The Specificity of Dimethylbenzylrifampicin as an Inhibitor of Viral Induced Transformation

(drug resistance/cell variants/Balb/3T3 cells/RNA-dependent DNA polymerase)

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ABSTRACT The effect of 2',6'-dimethyl-N(4')-benzyl-N(4')-[desmethyl]rifampicin on viral transformation induced by two unrelated oncogenic viruses was compared. Transformation of Balb/3T3 cells by the small, DNA-containing papova virus simian virus 40 was completely normal under conditions where transformation by the large, RNA-containing oncornavirus murine sarcoma virus was inhibited more than 150-fold. For these experiments a resistant variant of Balb/3T3 was selected that grows well in high concentrations of the drug, is not dependent on the drug for growth, and is probably not blocked at the level of drug uptake. These data show that dimethylbenzylrifampicin specifically inhibits oncornavirus-induced transformation rather than nonspecifically inhibiting cellular growth or transformation.

Certain derivatives of rifampicin (1-7) actively inhibit oncornavirus RNA-dependent DNA polymerase (reverse transcriptase) (8, 9). Two rifampicin derivatives, dimethylbenzylrifampicin (DMB) and rifazacyclo-16, inhibit viral reverse transcriptase (7) and at comparable concentrations also inhibit murine sarcoma virus (MSV) transformation of mouse cells in tissue culture (10-14). This correlation between biological and biochemical data suggests that these drugs block transformation by inhibiting the viral induced reverse transcriptase; alternatively the drugs may inhibit other cellular enzymes required for transformation. To further explore the latter possibility, we have compared the effect of DMB on transformation by MSV with its effect on transformation induced by simian virus 40 (SV40), a small DNA-containing virus which does not have reverse transcriptase activity. We found that SV40-induced transformation was completely normal under conditions where MSV transformation of the same cells was greatly inhibited.

MATERIALS AND METHODS

Cell Culturing. Balb/3T3 clone A31 cells (obtained from Dr. G. Todaro) were used as the parental cell stock. The cells were grown in Dulbecco's modification of Eagle's medium (Gibco no. H21) with 10% calf serum (Colorado Serum Co.) (complete medium). To obtain a drug resistant variant, 100 Balb/3T3 cells were plated into a 60-mm dish with complete medium containing 3 μ g/ml of DMB and allowed to grow for 3 weeks with biweekly fluid changes. The cells were passaged

one time to a Falcon flask containing complete medium plus 3 μ g/ml of DMB and then passaged 4 times in the same medium containing 20 μ g/ml of DMB. The cells that survived this treatment grew well in DMB and were considered resistant variants.

SV40 Virus. SV40 (small plaque variant) was prepared by innoculating Vero cells in Dulbecco's medium plus 2% fetal-calf serum with 1×10^{-4} plaque-forming units per cell of SV40 and harvesting the cell supernatant 2 weeks later at the time of maximum cytopathic effect. Viral stocks were titered on TC-7 monkey cells by means of a fluorescent antibody assay. Three days after infection with various dilutions of virus, the cells (grown on coverslips) were fixed and stained for V antigen (monkey anti-V serum was kindly supplied by Dr. H. Ozer. The fluorescein-conjugated antibody to monkey globulin was obtained from Sycco Co.).

SV40 Transformation. For the transformation assay, 60-mm tissue culture dishes of confluent resistant cells grown in complete medium plus DMB, 3 μ g/ml, were infected by removing the medium, adding 0.5 ml of either SV40 or mock-infected cell supernatants and incubating at 37° for 3 hr with periodic shaking. The virus or mock virus was removed and fresh complete medium added for 3 hr. Cells were treated with trypsin and plated at various dilutions; cells were allowed to become confluent or colonies were obtained as described (15). In brief, the petri dishes were fixed and stained with hematoxylin (Harris-Lillie). Transformants appeared on the confluent monolayer as darkly staining areas. Dishes plated with low concentrations of cells were allowed to grow until discrete colonies could be observed, which were then fixed and stained. The number of colonies present is a measure of the number of viable cells in the original sample. By counting the number of transformed colonies and knowing the number of viable cells in the original sample, one can calculate the percent of cells transformed:

$$100 \times (\text{No. of transformed colonies/no. of viable cells}) \\ = \text{percent transformed.}$$

MSV Transformation. Moloney strain of murine sarcoma virus was prepared by infecting a high passage Swiss mouse embryo cell line (HPME) with a 10^{-2} dilution of virus stock. Details of the assay have been published (16). Briefly, subconfluent cell monolayers were infected with MSV diluted in L15 medium with Polybrene (4 μ g/ml). After 1-hr adsorption,

Abbreviations: DMB, 2',6'-dimethyl-N(4')-benzyl-N(4')-[desmethyl]rifampicin; MSV, murine sarcoma virus; SV40, simian virus 40.

TABLE 1. MSV transformation of Balb/3T3 cells

Drug added	MSV-induced foci
None	288
DMB, 5 μ g/ml	205
DMB, 5 μ g/ml + amphotericin B, 1 μ g/ml	63

MEM (Minimal Eagle's Medium) with 10% fetal-calf serum or complete medium was added. After 3-days incubation, the culture fluids were changed and foci of transformed cells were counted 4-6 days after infection.

RESULTS AND DISCUSSION

In these experiments, DMB inhibited MSV transformation of Balb/3T3 cells to a greater extent when 1 μ g/ml of amphotericin B was included in the medium (Table 1). Previous reports (10, 13, 17) showed that DMB inhibited murine leukemia virus transformation of the mouse cell line UC1-B without the addition of amphotericin B, although it was subsequently shown that the inhibition was more reproducible and more complete when the amphotericin B was included (11). It was hypothesized that amphotericin B acted by altering cell membrane permeability, thus enabling the DMB to be taken up more readily by the cells.

The original experiments showing murine leukemia virus focus inhibition on Balb/3T3 cells were also complicated by possible toxicity effects caused by drugs (10). Furthermore, slight toxicity which would not be apparent in a 5-day MSV assay might affect a 2-week SV40 transformation study. To avoid this problem we first selected a variant of Balb/3T3 that was resistant to 10 μ g/ml of DMB (see *Materials and Methods* section). The resistant cells were selected using DMB without amphotericin B. All subsequent experiments were performed with DMB plus amphotericin B.

MSV-induced foci on the resistant cells and the standard Balb/3T3 cells were compared. With the same stock of virus, both lines produced similar numbers of foci (Table 2). The data in Table 2 also confirm previous observations (11) that addition of amphotericin B at a concentration of 1 μ g/ml had little effect on focus formation.

The plating efficiency of the resistant cells was unaffected by 10 μ g/ml of DMB plus 1 μ g/ml of amphotericin B, the drug concentrations which markedly inhibited growth of the parental Balb/3T3 cells (Table 3). The fact that the resistant

TABLE 2. Effect of amphotericin B on MSV-induced transformation

Cell line	Drug	MSV-induced foci
Standard Balb/3T3	None	58
	Amphotericin B 1 μ g/ml	51
Resistant variant Balb/3T3*	None	62
	Amphotericin B 1 μ g/ml	48

* Cells were maintained in complete medium containing 10 μ g/ml of DMB. Twenty-four hours before infection they were treated with trypsin and plated in complete medium lacking any drug. Immediately after infection, medium was added that contained the drugs indicated.

TABLE 3. Effect of DMB plus amphotericin B on cell plating efficiency*

	No drug	+DMB, 10 μ g/ml	+DMB, 10 μ g/ml + ampho. B, 1 μ g/ml
Standard Balb/3T3	28	14	0
Resistant variant Balb/3T3 passaged in 3 μ g/ml of DMB	3.3	3.3	3.4
Resistant variant Balb/3T3 passaged 3 times with no drug	4.2	4.0	4.0

Ampho. B is an abbreviation for amphotericin B.

* One hundred cells were plated in a 60-mm tissue culture dish and allowed to grow into colonies.

100 $\times$ (No. of colonies formed/no. of cells plated)
= cell plating efficiency.

cells also grew well in the absence of drug suggested that they had not become dependent on DMB for growth. After 3 passages without DMB, the plating efficiency of the cells was still unaffected by 10 μ g/ml of DMB plus 1 μ g/ml of amphotericin B, suggesting that the cells were resistant variants rather than standard Balb/3T3 cells adapted to the high drug concentration. The resistant variant plated equally well in DMB without amphotericin B as in complete medium; the standard Balb/3T3 showed slight toxicity in DMB even without the amphotericin B. Our experiments do not distinguish whether drug resistance in these cells is due to a stable alteration in gene expression or in the genetic material itself; therefore, we have not used the terminology "mutant." In the particular experiment shown in Table 3, the plating efficiency of the resistant variant was somewhat lower than that of the standard Balb/3T3 cells. This difference was not reproducibly observed and probably represents slight variations in technique from one experiment to another.

Table 4 compares viral transformation of the resistant cells in the presence and absence of drugs. MSV transformation of the resistant cells was inhibited in the presence of 10 μ g/ml of DMB plus 1 μ g/ml of amphotericin B. No foci were present at a concentration of virus that produced 162 foci in the absence of drugs. The fact that the drugs inhibit MSV transformation of the resistant variant suggests that the variants are not blocked in the uptake of DMB. Under the same growth

TABLE 4. Effect of DMB on viral induced transformation of DMB-resistant Balb/3T3 cells

Drug added	MSV-induced foci per flask	SV40-induced transformation*, %
None	162	4
DMB, 10 μ g/ml	161	2
DMB, 10 μ g/ml + amphotericin B, 1 μ g/ml	0	3

* The percent transformation was calculated from the efficiency of plating which, in the SV40-infected cells, was 11% with no drug added, 15% with DMB, and 11% with DMB plus amphotericin B (see *Materials and Methods* section).

conditions, SV40 transformation was essentially unaffected by 10 $\mu\text{g}/\text{ml}$ of DMB plus 1 $\mu\text{g}/\text{ml}$ of amphotericin. The variations in transformation frequencies observed (Table 4) are within the reproducibility and sensitivity of SV40 transformation assays in general. For each assay, at least 80 transformants were counted and the experiment has been repeated four times with essentially the same result. The SV40 virus stock used contained 10^8 plaque-forming units/ml and induced a transformation frequency of 2–6% on standard Balb/3T3 cells.

These data suggest that DMB inhibits oncornavirus-induced transformation rather than nonspecifically inhibiting cellular growth or transformation. Three different lines of evidence now suggest that the DMB is specifically inhibiting the reverse transcriptase itself:

- (1) The concentration of DMB needed to inhibit transformation is the same order of magnitude as that needed to inhibit enzyme activity (7).
- (2) Under conditions where transformation is inhibited, virus production is also inhibited (12). Recent evidence suggests that the reverse transcriptase is required for both viral production and transformation (18). Thus, if DMB were blocking only transformation, the data would suggest that the drug was not acting on the reverse transcriptase.
- (3) The present data show that DMB specifically inhibits transformation induced by virus containing reverse transcriptase.

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1. Gurgo, C. R., Roy, K., Thiry, L. & Green, M. (1971) *Nature New Biol.* **229**, 111–114.
2. Gallo, R. C., Yang, S. S. & Ting, R. C. (1970) *Nature* **228**, 927–929.
3. Yang, S. S., Herrera, F. M., Smith, R. G., Reitz, M. S., Lancini, G., Ting, R. C. & Gallo, R. C. (1972) *J. Nat. Cancer Inst.* **49**, 7–25.
4. Green, M., Bragdon, J. & Rankin, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1294–1298.
5. Gurgo, C., Ray, R. & Green, M. (1972) *J. Nat. Cancer Inst.* **49**, 61–79.
6. Gallo, R. C., Abrell, J. W., Robert, M. S., Yang, S. S. & Smith, P. G. (1972) *J. Nat. Cancer Inst.* **48**, 1185–1189.
7. Tischler, A. N., Joss, U. R., Thompson, F. M. & Calvin, M. (1973) *J. Med. Chem.* **16**, 1071–1077.
8. Baltimore, D. (1970) *Nature* **226**, 1209–1211.
9. Temin, H. M. & Mizutani, S. (1970) *Nature* **226**, 1211–1213.
10. Calvin, M., Joss, U. R., Hackett, A. J. & Owens, R. B. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1441–1443.
11. Hackett, A. J., Sylvester, S. S., Joss, U. R. & Calvin, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3653–3654.
12. Hackett, A. J., Owens, R. B., Calvin, M. & Joss, U. (1972) *Medicine* **51**, 175–180.
13. Ting, R. C., Yang, S. S. & Gallo, R. C. (1972) *Nature New Biol.* **236**, 163–166.
14. Wu, A. M., Ting, R. C. & Gallo, R. C. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1298–1302.
15. Todaro, G. J. (1960) in *Fundamental Techniques in Virology*, eds. Habel, K. & Salzman, N. P. (Academic Press, New York), 220–228.
16. Hackett, A. J. & Sylvester, S. S. (1972) *Nature New Biol.* **239**, 164–166.
17. Hackett, A. J. & Sylvester, S. S. (1972) *Nature New Biol.* **239**, 166–167.
18. Linial, M. & Mason, W. (1973) *Virology* **53**, 258–273.