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Amphotericin B-induced Sensitivity to Actinomycin D in Drug-resistant HeLa Cells[1]

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SUMMARY

HeLa cells, which were selected for resistance to actinomycin D on the basis of decreased penetration of the antibiotic into the cells, were treated with nontoxic concentrations of the polyene antibiotic amphotericin B. In the presence of amphotericin B, the cells became sensitive to the effects of actinomycin D, as demonstrated by loss of cell viability, typical morphological changes, and effects on the pattern of RNA synthesis. Furthermore, we were able to demonstrate that amphotericin B increased the amount of [^{3}H]actinomycin D incorporated into HeLa cells as determined by both radioactive counts and radioautography.

Amphotericin B might be useful in overcoming the resistance of tumors to antitumor agents when the resistance is based on decreased entry of the agents into cells.

INTRODUCTION

The resistance of tumors and many tumor cells to the antibiotic actinomycin D is often the result of a decreased uptake of that drug into cells (5, 6). An approach to circumvent this type of resistance was suggested by the observations that AMB[3] increased membrane permeability, thereby potentiating the uptake of second agents and even macromolecules into eukaryotic cells (7-9). Thus, it seemed possible that after cells developed impermeability to the antibiotic, AMB might help to restore their sensitivity. Here we report a successful test of this hypothesis.

MATERIALS AND METHODS

Materials. The resistant HeLa cells have been propagated for over 1 year in growth medium consisting of 0.4 μg of actinomycin D per ml, 10% fetal calf serum, and Dulbecco's or α-minimum essential medium (3). The cells were cultured in medium free of actinomycin D for 1 week prior to use in the experiments.

AMB in the form of Fungizone was purchased from E. R. Squibb & Sons, Inc., Princeton, N. J. It was dissolved in sterile water before use. Actinomycin D, obtained from Merck Sharp and Dohme, West Point, Pa., was dissolved in cold distilled H_2O and sterilized in Millipore filtration. [^{3}H]Actinomycin D (specific activity, 7.0 Ci/mmole) was purchased from Amersham/Searle, Arlington Heights, Ill. [^{3}H]Uridine (specific activity, 8 Ci/mmole) was purchased from Schwarz/Mann, Orangeburg, N. J. ^{14}C-Labeled 18 S and 28 S RNA's were isolated from HeLa cells labeled for 18 hr with 0.5 μCi [^{3}H]uridine per ml by Dr. S. Gotoh, of the Department of Microbiology, Washington University School of Medicine, St. Louis, Mo.

Studies of Cell Viability. The HeLa cells were harvested from Falcon tissue culture flasks using 0.25% trypsin and glass beads. The cells were washed and resuspended in fresh medium, and 1×10^5 cells/ml (3 ml) were incubated on 35-mm plastic Petri dishes with the various drugs for 18 hr at 37°. The cells were then harvested and counted in a hemocytometer with 0.2% trypan blue. Five hundred cells were then plated in 3 ml of medium on 35-mm plastic Petri dishes and observed for 14 to 21 days. Macroscopic colonies were stained with 1% gentian violet and counted.

RNA Determinations. HeLa cells (1×10^5/ml) were incubated in Falcon flasks with AMB (30 or 50 μg/ml); actinomycin D (1 or 2 μg/ml); or AMB (30 μg/ml) plus actinomycin D (1 μg/ml) for 4 hr. After 3 hr of incubation, the cultures were pulsed for 1 hr with [^{3}H]uridine, 2 μg/ml. Following this, the cells were harvested and the total RNA was extracted by the phenol method as described by Penman (10).

Polyacrylamide Gel Electrophoresis of the Extracted RNA. Acrylamide gel electrophoresis of RNA was carried out in 2.7% acrylamide for 3 hr after a prerun of 1 hr. The gels were run in 10-cm glass columns at room temperature with a constant current at 5 ma/gel. Bromphenol blue was used as a marker for the front, and ^{14}C-labeled 18 S and 28 S markers were run with each sample of RNA. Following this the gels were frozen in dry ice; 1.5-mm slices were incubated in scintillation vials with 0.3 ml concentrated NH_4OH for 5 hr at room temperature. Five ml Bray's solution were added, and samples were counted in a liquid scintillation counter.

Microscropic Studies. HeLa cells were dissociated with

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[3] The abbreviation used is: AMB, amphotericin B.

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0.25% trypsin, and 3 × 10^5 cells were plated onto a series of 35-mm plastic Petri dishes containing 22- x 22-mm No. 1 glass coverslips in stock medium without actinomycin D. Forty-eight hr later, the cells were refed with stock medium containing actinomycin D, AMB, or combinations of AMB and actinomycin D. The effects of these antibiotics were observed by phase contrast microscopy on living cells, and on cells grown on coverslips after fixation in 2% gluteraldehyde in Hanks' solution neutralized with 1 N NaOH to pH 7.4. The fixed cells resembled living cells in all details that could be resolved at the level of resolution of positive phase contrast microscopy.

Radiometric Studies. HeLa cells were dissociated with 0.25% trypsin, and 3 × 10^5 cells were plated in 3 ml of medium on 35-mm plastic Petri dishes. After 24 hr in culture, 0.5 μCi [^{3}H]actinomycin D per ml (1 μg/ml) was added to the cells, and half the cultures were also treated with 30 μg AMB per ml. At 1 and at 5 hr, cells were rinsed with unlabeled medium, harvested from the Petri dishes with a rubber policeman, washed 3 times by centrifugation, filtered, and washed. The filters were then dried and counted in standard toluene fluor in a scintillation counter.

For radioautography, the cells were plated on coverslips in 35-mm plastic Petri dishes. After 24 hr in culture, the medium was replaced with fresh medium containing 0.5 μCi [^{3}H]actinomycin D per ml (1 μg/ml), and one-half of the dishes were also treated with AMB (30 μg/ml). After 5 hr of exposure to the drug(s), the coverslips were washed with three 10-cu cm aliquots of Hanks' solution and the washed cells were fixed in a acetic acid:methyl alcohol (1:3) mixture. The coverslips were then dried, mounted on a glass slide, and dipped in Kodak Type NTB2 nuclear track emulsion. After 10 to 14 days of exposure at 4°, the slides were developed with Kodak D19 and stained with May-Grünwald-Giemsa.

RESULTS

Effects of Drugs on Cell Viability. AMB or actinomycin D alone had no effect on the viability of the resistant HeLa cells. The cell number increased by 50% during 18 hr of incubation. However, AMB in combination with actinomycin D resulted in a significant decrease in the number of viable cells as determined by trypan blue (Table 1). The differences in viability were even greater when the ability to form macroscopic colonies was tested. The plating efficiency of untreated, AMB- or actinomycin D-treated cultures was 20%. No colonies were seen on the plates seeded with the same number of cells treated with 30 μg of AMB and 1 μg actinomycin D per ml.

Table 1

Percentage of cells viable[a] after 18 hr of incubation

The percentage of sruviving cells from the experiments were averaged. All of the replicates were within 10% of each other.

Amount of actinomycin D (μg/ml)	% viable after incubation with AMB		
	0	30 μg/ml	50 μg/ml
0	100	100	100
1	96	50	43
2	100	31	27

[a] Measured by trypan blue.

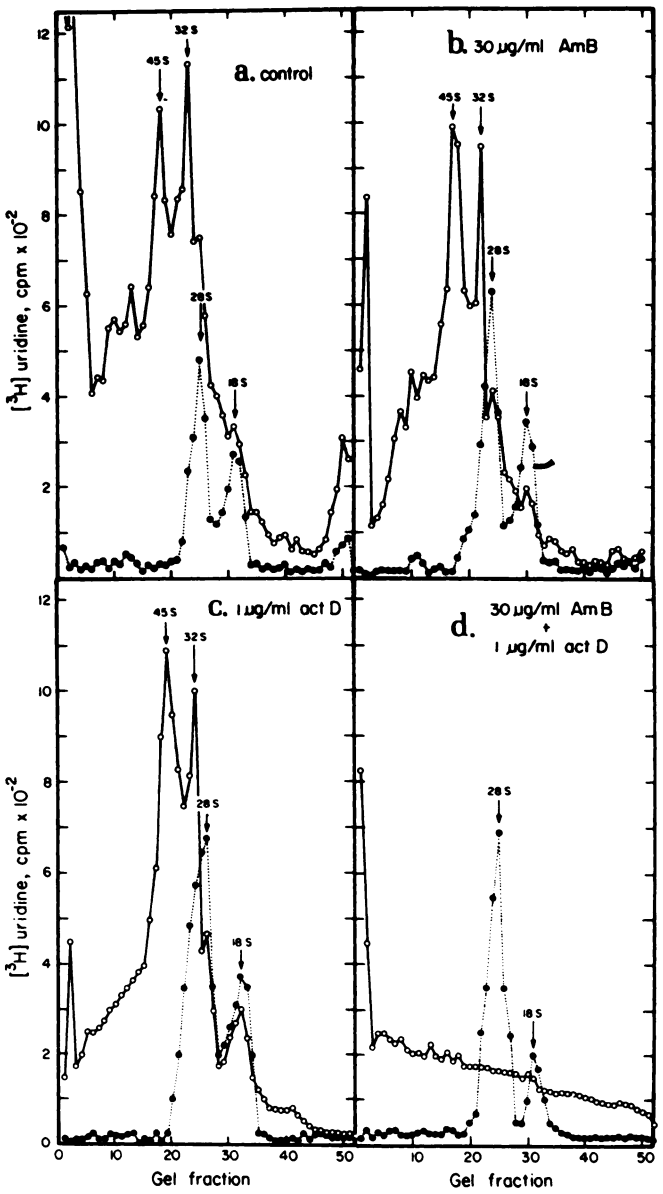

Chart 1. Acrylamide gel electrophoretic analysis of [^{3}H]uridine pulse-labeled RNA from untreated HeLa cells (*a*) and cells treated with AMB (*AmB*) (*b*) and actinomycin D (*act D*) (*c*) alone and in combination (*d*). The method of analysis is described in the text. (●) marker 18 S and 25 S RNA. (O) pulse-labeled [^{3}H]RNA.

RNA Synthesis. In the sensitive parental strain of HeLa cells, 1 μg of actinomycin D per ml inhibited RNA synthesis by 75%. In the resistant strain, total RNA synthesis was not affected by incubation in 30 to 50 μg or AMB or in 1 to 2 μg of actinomycin D per ml. However, when the resistant HeLa cells were treated with 30 μg of AMB per ml plus 1 μg of actinomycin D per ml, there was a 68% inhibition of total RNA synthesis.

The acrylamide gel patterns of pulse labeled RNA from resistant cells incubated in AMB (Chart 1*b*) or actinomycin D (Chart 1*c*) alone were indistinguishable from controls

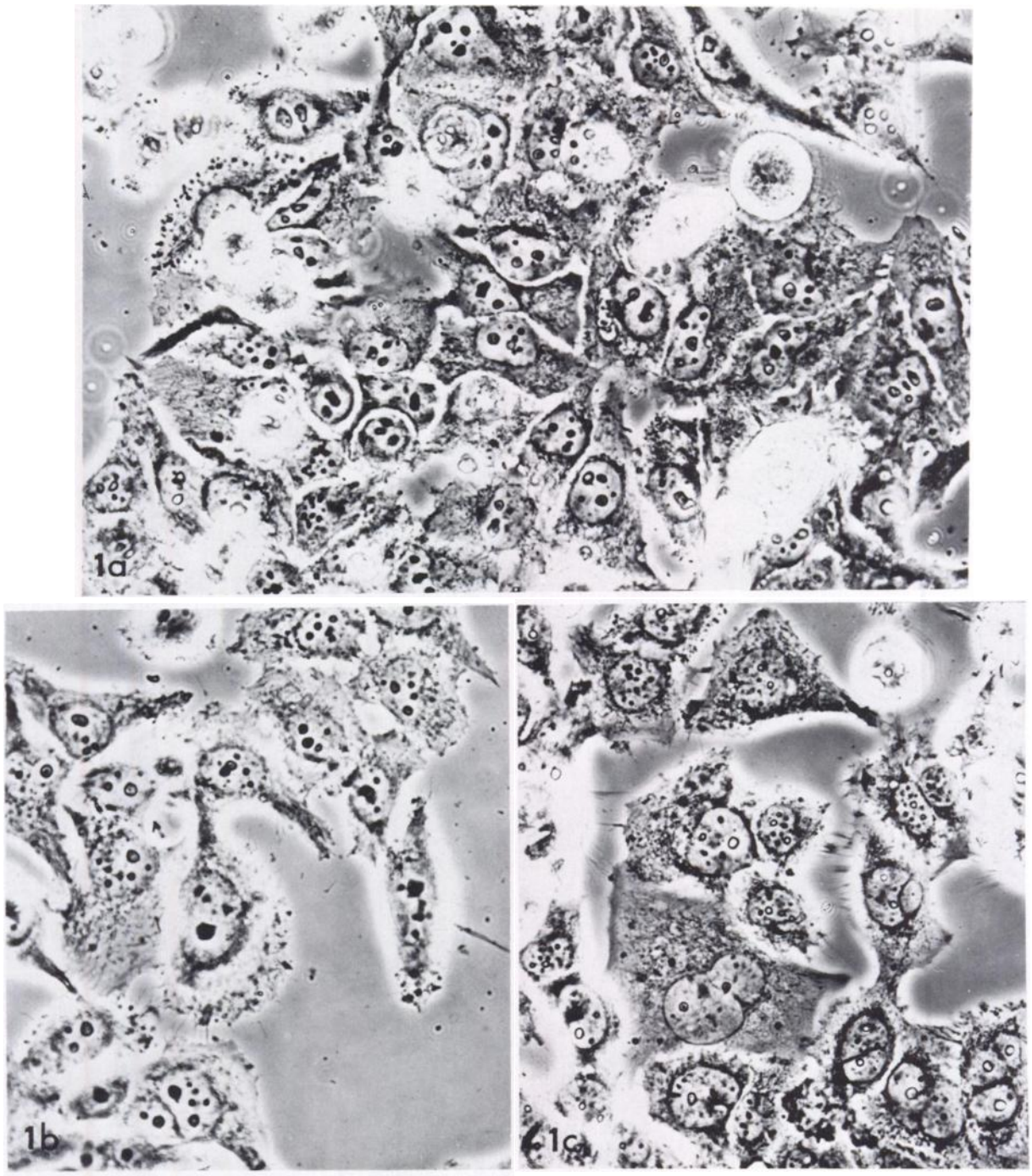

Fig. 1. Photomicrographs of resistant HeLa cells treated with AMB and actinomycin D alone and in combination. Resistant HeLa cells were grown for 24 hr in media with (*a*) 1 μg of actinomycin D per ml, (*b*) 25 μg of AMB per ml, (*c*) 1 μg of actinomycin D per ml and 25 μg of AMB per ml. They were fixed in 2% gluteraldehyde in Hanks' solution and photographed with positive phase contrast. Note that cells in 1*a* have large nucleoli and that there are many dividing cells. AMB (*b*) or actinomycin D (*a*) alone had no effect on the morphology of nucleoli or on cell division. The combination of both antibiotics in the medium resulted in cessation of growth and a marked effect on nucleolar morphology. Note that the nucleoli are smaller and some cells have fragmented nucleoli (*c*). × 420.

(Chart 1*a*) and from the standard patterns reported in the literature for rapidly labeled RNA. The contrast between these patterns and those of cells treated with AMB plus actinomycin D (Chart 1*d*) is clear. 45 S, 28 S, and 18 S RNA fractions were not present in cells treated with the combination, whereas synthesis of some heterogenous RNA continued. In other experiments, when graded doses of actinomycin D were used with AMB, the expected progressive inhibition of rRNA synthesis was observed (data not shown).

Microscopic Changes of AMB and Actinomycin D. Extensive changes in the nucleolar morphology of cells sensitive to

actinomycin D have been described (4). Drug-resistant cells do not show these changes after exposure to actinomycin D. The resistant HeLa cells, grown in 1 μg actinomycin D per ml for 1 to 4 days, did not show changes in the morphology of the nucleoli and retained nucleolar RNA as indicated by intense red-orange fluorescence after staining with acridine orange (Fig. 1*a*). The resistant cells grown in media with 30 μg of AMB per ml were sturcturally unchanged (Fig. 1*b*). At 50 μg/ml, the HeLa R-cells had ruffled cell membranes, but no obvious changes were noted in the structure of their nucleoli. However, when cells were grown in media containing 0.4 μg of actinomycin D per ml and 25 μg of AMB per ml (Fig. 1*c*), the nucleolar morphology was altered after 24 hr, and by 48 hr many of the cells had very small nucleoli, compared with untreated, AMB-, or actinomycin D-treated cells.

Table 2

Incorporation of [³H]actinomycin D into HeLa cells

	Radioactivity (cpm/ml/10⁵ cells)		
	Resistant HeLa cells		Sensitive HeLa cells
	Actinomycin D	AMB + Actinomycin D	Actinomycin D
1 hr	62	388	1350
5 hr	153	970	7988

Radioactive Studies. The amount of radioactive actinomycin D incorporated into the resistant cells is shown in Table 2. AMB increased the incorporation of [³H]actinomycin D into resistant cells by about 6-fold over the non-AMB-treated cells, but this was still considerably less than the amount of actinomycin D incorporated by sensitive cells.

We could also demonstrate an increased incorporation of [³H]actinomycin D into resistant cells in the presence of AMB by radioautography. The cells treated with 30 μg of AMB per ml had considerably more radioactive grains than the cells treated with [³H]actinomycin D alone (Fig. 2). It is also clear that the actinomycin D-treated cells have discrete nucleoli whereas there are no discernible nucleoli in the combination-treated cells.

AMB in the form of Fungizone was used in our experiments; 45% of the weight of Fungizone is sodium deoxycholate. Twice the concentration of sodium deoxycholate present in the amounts of Fungizone used in our experiments had no effect on the morphology of the HeLa cells and did not potentiate the effects of actinomycin D by any of the criteria we tested.

DISCUSSION

The viability, biochemical, morphological, and radioactive studies on the resistant HeLa cells indicate an ac-

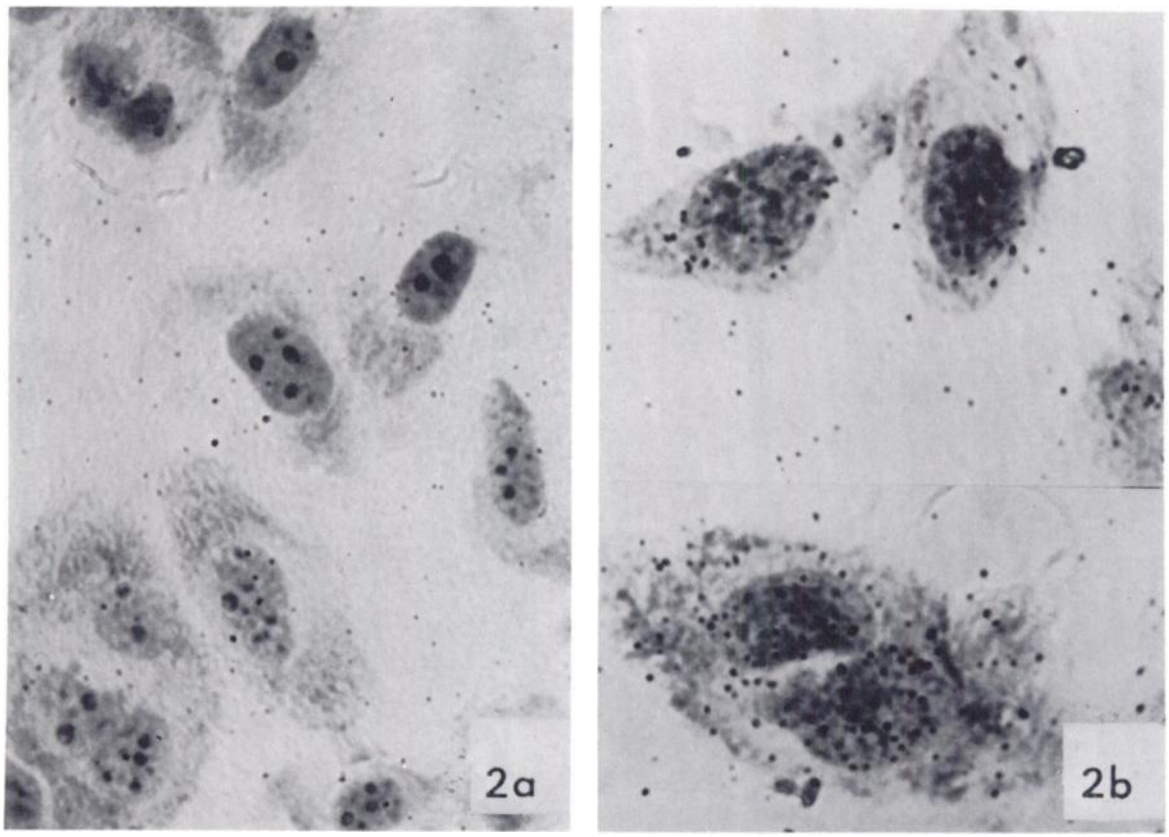

Fig. 2. Radioautographs of resistant HeLa cells treated with [³H]actinomycin D alone (*a*) and in combination with 30 μg of AMB (*b*). Note the small number of grains in *a* and the large nucleoli. In *b* there are a large number of intracellular and intranuclear g, and no nucleoli are visible. Cells are stained with May-Grünwald-Giemsa, × 600.

tinomycin D effect and not an AMB or nonspecific killing effect on the cells. This is consistent with our earlier data on AMB action (7–9) and also with previous work by Riehm and Biedler (12) with Tween 80. These studies have indicated that resistant cells take up less antibiotic than do sensitive cells, and that effects on the cell membrane can overcome this resistance.

The development of resistance to actinomycin D seemed to be based on relatively specific permeability changes (3), whereas the promotion of uptake by AMB was relatively nonspecific (7–9). If the *in vivo* resistance of tumors to certain antitumor agents is indeed based on a decreased uptake of these agents, then AMB may be effective in overcoming this resistance, as it has been in some antifugal therapy in animals and humans (1, 2, 11).

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