Title

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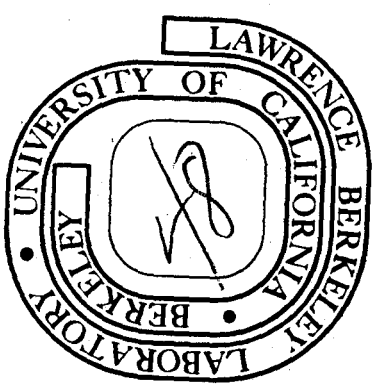

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DISCLAIMER

UPTAKE AND DISTRIBUTION OF DIMETHYLBENZYLDESMETHYLRIFAMPICIN (DMB) IN BALB/3T3 CELLS

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SUMMARY

Because of the demonstrated ability of dimethylbenzyldesmethylrifampicin (DMB) not only to inhibit RNA-instructed DNA polymerase (RDP) but focus formation by MSV on Balb/3T3 cells it was of interest to determine how it is taken up by such cells, both transformed and non-transformed. This was investigated using tritium-labeled DMB. It was found by radioautography that the drug is distributed throughout the cell with some concentration in the cytoplasm near the nuclear membrane. Cell fractionation showed that most of the material was indeed in the cytoplasmic components as opposed to the washed nuclei. A pulse labeling followed by chasing with unlabeled DMB demonstrated that the cell-associated DMB is about twice as great in the transformed as in the non-transformed cells.

INTRODUCTION

Some derivatives of Rifamycin SV have been found to be inhibitors of the RNA-instructed DNA-polymerase (RDP).[1-5] Some of them selectively inhibit sarcoma virus induced transformation of mouse cells in tissue culture.[6-8]

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One of these derivatives, cis-dimethylbenzyldesmethylrifampicin (DMB), was tested for its potency as an inhibitor for dimethylbenzanthracene (DMBA) induced tumors in rats and was found to have a significant tumor-delaying effect.[9] Therefore, it seemed worthwhile to undertake an investigation of the uptake and distribution of DMB in animal cells. A mouse cell line, A31[10,11] was used in this study. The distribution of DMB was investigated by autoradiography and cell fractionation, using tritium-labeled DMB as a tracer. We were able to show that cell-bound DMB is approximately two-fold higher in virally-transformed mouse cells than it is in non-transformed cells.

MATERIALS

^{3}H-DMB. This was prepared by a partial synthesis, condensing tritium-labeled 1-amino-2,6-dimethyl-4-benzylpiperazine with 3-formylrifamycin SV. The latter was prepared by the hydrolysis of rifampicin which was kindly supplied by Gruppo Lepetit S.p.A., Milan, Italy. The tritium was incorporated in the hydrazine in two ways: One, synthesis according to Pope[12] and Cignarella[13], starting with ring-labeled benzoic acid (New England Nuclear, specific activity 50 mC/mmole); Two, tritiated water in exchange labeling with the H[$AlCl_3OH$] complex using a modification of Mantescu's method.[14] The hydrazine and water-free aluminum chloride were mixed in the molar ratio 1:2 with argon protection. The reaction mixture was suspended in a ten-fold excess (by weight) of methylene chloride. After 5 minutes at room temperature, tritiated water (Lawrence Livermore Laboratory) (40 C/ml) was added at liquid nitrogen temperature in a molar ratio of 1:1 with the aluminum chloride. The reaction mixture was then warmed slowly and allowed to stand at room temperature for 3 hours. It was then decomposed by adding 10% KOH while cooling in liquid nitrogen. The hydrazine was extracted several times with chloroform. The activity in the labile posi-

tions was back-exchanged by alternating the pH between 2-14 several times and thus removed with the water phase. After drying and evaporation of the chloroform, the residue was reacted directly with 3-formylrifamycin SV in tetrahydrofuran (THF), yielding DMB. The DMB was isolated from the reaction mixture by chromatography on silica gel with chloroform-benzene as eluents and recrystallized from acetone. The specific activity of the resulting product was 1.01 C/mmole. Structural identity was determined by comparing the thin-layer chromatography (TLC), ultraviolet absorption, metabolism in animals[9] and inhibitory effect on viral-induced focus formation[6-8] of the ^{3}H-DMB with a sample of cis-DMB kindly supplied by Gruppo Lepetit. The exchange-labeled DMB was periodically checked by TLC and repurified by column chromatography, if necessary. Its hydrogenolysis shows that more than 90% of the tritium activity in the DMB is located in the benzyl group, which was trapped with toluene carrier and assayed by a gas chromatograph connected with a proportional counter.

Cells and Virus. The Balb/3T3, A31 cell line has been described previously in detail.[10] The virus stocks and the methods for infecting cells have been described.[10,11] Moloney sarcoma virus (MSV-M) was used to transform the A31 cells.

Autoradiographic Emulsions. The NTB2 emulsion for autoradiography was purchased from Eastman Kodak, Rochester, New York, and emulsion G5 and L4 were obtained from Ilford, Ltd., Essex, England.

METHODS

Uptake of DMB in Cells. Cells (1×10^{6}) were seeded into 250 ml Falcon flasks. After 24 hours, they were infected with 10^{7} infectious particles of MLV(MSV) stock virus and incubated at 37°C for 24 hr. The medium was changed to contain 3 µg/ml ^{3}H-DMB and incubation then continued for 2-140

hr. In some experiments ^{3}H-DMB was chased for 24 hr with 3 μg/ml of inactive DMB. The cells were washed six times (within 30 min) at 0°C with buffer A [0.25 M sucrose, 2×10^{-3} M $MgCl_2$ and 0.02 M Tris (pH = 7.9)]. The cells were trypsinized, counted in the hemocytometer, and an aliquot dissolved in Protosol for the determination of the tritium activity in a Packard Tricarb liquid scintillation counter.

Autoradiography. The autoradiography was done with cells fixed in situ as well as with sections cut from cell pellets. For the in situ fixation, cells were grown and treated as described in the section above. However, after washing, they were fixed in situ for 30 min with a solution of 2.5% glutaraldehyde in 0.1 N sodium cacodylate buffer. The flasks were rinsed with distilled water and cell bearing surface cut in strips the size of a microscope slide. These strips were dipped into photographic emulsion and after drying were stored at 4°C in a lead box for 1 to 4 weeks.

Cell pellets were obtained by scraping cells off the plastic after washing. The cell suspension was centrifuged and the pellet treated for 30 min with glutaraldehyde, stained 30 min with 1% OsO_4 (Dalton's), and 30 min with 0.5% uranyl acetate solution (Kellenberger). The cell pellet was dehydrated with alcohol and infiltrated with Epon.

The embedded pellet was cut in 1 micron sections, mounted on a grid, and covered with L4 emulsion, using a wire loop.

RESULTS AND DISCUSSION

Uptake of DMB by A31 Cells. Transformed and non-transformed cells were prepared as described. After exposure to ^{3}H-DMB for varying periods of time, the cells were washed, suspended with trypsin and counted. Although the flasks were seeded with the same cell number, after infection with MSV and transformation, the virus-yielding cultures show consistently fewer cells (Table I). Slight selective toxicity of DMB may account for this difference, or the rounded cells which are released into the medium during viral production by A31 cells were not included in the cell counts.

A concentration of DMB almost tenfold higher than that of the medium was found associated with the cells after trypsinization. After 100 hours exposure no apparent difference in the uptake of DMB was found in either cell type (Fig. 1 and Table I). Cell-associated ^{3}H-DMB (given in the number of ^{3}H-DMB molecules/cell) was obtained by calculating from the measured dpm and the known specific activity of the ^{3}H-DMB (2.68×10^{8} molecules/dpm).

Up to 92% of the ^{3}H-DMB is removed by trypsinizing and 60-70% can be eluted by chasing with cold DMB (Table II). Combination of both treatments removes 99% of the cell-associated DMB. The residual DMB concentration is almost twice as high in transformed cells ($6.3 \pm 0.5 \times 10^{5}$ molecules/cell) as in non-transformed cells ($3.8 \pm 0.3 \times 10^{5}$ molecules/cell).

Distribution of DMB in A31 Cells. Cell fractionation and autoradiography were used to localize the DMB in the cell. For the cell fractionation, cells were grown, as described in Methods, transformed with virus and exposed to DMB as described. After washing, the cells were suspended by scraping into buffer A and homogenized with a Teflon-glass homogenizer. The homogenate was clarified by low speed centrifugation (1000 g for 10 min) and 30% of the ^{3}H-DMB was found in the crude nuclei pellet. The pellet was washed twice with a

one-hundred fold volume of buffer A, resuspended and centrifuged through 2.4 M sucrose (50,000 g, 1 hr). The pellet obtained was composed of purified nuclei and now contained less than 1% of the activity present in the homogenate.

The low speed supernatant was centrifuged for 10 hr at 100,000 g. The pellet containing cytoplasmic organelles and membrane fragments yielded 40% of the activity in the homogenate. The remaining 30% of the activity was in the supernatant.

Autoradiography was done as described in "Methods". Figure 2 is the in situ autoradiogram of non-chased non-transformed A31 cells after 6 days of incubation with ^{3}H-DMB. It shows the lowest concentration of grains in the nucleus and the highest in the perinuclear region of the cytoplasm. Although transformed A31 cells showed the same pattern, the results were not as clear due to the heavy growth after six days.

The heavy grain density around the nucleus was not found in cut sections, very likely due to an extraction of ^{3}H-DMB during the embedding procedure. A significant protion of the cell-associated ^{3}H-DMB is already eluted by the glutaraldehyde fixation (70-90%). This fixation was used for cut sections as well as for whole cells. However, the embedding procedure was used for cut sections only. In this case, an additional 30-50% of the ^{3}H-DMB remaining in the fixed cells was eluted, mainly by ethanol used in the dehydration step. No apparent difference in grain density over the outer membrane was found by counting more than 3000 grains in cut sections.

The data obtained by autoradiography and cell fractionation suggest that only a very small portion of the DMB is localized in the nucleus. The concentration of DMB in the perinuclear region may be due to a physical solubilization of the drug in the lipid granules due to its lipophilic properties.[15,16] These granules accumulate in the perinuclear region with increasing age of the cell.

The only difference between non-transformed and transformed cells is found in the residual DMB concentration after trypsinizing and chasing, which is almost twofold higher in transformed cells. Experiments to localize and characterize the binding site with high affinity for DMB are in progress.

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Table I

The Uptake of ^{3}H-DMB by A31 Cells

Exposure to ^{3}H-DMB (Hr)	Morphology of Cell	No.Cells/Flask (x 10^{6})	Cell-associated ^{3}H-DMB after Trypsin, Molecules/Cell (x 10^{6})	Partition* Ratio
4	NT**	0.38	9	3.5
	T**	0.46	5	1.9
30	NT	0.75	5	1.9
	T	0.66	4	1.5
54	NT	3.2	4	1.6
	T	1.25	9	3.5
100	NT	6.0	23	9.2
	T	2.0	23	9.2
150	NT	6.5	25	9.9

* partition ratio = $\frac{\text{conc. of DMB assoc. with the cell}}{\text{conc. of DMB in the medium}}$

** NT = non-transformed; T = transformed

Table II

Effect of Trypsin Treatment and Chasing on Cell-Associated ^{3}H-DMB

Exposure of transformed cells to ^{3}H-DMB for 48 hr followed by chasing with cold DMB for 24 hr

	No Chase	Chase
No trypsin treatment	66.3* (100)**	25.3 (38)
Trypsin treatment	5.3 (8)	0.63 $\pm$0.05 (0.9)

* No. ^{3}H-DMB molecules per cell (x 10^{6})

** Percent cell-associated ^{3}H-DMB

Figure 1. Cell-associated ^{3}H-DMB in A31 cells expressed in the partition ratio $\frac{\text{conc. of } ^{3}\text{H-DMB inside the cell}}{\text{conc. of } ^{3}\text{H-DMB in medium}}$ (2×10^{7} cells = 30 µl) as function of exposure time to 3 µg/ml DMB in Eagle's minimal essential medium. (o–o) transformed; (Δ–Δ) non-transformed.

Figure 2. Autoradiogram of non-transformed A31 cells fixed with 2.5% glutaraldehyde in 0.1 N sodium cacodylate after an exposure time of 140 hr with 3 µg/ml ^{3}H-DMB. (In the control experiment, inactive DMB was used.) The sections were dipped into NTB-2 emulsion, exposed 2 weeks, stained with Giemsa's blood stain in 50% glycerol. The photograph was taken on Iodak High Contrast copy film with a Zeriss photomicroscope.

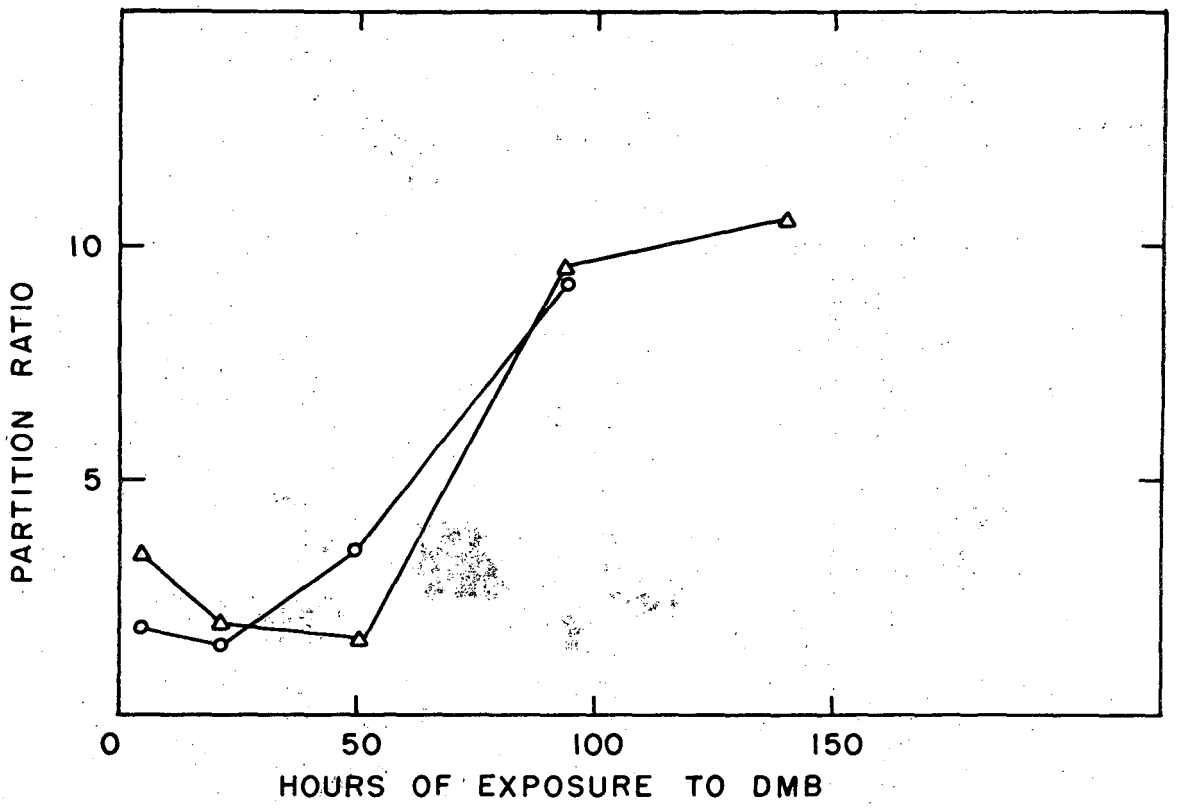

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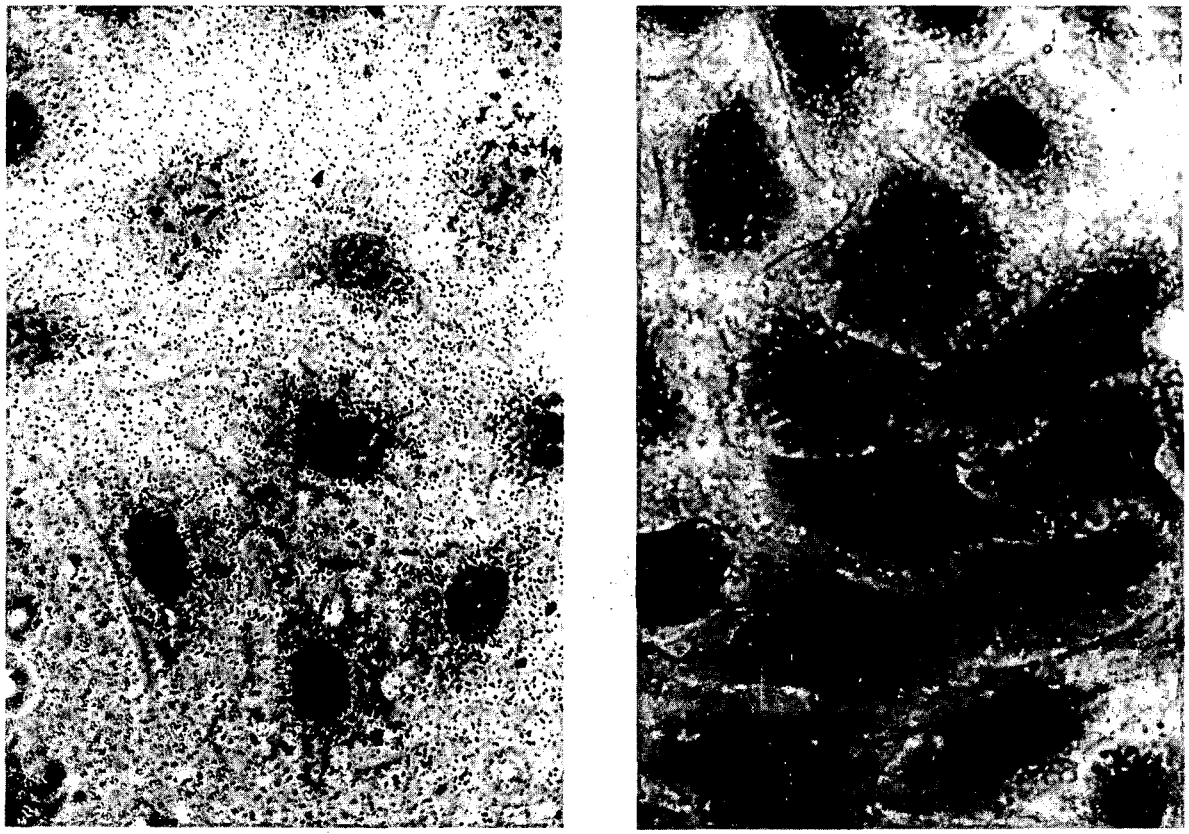
Figure 1.



Radioactive Control

Non-transformed Balb 3T3/A31 cells

5 μ

XBB 726-3254

Figure 2.

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