosidic linkage at C-7. Among the isolated metabolites [4–6], the MA 144-S1 (CS-13) and the MA 144-T1 (CS-15) are hydrolysis products (Fig. 1),

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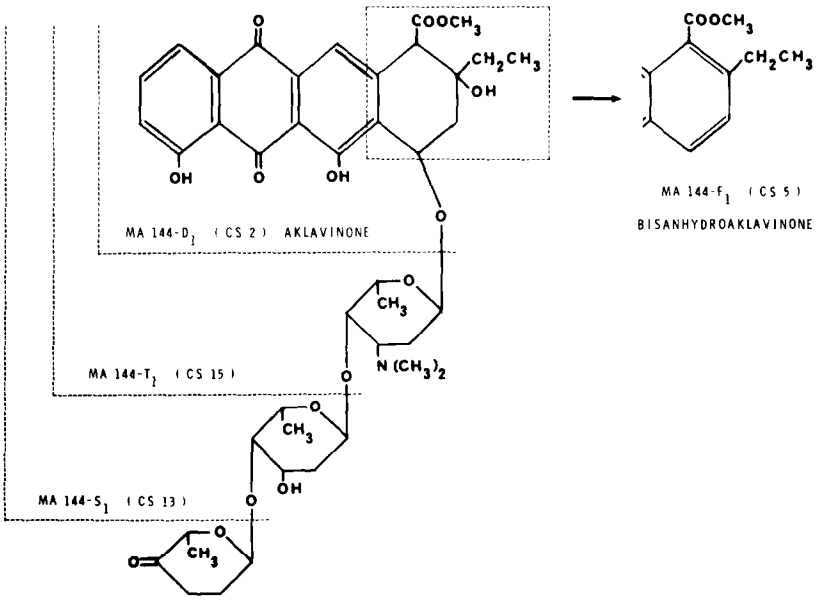

Fig. 1. Formula of aclacinomycin-A and the metabolites obtained by recurrent splitting of the hexopyranoses.

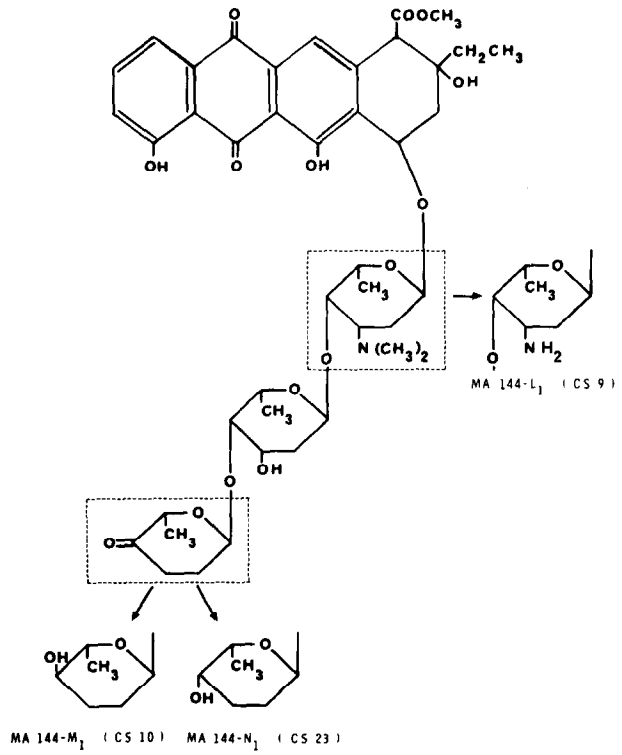

Fig. 2. Formula of aclacinomycin-A and the metabolites obtained by changes in sugar moiety.

whereas MA 144-L1 (CS-9), MA 144-M1 (M_1) and MA 144-N1 (N_1) correspond, respectively, to *N*-didemethyl aclacinomycin and to two isomers of hydroxyl ACM (Fig. 2). ACM, M_1, N_1, CS-13 and CS-15 are non-mutagenic in the salmonella test, whereas CS-9 is highly mutagenic by these criteria [7]. Some of these metabolites were also detected during quantitative determination of ACM in plasma samples of Mongrel dog [8], rabbits [9] and man (Tapiero *et al.*, unpublished data).

Moreover, in our previous study, we had shown that ACM is mainly incorporated into the nuclear fraction and hydrolyzed in the cytoplasmic fraction [10]. In the present report, we have analyzed the cytotoxic activity and the effect on macromolecular synthesis of these metabolites. A correlation between these activities, cellular uptake and nuclear distribution is discussed.

MATERIALS AND METHODS

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

Cell culture. Murine erythroleukaemia cells derived from a clone of Friend virus transformed cells 745 A were cultured as previously described [10].

Conditions of drug exposure and determination of intracellular drug concentrations. Unless otherwise mentioned, 5×10^6 cells were exposed to 10 nmole of ACM or each metabolite in 1 ml medium. After incubation at 37°, cells were washed twice with ice-cold Hank's balanced salt solution (Institut Pasteur, Paris, France) and resuspended in 0.2 ml Hank's balanced salt solution containing 0.1% NP40. Separation of the cytoplasmic and nuclear fraction was carried out as previously described [10]. The incorporated drugs were extracted by addition of 6 volumes of ethyl acetate (Merck, Darmstadt, F.R.G.) and agitation at room temperature. The organic phase was separated by centrifugation and evaporated to dryness. The residue was redissolved in 300 μl of the mobile phase and then injected into the HPLC column.

A Waters liquid chromatograph (Waters Associated Milford, MA) with a WISP injector 710B, dual model 6000 A pumps, model M 720 solvent programmer and data module M 730 was used with a Schoeffel FS 970 fluorometer (Schoeffel Instruments, Kratos Inc., U.S.A.). This system was equipped with a Merck lichrosorb SI 60 stainless-steel column [4 mm (inner diameter) × 25 cm]. The elution was performed with chloroform–methanol–acetic acid–water–triethylamine (60:20:10:2:0.01 v/v) at a flow rate of 1 ml/min and at ambient room temperature of 20°. Reagents were prepared daily and filtered through a 0.5 μm millipore FH filter (Millipore corporation, Bedford, MA). Fluorescence excitation was carried out at 435 nm and emission above 500 nm was selected with a cut-on filter.

Labelled thymidine, uridine and amino acid incorporation studies. Friend leukaemia cells (FLC) in log phase were harvested by centrifugation at 30 *g* for 10 min at room temperature in pre-warmed buckets. Cells were resuspended to a final concentration of

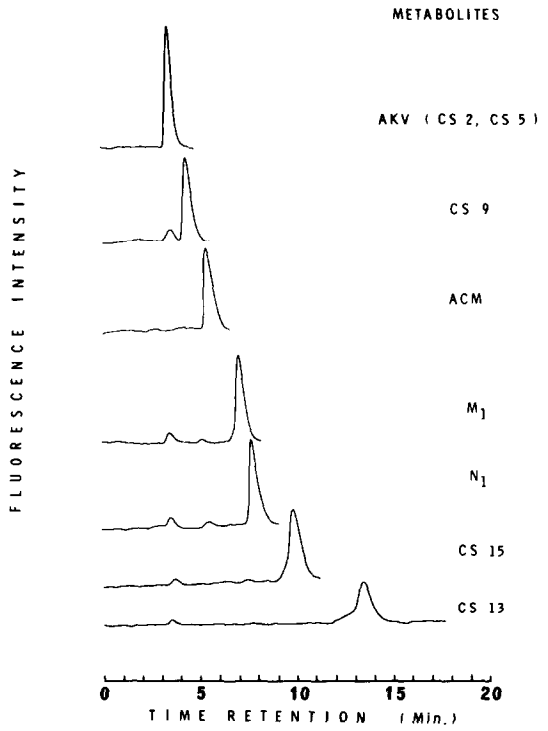

Fig. 3. Elution profiles of aclacinomycin metabolites. Aliquots of 500 pmole, dissolved in the mobile phase, were injected into the HPLC column. Fluorescence was monitored as described in Materials and Methods.

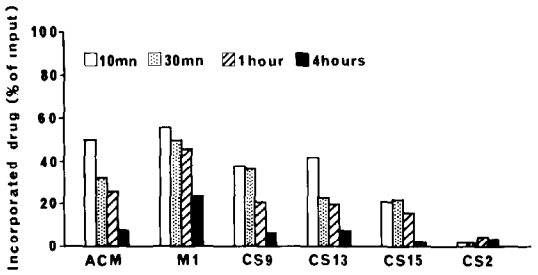

Fig. 4. Time-dependent incorporation of ACM and its metabolites into FLC. Samples of 5×10^6 cells were resuspended in 1 ml medium containing 10 nmole ACM or its metabolites and incubated at 37° at the indicated times. Cells were then washed twice and the amount of extracted drugs was determined by HPLC analysis as described in Materials and Methods.

5×10^6 cells/ml in a modified Eagle's spinner medium (GIBCO) supplemented with 10% fetal calf serum and 25 mM Hepes to adjust the pH to 7.5, or in Hank's balanced salt solution for amino acid incorporation. Samples were pre-incubated at 37° for about 20 min. One ml of cell suspension was added to individual tubes containing the appropriate amount of drug and incubated at 37° for different times. After this initial incubation, aliquots of 200 µl were diluted to 2.0 ml with pre-warmed medium containing either 2.5 µCi/ml of [methyl-^{3}H] thymidine (53 Ci/mmole; Amersham, U.K.) or 5 µCi/ml of ^{3}H uridine (40 Ci/mmole; Amersham, U.K.) or 0.5 µCi/ml of [U-^{14}C]protein hydrolysate (50 mCi/mg atom; Amersham, U.K.). The cell suspensions (0.5×10^6 cells/ml) were incubated for additional 5 min for [^{3}H]uridine and 10 min for [^{3}H]thymidine, or [^{14}C]protein hydrolysate. After the second incubation, 10 ml of chilled 0.15 M NaCl was added and the cells were centrifuged 2500 rpm/2 min. The cells were resuspended in 0.25 ml Hank's saline buffer containing 0.1% NP40, and 6 ml of 5% cold trichloroacetic acid (TCA) was added. After 5 min incubation in an ice bath, the acid-precipitable material was collected by filtration through Whatman glass fibre filters GF/B. The filters were rinsed with 20 ml 5% TCA and 5 ml ethanol, dried and the radioactivity was determined in a liquid scintillation spectrometer. Experiments were done in duplicate or triplicate with a control without antitumour drug.

RESULTS

Normal phase high performance liquid chromatography (HPLC) was used for the analysis of ACM and its metabolites. These compounds, which were characterized by their time of retention, were stable at 37° for more than 4 hr. Nevertheless, slight contamination (mainly with the inactive degradation product CS-2), not exceeding 10%, was observed in

Table 1. Subcellular distribution of aclacinomycin metabolites in Friend leukaemia cells

ACM derivatives	Nuclear fraction (N) (pmole/10^6 nuclei) (means* ± S.D.)	Cytoplasmic fraction (C) (pmole/10^6 cells) (mean ± S.D.)	N + C	$\frac{N}{C}$
ACM	740 ± 140	270 ± 50	1010	2.74
M_1	820 ± 140	300 ± 60	1120	2.73
N_1	780 ± 120	320 ± 30	1100	2.43
CS-9	400 ± 60	360 ± 70	760	1.11
CS-13	560 ± 60	280 ± 28	840	2.0
CS-15	240 ± 40	180 ± 7	420	1.33
CS-2	n.d.†	64 ± 9	64	—
CS-5	n.d.	100 ± 9	100	—

Exponentially growing FLC (5×10^6 cells) were incubated 10 min at 37° in 1 ml medium containing 10 nmole aclacinomycin or its metabolites. Cells were then washed, nuclei and cytoplasm separated, and the drug was extracted and subjected to HPLC analysis as described in Materials and Methods.

* Mean of three determinations.

† n.d. = Not detected.

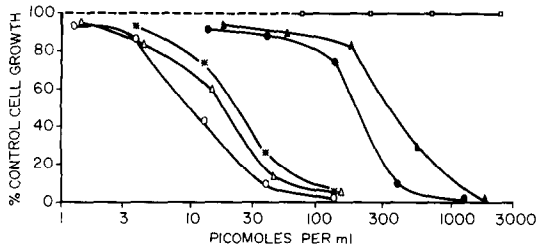

Fig. 5. The effect of aclacinomycin metabolites on Friend leukaemia cell growth. Logarithmically growing FLC, seeded at 0.1×10^6 cells per ml, were grown in medium supplemented with various concentrations of ACM (○—○), CS-13 (*—*), M_1 (△—△), CS-9 (●—●), CS-15 (▲—▲), and aklavinone (CS-2) (□—□). Cell counts of trypan blue excluding cells were performed after 72 hr in triplicate and compared to control culture. All counts were expressed as a percentage of control cell growth.

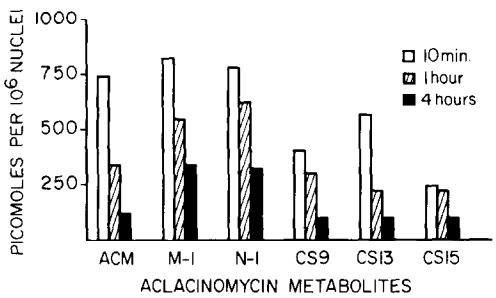

Fig. 6. Outflow of aclacinomycin metabolites from the nuclear fraction. Exponentially growing FLC were exposed to 10 nmole ACM or its metabolites (see Table 1) in 1 ml medium and incubated 1 or 4 hr at 37°. The metabolites extracted from the nuclear fraction were subjected to HPLC analysis as described in Materials and Methods.

some metabolites (Fig. 3). The kinetics of uptake and the subcellular distribution of each compound were studied at 37° by exposing 5×10^6 cells to 10 nmole/ml. The maximum uptake of ACM and its metabolites was generally reached in about 10–30 min. However, the amount incorporated varied according to the metabolite analyzed (Fig. 4). It was higher than 50% of the input with ACM, M_1 and N_1; about 40% with CS-13 and CS-9; 20% with CS-15; and less than 5% with CS-2 and CS-5. The subcellular distribution also varied and the ratio of the amount incorporated into the nuclei to that found in the cytoplasm was higher than 2 with ACM, M_1, N_1 and CS-13, but about 1 with CS-9 and CS-15. Aklavinone (CS-2) and bis-anhydroaklavinone (CS-5) were not detected in the nuclear fraction (Table 1).

The mechanism by which ACM and its derivatives act is still unclear. If their cytotoxic activity is related to their inhibitory effect on RNA and DNA synthesis, one might expect to find a higher cytotoxicity with those compounds which were preferentially distributed in the nuclear fraction. The data in Fig. 5 confirm this expectation. Compounds with high nuclear affinity, such as ACM, M_1, N_1 and CS-13, inhibited cell proliferation at a concentration 20–30 times lower than those with low nuclear affinity (CS-9 and CS-15).

Aklavinone (CS-2) and bis-anhydroaklavinone (CS-5), which were not incorporated into the nuclei, did not inhibit cell proliferation. After the maximum incorporation was reached, an outflow from nucleus and cytoplasmic degradation to AKV occurred, as already observed for ACM [10]. After 4 hr the amount that remained in the nuclear fraction was similar for compounds such as ACM, CS-13 and CS-9, which have a different inhibitory effect on cell growth (Fig. 6).

According to these results, and also because exposure of FLC to a cytotoxic drug concentration (10 nmole ACM or ACM metabolites per ml) does not inhibit protein synthesis, it is concluded that the cytotoxic effects of ACM or its active metabolites are the consequence of their nuclear localization.

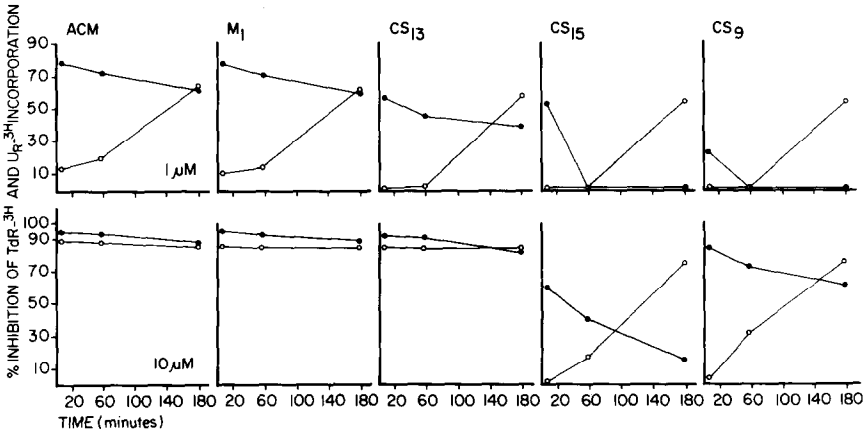

Fig. 7. Inhibitory effect of aclacinomycin and its metabolites on RNA and DNA syntheses. Friend leukaemia cells (5×10^6) were exposed to 1 or 10 nmole of drugs in 1 ml medium at 37°. After various time exposure, cells were labelled with [^{3}H]uridine (●—●) and [^{3}H]TdR (○—○) as described in Materials and Methods. Results are expressed as per cent of inhibition relative to untreated cells.

Table 2. Growth inhibition of Friend leukaemia cells exposed for 3 hr to aclacinomycin and its metabolites

Drug concentrations (nmole/ml)		Cell growth (% of control)
	Untreated	100
ACM	1	100
	10	7.4
M_1	1	100
	10	18.5
CS-13	1	92.6
	10	7.4
CS-15	1	90.8
	10	83.5
CS-9	1	98.2
	10	71.3

Exponentially growing FLC were exposed to ACM or its metabolites as indicated in Fig. 6. After 3 hr incubation at 37°, cells were washed with medium, diluted and seeded at 0.1×10^6 cells/ml. They were allowed to grow for 72 hr at 37° in a CO_2 incubator. Cells excluding trypan blue were counted. Untreated cells (2.7×10^6 cells/ml).

Induction of thymidine and uridine incorporation into FLC nucleic acids

The inhibition of incorporating appropriate precursors into RNA and DNA of intact FLC by ACM or by its metabolites varied with the drug concentrations and with the exposure time. When 5×10^6 cells were exposed to a non-lethal drug concentration (1 nmole/ml) for a short time (5 min), the incorporation of tritiated uridine was inhibited to a greater extent than that of tritiated thymidine. The rate of inhibition varied according to the metabolite studied. After a longer exposure time (180 min) the incorporation of tritiated thymidine was inhibited to about the same degree with ACM and with its metabolites, and to a greater extent than the inhibition of tritiated uridine incorporation into RNA.

When cells were exposed to 1 μM of ACM or its metabolites for 3 hr and further cultivated without drug, the DNA synthesis inhibition which was previously observed disappeared after 24 hr. When the cells were exposed to higher drug concentrations (10 μM), RNA and DNA syntheses were inhibited to the same extent even after a short time exposure (Fig. 7). But further cultivation without drug allowed DNA synthesis recovery after 48 hr only when the cells were previously exposed to CS-9 and CS-15. According to our results, we suggest that ACM and its metabolites are probably acting by the same mechanisms but to a smaller extent for CS-9 and CS-15 since they are less incorporated into the nuclei. Since the early effect of the low drug concentrations is on the RNA synthesis, the further inhibition of DNA synthesis could result from this effect. However, the cytotoxic effect which occurs at higher drug concentrations (Table 2) may rather be due to direct action on the cellular genome. Therefore cytotoxicity and nucleic acid synthesis inhibition may both be related to the rate of drug incorporation into the nuclei.

DISCUSSION

ACM was selected for clinical trial on the basis of its wide spectrum of activity against experimental tumours [11, 12] and its low cardiotoxicity in animal models [13, 14]. When administered to dogs [8], rabbits [9] or man as a rapid intravenous injection, metabolites such as the reduced carbamyl group of L-cinerulose (M_1 and N_1), the splitting terminal L-cinerulose (CS-13), the *N*-didemethyl aclacinomycin (CS-9) and aklavinone (CS-2) were recovered from plasma samples (Tapiero, unpublished data). The process that leads to the cytotoxic activity of ACM is still unknown. The present results show that cytotoxicity is related to the amount of drug incorporated in the nuclear fraction. Cytotoxic effects and nuclear incorporation are both influenced by changes in the sugar moiety configuration. Recurrent splitting of the ACM sugar chain, which successively produces the metabolites CS-13, CS-15 and CS-2, showed a progressive decrease in nuclear incorporation and, consequently, reduced cell growth inhibition. When no sugar was linked to the aklavinone (CS-2), nuclear incorporation and cytotoxicity were not observed. Reduced cytotoxicity and low nuclear incorporation were also observed with the *N*-didemethyl aclacinomycin (CS-9). This derivative was about 20 times less cytotoxic and its nuclear incorporation was reduced by 45% compared to ACM. Our results show that the cytotoxic effect of these metabolites is associated with cellular uptake and nuclear incorporation. Previous results with adriamycin, daunorubicin [15, 16] and ACM [10] have suggested that uptake occurs via a passive process.

In the present study, the possibility that the cell surface and the nuclear membrane components interact differently with ACM metabolites according to their chemical structure cannot be excluded. The present results also show that the efflux from the nuclei and the deglycosidation at C-7 which occur in the cytoplasm are responsible for the decreased level of incorporated metabolites.

Previous reports of the effect of ACM on nucleic acids have shown that ACM inhibits RNA synthesis and preferentially nucleolar RNA synthesis at drug concentrations lower than those required to inhibit DNA synthesis [17]. ACM is thought to interact directly with DNA because it stabilizes DNA against thermal denaturation and demonstrates different association constants for single- and double-stranded DNA [18]. Moreover, following treatment of cells with ACM, the cell-cycle progression was reported to be affected depending on the cell type and drug concentration. The lower drug concentration led to an early accumulation of G_2-phase cells which are able to divide eventually, whereas at higher drug concentration, cell transit through S-phase was prevented [19]. The rate of cell exit from G_1 and the proportion of cells progressing through S-phase were linked to the RNA content [20, 21]. In the present studies, we have shown that the incorporation of nucleoside precursors into nucleic acid depends on the dose, the ACM metabolite and the exposure time. Thus the low drug concentration, which was non-lethal, leads to early inhibition of RNA synthesis. The degree of inhibition varied with the metabolite studied and was correlated to the amount of cellular uptake. The rate of RNA synthesis inhibition was reduced after long exposure, whereas that

of DNA synthesis inhibition increased to the same extent for all metabolites. At higher drug concentrations, cytotoxicity was observed for cells exposed to ACM metabolites with high nuclear incorporation rate (ACM, M_1 and CS-13). In this case early inhibition of the RNA and DNA syntheses was observed. However, when cells were exposed to metabolites with lower nuclear incorporation (CS-15 and CS-9), and consequently with less cytotoxicity, DNA synthesis inhibition was then secondary to its action on RNA synthesis.

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