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March 1, 1971

AEC Contract No. W-7405-eng-48

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THE EFFECT OF RIFAMPICIN, AND TWO DERIVATIVES, ON CELLS INFECTED WITH MOLONEY SARCOMA VIRUS

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Summary

It is shown that rifampicin, and especially its relative dimethyl-N-benzyl-N-desmethyl rifampicin, can inhibit focus formation by Moloney sarcoma virus on BALB/3T3 tissue cultures. At a dose level of 10 μg/ml DMB appears to totally inhibit focus formation while reducing virus replication by at least a factor of fifty and cell proliferation by only a factor of three. These observations, taken together with those of others, suggest a role for the hybrid RNA-DNA dependent DNA polymerase and the gene for its synthesis both in normal cell processes and in the transformation process.

Rifamycin and its derivatives are a group of antibiotics which have been developed, particularly for use against mycobacterium, and the mode of action involves the bacterial RNA polymerase (1). Following the discovery that these drugs could also inhibit the replication of certain viruses, particularly adenovirus and vaccinia (2,3,4), it became of interest to explore the extent and possibly determine the nature of this antiviral activity. Toward this end, we obtained some samples of these materials in April of 1970* whose structures are shown in Figure 1.

There followed the discovery of the RNA-dependent DNA polymerase in oncogenic RNA virus by Temin (5) and Baltimore (6). An extensive discussion ensued about the distribution of this enzyme (particularly in virions and in cells from a variety of tumors) and the inhibition of the enzyme by some of the same antibiotic derivatives (7-10).

It thus became clear that the possibility was real that one or more of these rifamycin derivatives could inhibit the transformation of cells from the normal into the neoplastic state. We therefore undertook immediately to determine whether or notsuch a transformation could be affected by some of the derivatives which we had available.

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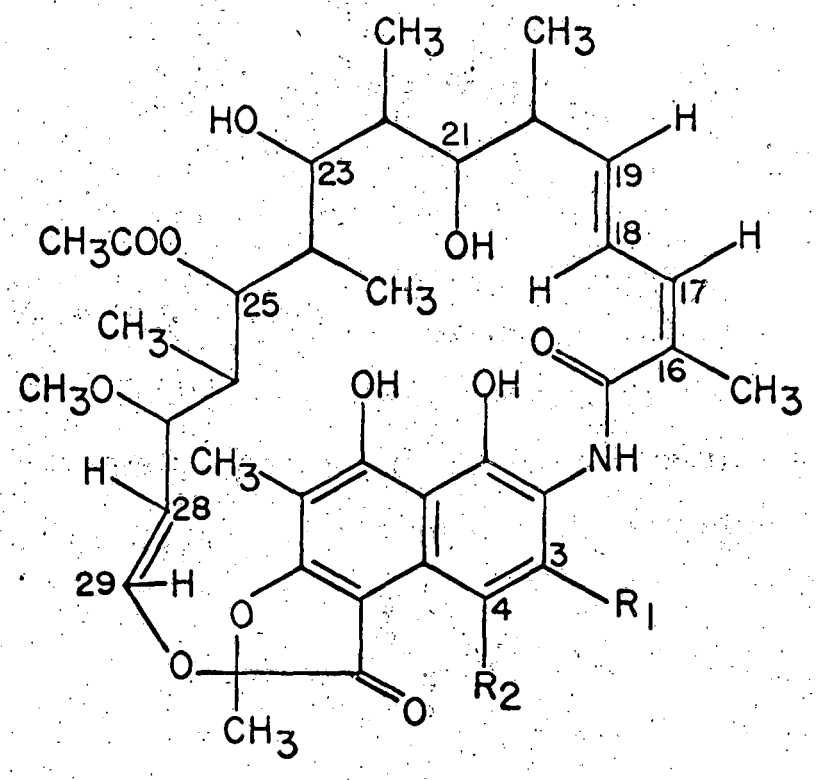
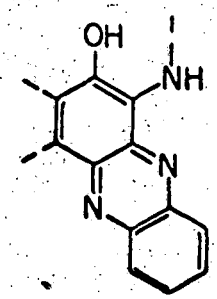
* The samples of rifamycin and its derivatives were kindly supplied by Drs. P. Sensi and G. Lancini of Gruppo Lepetit, Milan, Italy

Abbreviations used: R - rifampicin; DMB-dimethyl-N-benzyl-N-desmethyl rifampicin; Rz-rifazine; MSV-murine sarcoma virus (Moloney)



COMPOUND	R_1	R_2
Rifampicin (R)	$-CH{=}N-N\ \ N-CH_3$	—OH
DMB	$-CH{=}N-N(CH_3)\ \ N(CH_3)-CH_2-C_6H_5$	—OH
Rifazine		

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Calvin, et al. FIGURE 1

Such a possibility had already been suggested by the experiments of Diggelmann and Weissmann (11) in which Rous sarcoma virus transformation of chick fibroblast monolayers had been inhibited by rifampicin at a level of 60 μg/ml. The evidence of Green, presented at the Paris meeting on oncogenic viruses in November 1970, suggested that some of the derivatives of rifamycin might be more effective (9). We were interested not only in the possibility of preventing the transformation, but, ultimately, of affecting the transformed cells as well.

MATERIALS AND METHODS

Cell Cultures

BALB/3T3 cells were kindly sent to us by R. Gilden, Flow Laboratories, Inc., Rockville, Md. Cultures were grown in 250 ml plastic flasks in growth medium (GM) consisting of Eagles minimal essential medium with 10% fetal bovine serum. Cell counts were made with a Coulter counter after suspending the cells with trypsin-versene and diluting in GM.

Virus Stock

Moloney murine sarcoma virus (MSV) was obtained from J. Moloney, National Institutes of Health, as a tumor homogenate. It has been passaged four times in a Swiss-derived high passage mouse embryo cell line and assayed for focus-forming units (FFU) in BALB/3T3 cells. The virus pool used in these experiments titered 8.5×10^6 FFU/ml.

Assay of MSV

A modification of the method described by Hartley and Rowe (12) was used for the focus assay.

Flasks were seeded with 1-2 x 10^6 cells in 25 ml of GM and incubated at 37°C for 24 hr. Following the removal of fluids, virus was introduced in 0.5 ml of GM and allowed to adsorb on the monolayer for 90 min at 37°C. Twenty-five ml of GM was then added and the cultures returned to the incubator. After 3 days the cultures were fluid-changed, and foci of transformed cells counted at day 7.

The antibiotics were dissolved in dimethylsulfoxide at 1 mg/ml.

RESULTS AND DISCUSSION

Our first exploratory experiments defined the concentration region of useful activity of the drugs in the tissue cultures. Levels of drug above 20 μg/ml, particularly of DMB, produced grossly visible toxic effects. It was noted in the preliminary observations that levels of 10 μg/ml of DMB seemed to have a profound effect on cells which had been transformed by Moloney sarcoma virus, by inducing them to form syncytia to a degree not observed either with nontransformed cells or in the transformed cells without the drug. This observation is being explored further. Having defined the levels of drug usefulness, we then proceeded with the transformation experiment.

The results of one experiment are shown in Table 1. It is quite clear that the DMB compound is a potent inhibitor of the transformation process at 10 μg/ml. No foci were visible even though the control shows over 1000 foci/flask. It is also clear that the drug exhibits a slight inhibition of focus formation as well as some inhibition of virus replication. A more explicit and broader experiment was then performed, using a single dose of drug at a concentration of 10 μg/ml, the results of which are shown in Table 2. Here it is again quite clear that the most potent drug we have so far studied is the DMB compound which totally inhibits focus formation at 10 μg/ml. That not all RNA viruses are subject to this inhibition is demonstrated by the fact that vesicular stomatitis virus, which is not oncogenic but cytolytic and does not carry the R-DNA dependent DNA polymerase (13), is in no way affected by this drug in its ability to replicate on BALB/3T3 cell tissue culture. A 48 hr control showed 1.9×10^8 plaque forming units/ml (PFU), while the system containing 10 μg/ml of DMB showed 2.2×10^8 PFU/ml.

It is also important to note that cell proliferation itself is somewhat inhibited at this level, although only of the order of sixty percent. It is unlikely that a net increase in cell number occurred during the 24 hour period between seeding (14) and introduction of the drug. The

Table 1

Inhibition of focus formation and MSV replication by rifampicin and its dimethylbenzyl derivative

Group		Virus yield per flask x 10^4 (FFU)	FFU per flask	% FFU of control
Antibiotic	μg/ml			
None		308	1215	100
R	5	401	1490	120
	10	141	683	56
DMB	5	11	240	20
	10	.09	0*	< 1

BALB/3T3 cultures, seeded with $1x10^6$ cells 24 hr previously were infected with approximately 1200 FFU/flask. Following a 90 min adsorption period, freshly prepared rifampicin (R) or DMB were added at 5 and 10 μg/ml in GM. At 3 days post-inoculation, the cultures were fluid-changed with the same medium. Foci appearing in the cultures at day 6 were counted and expressed as FFU/flask. In addition, the supernatant fluid was assayed for the yield of infectious virus. The figures are an average of 2 flasks per group.

* While we saw no identifiable foci, there may have been some too small to recognize.

Table 2

Effect of rifampicin and two of its derivatives on cellular and viral replication in BALB/3T3 cells infected with Moloney sarcoma virus

Group	No. of cells per flask ($\times 10^6$)	No. of cells % of control	FFU per flask	% FFU of control	Yield MSV (FFU) per flask ($\times 10^4$)	Yield MSV (FFU) per cell
Uninfected control	9.9	100	--	--	--	
Infected control	8.4	100	446	100	53	.06
R	5.9	60	--	--	--	
R & MSV	6.4	76	201	45	10	.015
Rz	6.3	63	--	--	--	
Rz & MSV	5.8	69	332	75	69	0.1
DMB	3.1	32	--	--	--	
DMB & MSV	3.5	46	0*	< 1	1	.003

The same procedure as described in Table 1 was utilized to infect BALB/3T3 cultures with an estimated dose of 500 FFU of MSV. The antibiotics were added to the growth media (including fluid change) at a final concentration of 10 μg/ml. FFU were counted, the supernatant fluid was assayed for yield of infectious virus, and the number of cells per flask was counted at day 7. These data are from the last of 8 separate experiments conducted. While the figures varied between experiments, the data have followed a consistent pattern.

* See Table 1.

three-fold increase in cell numbered measured at day 7 probably occurred after introduction of the drug and this is supported by the fact that 10^4 FFU were produced in these cultures. Whether the inhibitory action of DMB on focus formation and production of infectious virus is solely a function of the reduced number of cellular divisions is not yet clear. Since cell number has increased three-fold, even in the presence of the strongest focus inhibitor (DMB) while focus formation has apparently been totally inhibited, it would appear that this is not the case. Since the drug was added only 24 hours after initial seeding, this three-fold multiplication would nothave had time to take place in that short period, following an inoculation with trypsinized cells (14). A more detailed exploration of this effect, both in time and in quantity, must be made and eventually related to the molecular effects of the drug on the enzyme involved.

It is interesting to note in this connection that Todaro has recently reported the presence in the nontransformed BALB/3T3 cells of a small amount of enzyme which responded to the rADT template (15). This in itself might be enough to account for our observation of reduced cell multiplication in the presence of the drug. However, it is altogether likely that other crucial enzymes are also inhibited which might participate in this reduction in the cell multiplication in the uninfected case.

Another interesting observation reported by Spiegelman (16) is that a monocytic leukemia carried in an ascitic form in a rat and induced by treatment with dimethylbenzanthracene has an enzyme very similar to the one found in human leukemic cells. Spiegelman has also reported the presence of a similar activity in a variety of embryonic tissue (17,18). It seems that this hybrid double-stranded R-DNA-dependent DNA polymerase enzyme may be common to those cells which are growing and dividing rapidly. In fact, it may be an especially facile supplementary route for replacing DNA via the DNA to RNA to DNA route, particularly in view of the questions which are being raised regarding the function of the Kornberg enzyme in DNA replication (19).

All of this tends to support the notion of a gene for this enzyme, and for other aspects of transformed cells, which may very well be present in an unexpressed form in what we believe to be normal cells (20). Expression of such genes, then, may be triggered either by chemicals, perhaps even by radiation, and by virus, with the last one possibly introducing new information into the cell as well. It remains to be seen how far such hypotheses can be developed in molecular terms.

Acknowledgment: The work described in this paper was sponsored, in part, by the U.S. Atomic Energy Commission and, in part, by Contract No. 436313 between the Regents of the University of California and the National Cancer Institute (Special Virus Cancer Project). One of us, URJ, is a fellow of the Stiftung für Stipendien auf dem Gebiete der Chemie, Basle, Switzerland.

REFERENCES

1. "RNA Polymerase and Transcription", 1st Lepetit Colloquium, November 1969. North-Holland Publishing Co., Amsterdam, The Netherlands (1970), 339 pp.
2. Heller, E., M. Argamon, H. Levy and N. Goldbaum, Nature, 222, 273 (1969).
3. Subak-Sharpe, H., M. C.Timberry and J. F. Williams, Nature, 222, 341 (1969).
4. Lancini, G., R. Cricchio and L. Thiry, J. Artibiotics, 24, 64 (1971).
5. Temin, H.M. and S. Mizutani, Nature, 226, 1211 (1970).
6. Baltimore, D., Nature, 226, 1209 (1970).
7. "Biology of Oncogenic Virus", 2nd Lepetit Colloquium, November 1970. North-Holland Publishing Co., Amsterdam, The Netherlands, in press.
8. Gallo, R.C., S. S. Yang and R. C. Ting, Nature, 228, 927 (1970).
9. Gurgo, C., R. K. Ray, L. Thiry and M. Green, Nature, 229, 111 (1971).
10. Spiegelman, S., A. Burny, M. R. Daz, J. Krydar, J. Schlom, M. Travnicek and K. Watson, Nature, 228, 430 (1970).
11. Diggelmann, H., and C. Weismann, Nature, 224, 1277 (1969).
12. Hartley, J. W. and W. P. Rowe, Proc. Nat. Acad. Sci. 55, 780 (1966).
13. Baltimore, D., A. S. Huang and M. Stampfer, Proc. Nat. Acad. Sci. 66, 572 (1970).

14. Puck, T. T., P.I. Marcus and S. J. Cieciura, J. Exp. Medicine, 103, 273 (1956).

15. Scolnick, E. M., S. A. Aaronson, G. J. Todaro and W. T. Parks, Nature, 229, 318 (1971).

16. Reference 7, page ____.

17. Spiegelman, S., in proceedings of "Third Annual Biochemistry PCRI Winter Symposia", January 1971, Miami, Florida; to be published.

18. New Scientist, 49, 230 (1971).

19. Nature (New Biology), 229, 65 (1971) and references cited therein.

20. Huebner, R. J., and G. J. Todaro, Proc. Nat. Acad. Sci. 64, 1087 (1969).

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