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Effect of Rifamycin and Tilorone Derivatives on Friend Virus Leukemia in Mice[1] (38968)

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RNA tumor viruses (oncornaviruses) contain within their cores an enzyme, RNA-directed DNA polymerase (reverse transcriptase) which transcribes their RNA to a corresponding DNA (1, 2). Integration of the newly formed viral DNA into the DNA of susceptible animal cells leads to the permanent introduction of viral genetic material that is probably necessary for viral replication, and is essential for the malignant transformation of normal cells by oncornaviruses. Intensive studies of the complex molecular events involved in viral DNA synthesis are reviewed in references (3–5).

Several agents, including derivatives of rifamycin (6) and analogs of tilorone and fluoranthene (7) are potent *in vitro* inhibitors of the oncornavirus RNA-directed DNA polymerase and block either the malignant transformation *in vitro* of normal cells by oncornaviruses or the replication of the transformed cells, or both (reviewed in 5). Since these agents also inhibit DNA polymerases of normal cells, one cannot assess the importance of viral enzyme inhibition alone. The rifamycin derivatives apparently act by binding to the viral enzymes (7, 8), while the tilorone–fluoranthene agents act by binding to the nucleic acid template (7).

In living animals rifampicin, a rifamycin derivative inactive on oncornavirus RNA-directed DNA polymerase, has been found to inhibit adenoviral oncogenesis in male, but not in female hamsters (9). This agent inhibited the hamster H-1 virus (a nononcogenic virus, latent in adult hamsters) but did not inhibit seven other viruses (10). Rifampicin, mixed with food, also caused 62–90% inhibition of splenomegaly in Rauscher virus-induced murine leukemia (11). Since rifampicin has negligible inhibitory activity against the viral enzymes, its effect must be produced by some other mechanism.

More importantly, perhaps, AF/ABDMP [for chemical structure, see reference (6)] a rifamycin derivative of high potency against oncornaviral enzymes, when emulsified and injected ip, was found to greatly delay the incidence of rat mammary cancers induced by intravenous injections of dimethylbenzanthrancene (12).

Members of the tilorone–fluoranthene group have been found to inhibit the Ehrlich carcinoma solid tumor and Friend virus leukemia (13). Tilorone HCl has shown definite anti-tumor activity against Walker carcinosarcoma and A-RCS reticulum sarcoma, but not against several other rodent tumors, including leukemia L1210 and P388 (14). Since these agents stimulate interferon synthesis and cause general immunologic enhancement in variable degrees (13) it is not possible to ascribe their inhibitory effect specifically to the blocking of viral enzymes.

We show here the effects of several agents of each group on the progress of Friend virus leukemia. Reports of the effect of most of these agents on tumors in living animals have not been found in the literature.

Materials and Methods. Mouse serum containing Friend leukemia virus (titer 3.71 spleen enlarging units/ml) was furnished by courtesy of Dr. Gerard Spahn, Microbiological Associates. Rifamycin derivatives were kindly furnished by Drs. W. Kump, H. Bickel, and Hans Heymann, Ciba-Geigy Corporation; and the tilorone–fluoranthene agents by Merrell Drug Co. (Cincinnati, Ohio). Methotrexate (parenteral) came from Lederle laboratories. BALB/c mice weighing 18–20 g were purchased from Microbiological Associates.

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TABLE I. INHIBITION BY RIFAMYCIN DERIVATIVES OF SPLENOMEGALY IN FRIEND VIRUS LEUKEMIA

Agent	Number of experiments	Number of mice scored	% Inhibition in each experiment	Average % inhibition
Methotrexate[a]	4	37	90, 92, 92, 91	92
C23[b] (48514)[c]	5	48	40, 42, 58, 59 56, 38	49
C20 (48411)	3	26	48, 20, 69	46
C11 (48623)	2	17	37, 24	31
C31 (48761)	3	18	18, 10, 0	9
C22 (48414)	1	9	20	
C27 (48733)	1	10	16	
C10 (48273)	1	9	0	
Rifampicin[d]	1	8	0	

[a] A folic acid inhibitor, inactive against the viral enzymes, used as a positive control.
[b] For chemical structure, see reference 6.
[c] Manufacturer's number.
[d] Obtained from Calbiochem, La Jolla, Calif.

In each experiment, ten mice served as normal spleen-weight controls, ten were given virus alone, and other groups of ten received virus plus the agents to be tested. Mice were infected by the injection of 0.05 ml of infected serum in the retroorbital plexus. Rifamycin derivatives were dissolved in dimethylsulfoxide (DMSO) and injected sc in the back at the level of 13.5 mg/kg approx equal to 20 ng/ml in the fluid component of the mice). Tilorone-fluoranthene agents were given sc in aqueous solution at 50 mg/kg. Methotrexate, 1.4 mg/kg, was used as a positive control. Rifamycin injections were given in 0.05 ml of fluid; tilorone–fluoranthene derivatives and methotrexate in 0.1 ml. Injections were given on days 0, 1, 2, and 14, 15, 16. Drugs were usually injected 30 min before or 30 min after the virus. Experiments were terminated by sacrificing animals with ether on days 20–30, at which time all spleens were weighed, and the percentage of inhibition of splenomegaly in each treated group was determined according to Munson *et al.* (13).

One experiment was designed to study the effects of two adjuvants (*Mycobacterium butyricum* and pertussis vaccine) alone and in combination with active derivatives. In another experiment, the effects of combined therapy with two active agents were investigated.

Results and Discussion. Mice given virus alone (virus control group) had average spleen weights varying from 520 to 1100 mg depending largely on the time each experiment was terminated. (Average weights of normal spleens were 80–90 mg.)

None of the agents evaluated in Tables I and II showed evidence of toxic effects, and the rate of weight gain of treated animals was comparable to that of the virus control group. About 1 in every 15 animals died during the course of the experiment, but deaths were not more numerous in the treated than in the virus control group. Animals which died were not scored unless death occurred a few hours before the planned termination of the experiment. All but 1 of 542 virus-injected animals, treated or untreated, showed evidence of leukemia (with the possible exception of some in the methotrexate-treated groups). Spleen weight inhibition was greatest when experiments were terminated on days 20–25, and was relatively low by day 30.

The positive control (methotrexate treated) mice uniformly showed 90–92% inhibition of splenomegaly. With the rifamycin derivatives, more variable results were obtained (Table I). However the inhibition of splenomegaly by C20, C23, and C11 is clearly significant. The low figures obtained with some of the other agents would perhaps have been higher if more experiments had been done. Even more variable results were obtained with the tilorone–fluoranthene group (Table II), but it is clear that tilorone HCl, M5, and

TABLE II. INHIBITION BY TILORONE–FLUORANTHENE GROUP OF SPLENOMEGALY IN FRIEND VIRUS LEUKEMIA

Agent	Number of experiments	Number of mice scored	% Inhibition in each experiment	Average % inhibition
M1[a] (10008)[b] (Tilorone HCL)	5	44	67, 25, 25, 23, 53	39
M5(100024)	3	27	69, 9, 19	32
M7(10635)	2	9	61, 35	48
M8(10874)	1	10	35	
M2(2557)	1	9	23	
M3(9563)	1	8	18	
M6(10233)	1	9	18	

[a] For chemical structure, see reference 6.
[b] Manufacturer's number.

M7 significantly inhibited, or more likely only retarded, the development of splenomegaly.

Correlation of in vivo *with* in vitro *effects.* It will be noted that C23 and C20, which most clearly inhibited splenomegaly, were among the five derivatives of rifamycin found most active against the viral enzymes at 20 ng/ml (6). Rifampicin and C10, the weakest members of the group in inhibiting mammalian cell DNA polymerase *in vitro* at 20 ng/ml (6) gave the only completely negative results *in vitro.*

Of the tilorone–fluoranthene group, M1 (tilorone HCl), M5, and M7, which most clearly inhibited splenomegaly, were among six out of the ten derivatives studied (7), which completely inhibited avian myeloblastosis virus DNA polymerase *in vitro* at 50 ng/ml. M8, which was probably effective *in vivo* (Table II) was another one of the six.

Adjuvants. 100 mg of *M. butyricum* was emulsified with 10 ml of saline and 0.2 ml/mouse was injected sc on the day of virus injection. Pertussis Vaccine (Lilly) 0.15 ml/mouse was given similarly. These adjuvants reduced the percentage of inhibition of splenomegaly by all agents tested except in the case of *M. butyricum* plus methotrexate (Table III). Each adjuvant given alone apparently enhanced splenomegaly.

Combined therapy. C23, C20, and M5, when each was combined in full dosage with methotrexate, gave 81, 70, and 57% inhibition of splenomegaly. Although methotrexate alone was not used in this experiment, there is obviously no synergistic reaction, and probably a lowering of the inhibition which would be expected by methotrexate alone.

TABLE III. EFFECT OF ADJUVANTS ON INHIBITION OF SPLENOMEGALY[a]

Adjuvant	Agent	% Inhib. alone	% Inhib. with adj.
M. butyricum	Methotrexate	91	96
M. butyricum	C23[b]	56	18
M. butyricum	C31	10	0
M. butyricum	M7[c]	61	32
M. butyricum	M1	53	18
Pert. vaccine	Methotrexate	91	78
Pert. vaccine	M1	53	46
M. butyricum			+12[d]
Pert. vaccine alone			+17[d]

[a] All figures are from a single experiment.
[b] For chemical structure, see reference 6.
[c] For chemical structure, see reference 7.
[d] % Enhancement when given alone.

Rifamycins given before and after the virus. In one experiment, C23 was given 3 hr before the virus in one group and 3 hr after virus in another. Spleen weight inhibition was 40 and 38%, respectively. Since it has been shown (15) that 3 hr after Friend virus injection the hemopoietic cells in the spleen are already transformed, our result suggests that viral enzyme inhibition is not the major mechanism by which C23 inhibited splenomegaly. Gielkens *et al.* (11) found that rifampicin (a rifamycin derivative with low viral enzyme inhibition) when given in a mixture

with mouse feed, was almost as effective (85%) in inhibiting splenomegaly when started 4 days after virus injection as when started on the day of virus injection (90%).

Comparison with results by others. Rifampicin, as noted above, caused 62–90% inhibition of splenomegaly in Rauscher leukemia (11) when mixed with food to give serum levels of 100 ng/ml. Our negative results at about 20 ng/ml are not inconsistent with this finding. AF/ABDMP, which was found to delay chemically induced rat mammary tumors (12) was not tested by us. *In vivo* results from the other seven rifamycin derivatives which we studied have not been found in the literature.

In the tilorone–fluoranthene group, Munson *et al.* (13) found that tilorone HCl at 50 mg/kg gave 86% inhibition in the four (out of eight) surviving mice (our average figure was 39%). Their figure for M7 at 50 mg/kg was 70% inhibition (our figure was 48%). Their figure for M6 at 100 mg/kg was 36% inhibition (our figure at 50 mg/kg was 18%). Their figure for M3 was 0% at 50 mg/kg (our figure was 18%). Figures are not available for comparison with our *in vivo* results using M5, M8, and M2.

The only agent reported to produce prolonged (2 mo) remission in Friend virus leukemia is statolon (16) which in addition to being a potent stimulator of interferon, apparently restores immunocompetence and stimulates cytotoxic antibody. Our results with adjuvants may be explained by the stimulation of serologic antibody, which under some conditions blocks the activity of immunocytes (immunologic enhancement).

The inhibitors used in this study will also block the activity of the cell DNA polymerases (5–7). Therefore we cannot conclude from our study that inhibition of the viral RNA-directed DNA polymerase plays an important role in the inhibition of splenomegaly.

Summary. Several derivatives of rifamycin, and analogs of the tilorone–fluoranthene group were tested for inhibition of splenic enlargement in Friend virus leukemia. At least three members of the rifamycin group caused significant inhibition (31–49%) as did at least three membes of the tilorone group (32–48%). These six compounds are among those found by others (6, 7) to be most inhibitory *in vitro* to the RNA-directed DNA polymerase of oncornaviruses. However our studies do not furnish direct evidence for or against a role of inhibition of the viral enzyme in the suppression of splenomegaly. None of the agents was as effective as methotrexate, which caused 90–92% inhibition. The activity of five of the agents was reduced, rather than enhanced by the injection of adjuvants (*M. butyricum* and pertussis vaccine). Three of the agents had a subtractive, rather than an additive effect on the inhibition caused by methotrexate alone.

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