Title

EFFECT OF DIMETHYLBENZYLDESMETHYLRIFAMPICIN(DMB) ON CHEMICALLY-INDUCED MAMMARY TUMORS IN RATS

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Publication Date

1972-09-01

Submitted to Nature

LBL-1318
Preprint

EFFECT OF DIMETHYLBENZYLDESMETHYLRIFAMPICIN (DMB) ON CHEMICALLY-INDUCED MAMMARY TUMORS IN RATS

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September 1972

AEC Contract No. W-7405-eng-48

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Effect of Dimethylbenzyldesmethylrifampicin (DMB) on Chemically-Induced Mammary Tumors in Rats

Several derivatives of rifamycin SV have been shown to inhibit virally-induced transformation in tissue culture. Transformation of chick fibroblasts induced by Rous sarcoma virus (RSV) is inhibited by rifampicin at levels of 20-60 μg/ml,[1-3] and the transformation of Balb/3T3 cells induced by Moloney sarcoma virus (MSV) is inhibited more than 90% by dimethylbenzyldesmethylrifampicin (DMB) at levels of 6 μg/ml.[4,5]

At the animal level, an antitumor activity by rifampicin against Walker 256 carcinosarcoma,[6] a selective inhibition of RLV-induced splenomegaly by a streptovaricin complex[7], and inhibition of an adenovirus-induced tumor[8] have been reported. These findings, together with the fact that DMB is a strong inhibitor of the RNA-instructed DNA polymerase enzyme activity (RIDP) in crude viral extracts and the report that an RIDP functionality might be present in a chemically-initiated rat tumor carried as an ascites[9] encouraged us to test DMB for antitumor activity on the animal level when the tumor is chemically initiated. Also, the reported presence of the RIDP in certain rapidly growing cells, such as found in embryonic tissue, suggested to us that this functionality might be cryptically present in a variety of cells and be in some way activated (or released) by the action of chemicals. The particular method of chemical carcinogenesis was chosen because of the high incidence of tumors generated by the two carcinogens used, namely, 7,12-dimethylbenzanthracene (DMBA) and 7,8,12-trimethylbenzanthracene (TMBA).[10] These two carcinogens produced 100% tumors in rats within ten weeks.

Prophylactic prevention of chemically-induced tumors by DMB would then add evidence to the hypothesis that chemical carcinogenesis involves the triggering of information already in the cell[11,12] and entailing some gene duplication.[13] We chose DMB for the inhibition studies because it is one of the most potent RIDP and focus inhibitors so far known, and, at the same time, one of the least toxic drugs tested on the tissue culture level.

Rats injected with a carcinogen were treated with various doses of DMB. The development of tumors in the treated animals was compared with that in animals receiving the chemical carcinogen only. Blood and tissue levels of DMB were determined using tritium-labeled DMB, or, in certain tissues, optically by comparing the absorption of tissue extracts with DMB standard.

Materials

DMB* was kindly supplied by Gruppo Lepetit, S.p.A., Milano, Italy.

Tritium-labeled DMB, with a specific activity of 1 C/mmole, was synthesized in this laboratory; details of its synthesis will be published elsewhere.[14] For the experiments described in this paper, it was diluted to a specific activity of 10 mC/mmole, with inactive DMB. All the activity is located in the side chain (2,6-dimethyl-4-benzylpiperazine hydrazone residue), and more than 90% in the benzene ring of the side chain of the molecule. Its decomposition due to radiolysis was checked periodically by thin-layer chromatography, and it was repurified by column chromatography, if necessary.

The carcinogens were supplied in stabilized oil-water emulsions.[15]

We are grateful to Dr. Howard Mel of the Department of Medical Physics, University of California, Berkeley and Dr. Charles Huggins of the University of Chicago for the TMBA, and to the Upjohn Company, Kalamazoo, Michigan, for the DMBA.

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* DMB is 2',6'-dimethyl-N(4')-benzyl-N(4')-[desmethyl]rifampicin.

Animals. Female-Sprague-Dawley rats (45-50 days old) for the experiments involving DMBA were purchased from the Holtzman Company, Madison, Wisconsin. Female Long-Evans rats (45-50 days old) for the experiment using TMBA were purchased from Simonson Laboratories, Gilroy, California. The animals were maintained between 23° and 25°C with food and water ad lib.

Methods

Administration of carcinogens and DMB. A total of 6 mg DMBA in three doses at three-day intervals, or a total of 18 mg TMBA in four doses with two week intervals between doses, was injected intravenously.

The DMB was administered either i.p. or orally as a 3% solution in a mixture containing 5% purified egg-Lecithin (Schwartz/Mann) in Wesson oil, which was emulsified for the i.p. injection with a six-fold volume of a 0.3% aqueous solution of Pluronic F-69 (Wyandotte Chemical Company). For the oral administration, the oil-lecithin solution was measured with an automatic sampling syringe.

Sample collection and monitoring. Blood samples were taken from the tail vein. Tissue samples were taken immediately after the animals were sacrificed (with ether) and soaked for two hours in normal saline, then blotted dry on paper towels. Blood and tissue samples were prepared for scintillation counting either by dissolving in Protosol (New England Nuclear Company) or by combustion in a Packard TriCarb sample oxidizer. Both methods of preparation gave the same tritium activities within 10% error.

For the extraction of DMB from tissue, the latter was homogenized in a five-fold weight of dimethylsulfoxide (DMSO) in a homogenizer using a teflon plunger. After 15 minutes, the homogenate was centrifuged at

48,000 g for 10 min. Longer extraction times resulted in an increased background for the optical assay for DMB due to decomposing blood pigments. The optical assay for DMB was done by taking the absorption spectrum of the supernatant in the region of 350-700 nm, using DMSO as a blank, and comparing the OD of the peak around 486 nm with a standard solution which had an OD of 0.3 at 486 nm, which corresponds to a concentration of 20 µg/DMB/ml DMSO. For the tritium assay of the extractable DMB, an aliquot of supernatant was counted.

Animals were examined weekly by palpation for tumors. Tumors were graded in three sizes: small for tumors less than 1 cm in diameter, medium for tumors around 2 cm in diameter, and large, usually necrotic tumors. Animals were sacrificed when tumors became ulcerated.

Results

The results given in Tables 1 and 2 and Fig. 1 indicate that i.p. administration is more effective in maintaining blood and tissue levels than oral administration for the following reasons: (1) As the blood levels show (Fig. 1) drug uptake corresponding to DMB, measured in radioactivity, for the oral administration is only 80% of the i.p. administration. (2) The similarity of the optical assay and radioactivity assay shown in Table 1 indicates that with i.p. administration the DMB in fat, muscle, liver and intestines seems to remain intact three days after injection; however, with oral administration DMB as such is found only in the fat.

It is evident from Figs. 2 and 3 that DMB, while not completely inhibiting tumor growth, does delay the onset of tumors. It also slows the progression from small nodules to large necrotic tumors. Even after withdrawal of the drug, the growth of tumors is delayed. It should be

mentioned that the drug had no apparent effect upon the growth of any of the animals as measured by weight gain.

In the group of animals receiving DMB by mouth (Fig. 4), there is very little difference in the onset of palpable tumors between control and DMB-treated animals. The growth of the tumors was inhibited only slightly in the treated animals. This might be expected from the metabolism studies indicating only a significant level of DMB/in fat and less than 2 μg/g in the other organs.

In the group of Sprague-Dawley rats injected with DMBA and treated i.p. with DMB, 2 animals receiving DMB every 7 days and 3 animals receiving DMB every 4 days developed grossly enlarged livers; the spleens were also enlarged in some of these animals. None of the rats receiving carcinogen only showed such pathology. In the group of Long-Evans rats injected with TMBA, 4 DMB-treated animals and 1 control developed enlarged livers and spleens.

In the group of Sprague-Dawley rats injected with DMBA and given DMB orally, 2 receiving 3 mg/2 days and 2 animals receiving 6 mg/2 days developed enlarged livers and spleens. No such pathology was found in animals receiving the carcinogen only.

The above-reported pathology is typical of leukemia produced by carcinogens.[16] Huggins[10] reports that in Sprague-Dawley rats DMBA selectively produces mammary tumors. Recently, Murad and von Haam have reported a few cases of leukemia in conjunction with mammary tumors in Sprague-Dawley rats.[17] In our experiments we have not found leukemia in any of the control rats of this strain. Those DMB-treated animals which developed these symptoms did not develop mammary tumors.

Histological examination of livers and spleens from the treated animals is underway.

This work was supported, in part, by the U.S. Atomic Energy Commission. One of us (U.R.J.) is a fellow of the Elsa U. Pardee Foundation for Cancer Research.

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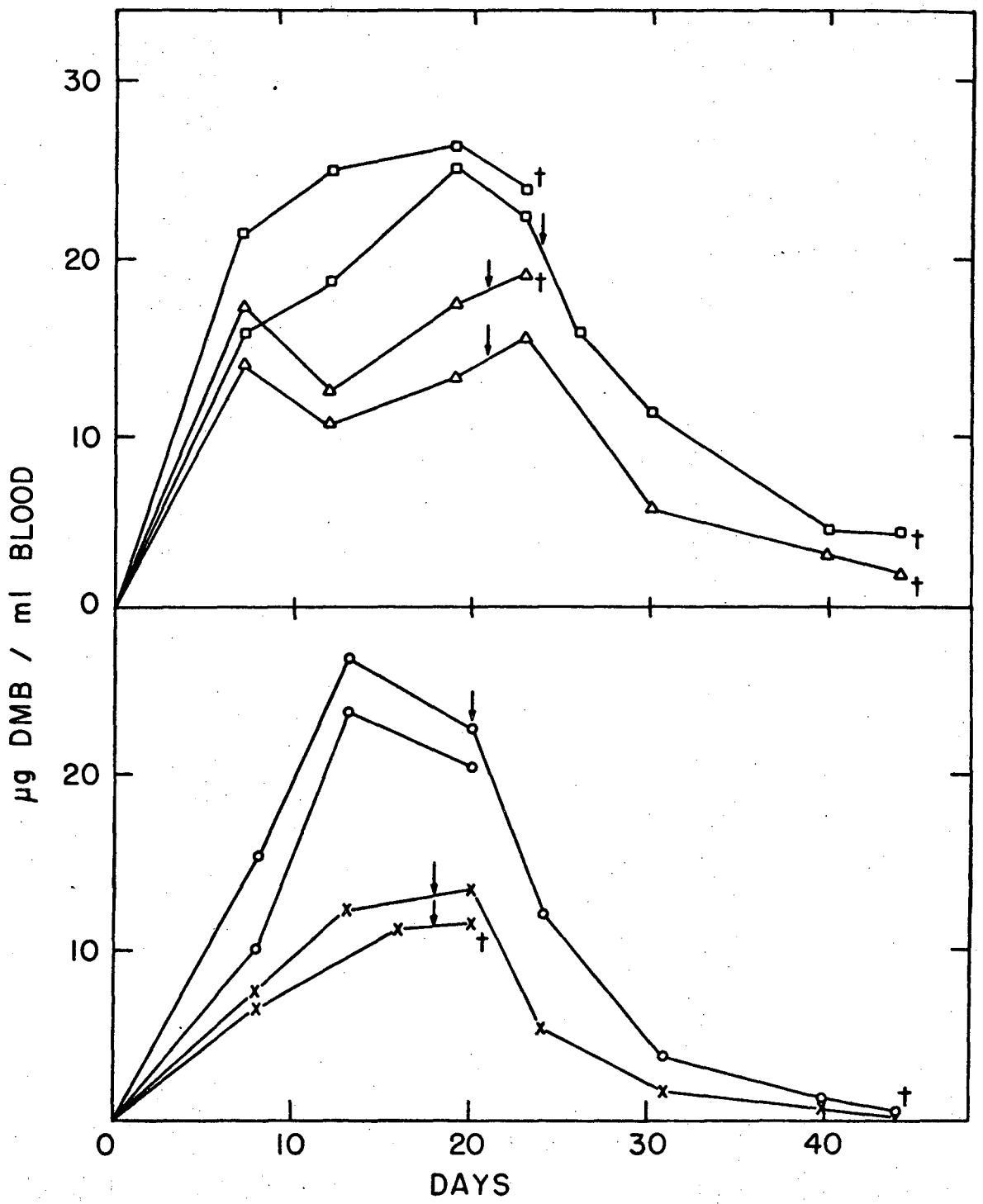
Figure Captions

Figure 1. Radioactivity measured in blood (taken from tail vein) and expressed in equivalents of DMB. The drug was given i.p. or orally in varying amounts. Two animals were used for each dose and method of administration. One animal was sacrificed three days after the last dose of DMB and in the other animal the blood assays were continued for three weeks after the last dose. The samples were taken between doses, but not earlier than 36 h after administration of a dose.

With injections of 10 mg DMB every seven days (Δ—Δ) tritium levels corresponding to 10-17 µg DMB/ml blood were maintained as long as the drug was continued. If the same dose was injected every 4 days (□—□) a level of 21 to 26 µg/ml was maintained.

The easier method of oral administration yielded blood levels corresponding to 8 to 13 µg/ml if 3 mg/2 days (X—X) and 20 to 26 µg/ml if 6 mg/2 days (o—o) were given. As soon as the DMB treatment was stopped, the blood levels dropped to less than 50% of the original level within one week and to less than 10% after three weeks for animals with oral administration. The levels of i.p. treated animals seemed to drop off somewhat more slowly. After three weeks, around 20% of the tritium activity measured at the time of the last dose was still found.



XBL 728-4737

Figure 2. Effect of DMB on the growth of DMBA-induced mammary tumors in female rats. This gives a comparison of the development of tumors in animals receiving 10 mg DMB i.p. every 7 days with control animals. At 8 weeks, 60% of the treated animals were without tumors, compared to only 10% of the control animals. At 16 weeks, 60% of the control animals had been sacrificed, due to mammary tumors, while only 10% of the DMB-treated animals had been sacrificed, and 10% showed no tumor. Not only does DMB retard the first appearance of tumors, but it slows the subsequent growth of those tumors which do appear. For instance, at 10 weeks, 6 of the 8 treated animals which had developed tumors had only small nodules, while 4 of the controls were already sacrificed and 2 others had large necrotic tumors. At 20 weeks (4 weeks after the cessation of DMB treatment) only 3 of the treated animals had been sacrificed because of tumors and 1 had a large necrotic tumor; 6 of the controls had been sacrificed and 2 had large necrotic tumors.

Code: (one symbol = 1 rat)

 = no tumor

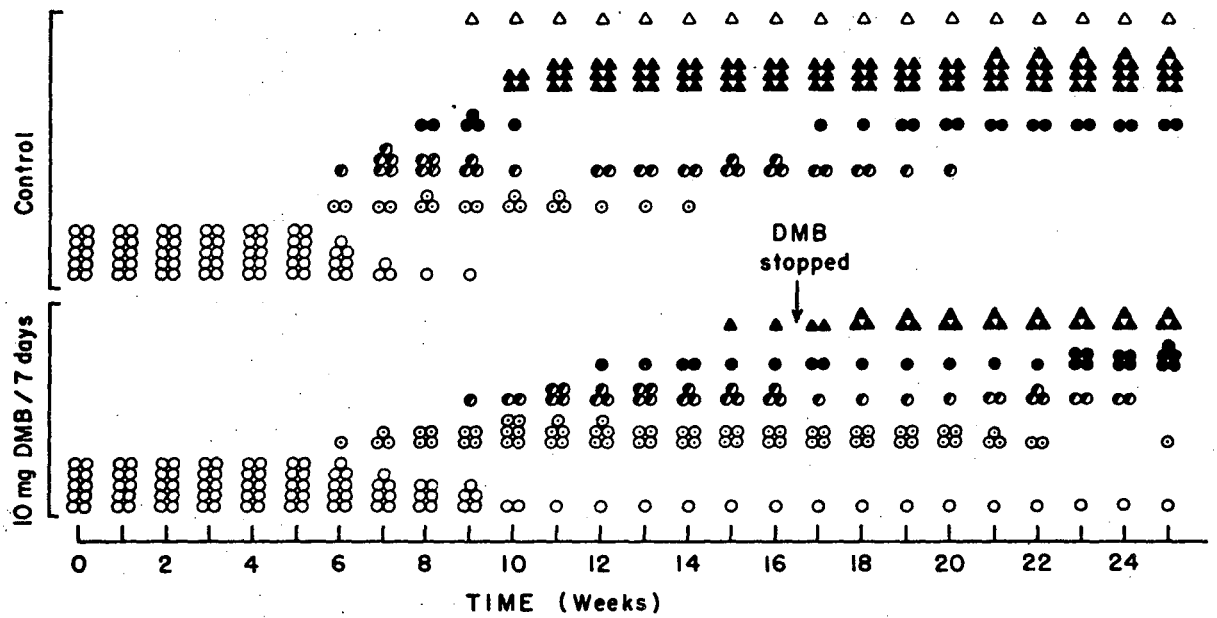
 = small tumor <1 cm

 = medium tumor ~2 cm

 = large tumor, usually necrotic

 = sacrificed due to mammary tumor

 = dead due to non-tumor-related causes


Control
10 mg DMB / 7 days
DMB
stopped
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (Weeks)

XBL 728-4716

Figure 3. Prophylactic effect of DMB (administered i.p.) against DMBA and TMBA. (Percentage of the total population unaffected by the carcinogen is plotted as a function of time.)

○ = control

△ = 10 mg DMB/7 days

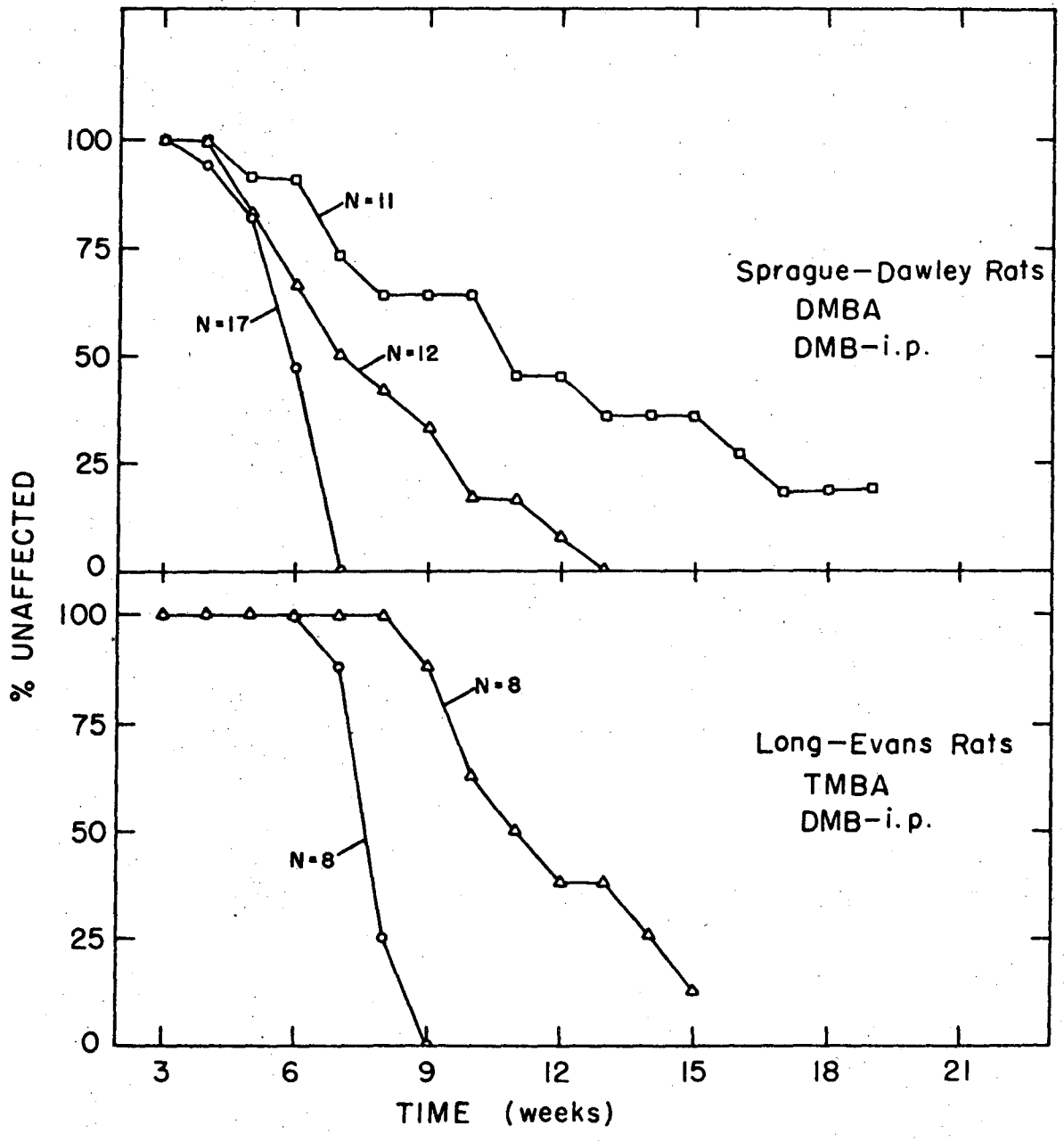
□ = 10 mg DMB/4 days

Figure 4. Prophylactic effect of DMB against DMBA administered orally. (Percentage of the total population unaffected by the carcinogen is plotted as a function of time.)

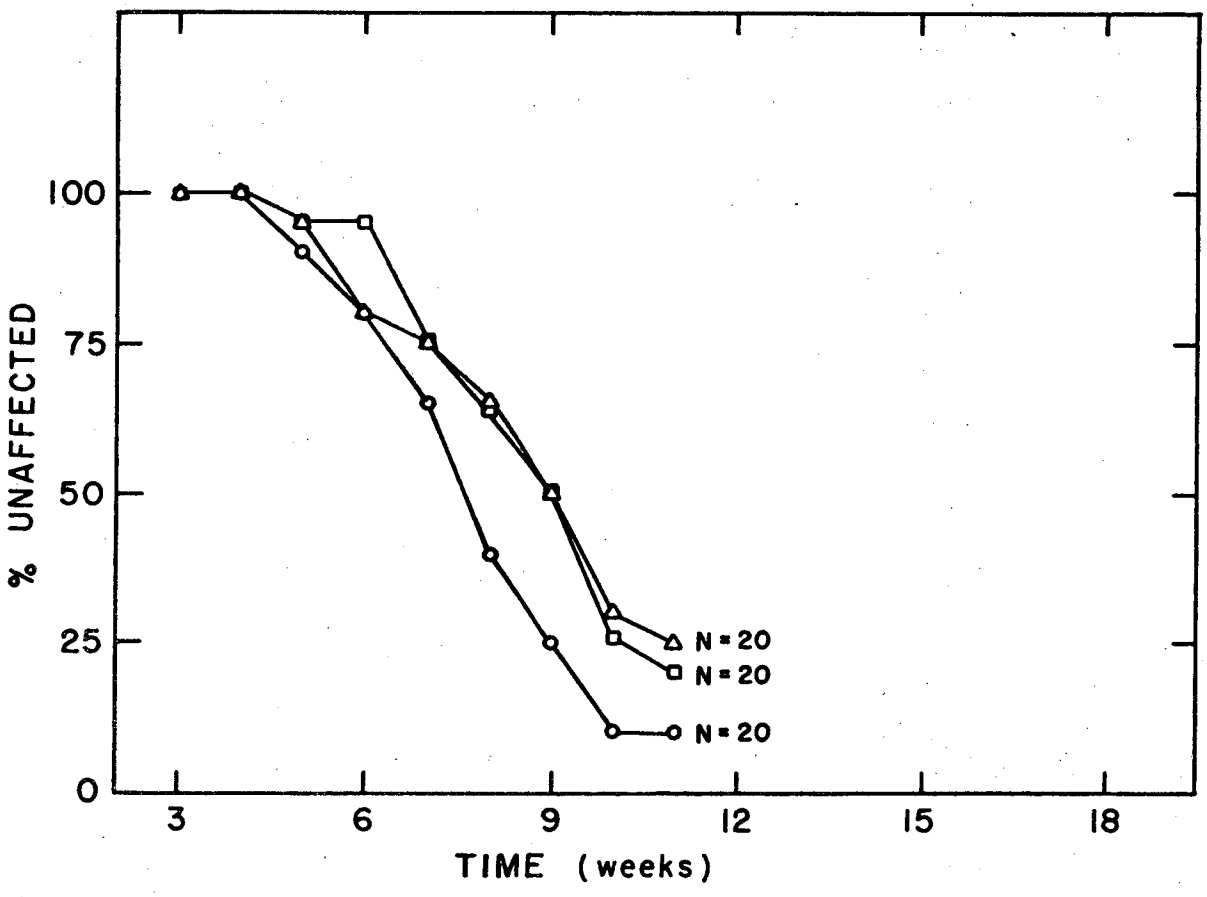
○ = control

△ = 3 mg DMB/2 days

□ = 6 mg DMB/2 days


100
75
50
25
0
% UNAFFECTED
N=11
N=17
N=12
Sprague-Dawley Rats
DMBA
DMB-i.p.
100
75
50
25
0
N=8
N=8
Long-Evans Rats
TMBA
DMB-i.p.
3
6
9
12
15
18
21
TIME (weeks)

XBL728-4738



XBL728-4739

Table 2. Radioactivity measured and expressed as µg DMB in various tissues of 4 rats with oral and i.p. administration in which blood levels were followed, after cessation of DMB administration. Tissue levels were determined by combustion at 3 weeks after the last dose. No optical absorption different from the one of untreated rats could be found in the visible region.

	oral		i.p.	
Dose given	3 mg/2 days	6 mg/2 days	10 mg/7 days	10 mg/4 days
Blood	0.2	0.6	1.6	4
Fat	< 0.2	< 0.4	1.1	6.3
Liver	1.4	8.4	28	86.8
Muscle	0.6	1.3	0.8	1.5
Intestine	0.4	0.75	5.2	5.7

Values expressed in µg/gram

Table 1. Radioactivity and optical absorption expressed in equivalents of DMB in µg/g in various tissues of one i.p.-treated and one orally-treated rat. The first column gives the values of the total DMB content measured in tritium activity. The second and third columns give the values of DMSO-extractable DMB measured by tritium activity and absorption at 486 nm. The two assay methods correspond well for the liver, fat, muscles and intestines of the i.p.-treated rat and for the fat of the orally-treated rat. This suggests that the DMB molecule, consisting of the basic rifamycin frame and the tritiated substituent in position 3, is still intact in these tissues. The optical assay indicated that there was no DMB in the liver, muscles and intestines of the orally-treated rat. However, absorption peaks around 400 nm and 520 nm were observed in the muscle extract of the orally-treated rat. These peaks do not occur in any of the tissue samples of a rat not given DMB. This suggests that these additional peaks stem from catabolic products of DMB. The spleen, kidney and blood extracts were strongly colored, and it was not possible to determine the absorption at 486 nm.

Table 1

	10 mg/4 days i.p.			3 mg/2 days orally		
	Tritium assay combust.	Tritium assay extract.	OD-assay extraction	Tritium assay combust.	Tritium assay extract.	OD-assay extraction
Blood	15.6		Not measurable	11.4	no data	Not measurable
Serum	0.5	0.5	< 2	0.5	"	"
Spleen	96.5	26	Not measurable	11.2	"	"
Kidney	43.2	10.3	"	16.4	"	"
Liver	132	17.8	20 ± 5	35.0	6.8	< 2
Fat	10.6	8.0	6 ± 2	9.0	9 ± 0.5	10 ± 1
Muscle	13.0	1.5	3 ± 2	4.9	1.0	< 2
Intestines	59.5	5.9	9 ± 4	20.0	7.6	< 2

values expressed in µg/gram

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