The Influence of Homologs of Riboflavin on the Growth of Walker Rat Carcinoma 256

YOON SOO KIM, MARY M. APOSHIAN, AND J. P. LAMBOOY

Department of Biochemistry and the Eugene C. Eppley Institute for Cancer Research, University of Nebraska, College of Medicine, Omaha, Nebraska

Summary

7,8-Diethyl-10-(1'-D-ribityl)isoalloxazine (diethyl riboflavin), a potent antagonist of riboflavin in the rat, has been found to be nonspecific in the inhibition of the growth of Walker rat Carcinoma 256. 7-Ethyl-8-methyl-10-(1'-D-ribityl)isoalloxazine (7-ethyl-8-methylflavin) and 7-methyl-8-ethyl-10-(1'-D-ribityl)isoalloxazine (7-methyl-8-ethylflavin) have been found to replace riboflavin for the growth of and the efficiency of utilization of food by the riboflavin-deficient Sprague-Dawley rat. These 2 flavins do not, however, show the same interchangeability with riboflavin in the growth of Walker rat Carcinoma 256 in this rat. The growth rate of the tumor in the presence of 7-methyl-8-ethylflavin is indistinguishable from that observed in the presence of riboflavin. The growth rate of the tumor in the presence of 7-ethyl-8-methylflavin is 0.5 that observed in the presence of riboflavin.

Introduction

The importance of the tissue level of riboflavin in the metabolism of cancer was first pointed out by Morris and Robertson (15). They found that the growth rate of spontaneous mammary carcinoma in C3H mice was decreased by riboflavin deficiency. Later, Morris (14) showed that the growth of mammary adenocarcinoma was also retarded by the deficiency. These observations and the growing interest in the chemotherapeutic potential of antimetabolites stimulated efforts to synthesize antagonists of riboflavin. Conceivably, such antagonists could be used for the purpose of inducing riboflavin deficiency in cancer tissue when administered to a tumor-bearing animal.

Stoerk and Emerson (16) reported the regression of established lymphosarcoma (6C3H) in mice by the use of a riboflavin-deficient diet, but the effect could be brought about more promptly by the administration of 1 or the other of the relatively weak riboflavin antagonists, 6,7-dimethyl-10-(1'-D-ribityl)isoalloxazine (isoriboflavin), or 7,8-dimethyl-10-(1'-D-dulcitol)isoalloxazine (galactoflavin). Shortly after the appearance of the above report, Holly and associates (4) reported that 7,8-dichloro-10-(1'-D-sorbitol)isoalloxazine was particularly effective in bringing about the regression of the same lymphosarcoma in mice.

The synthesis (5) of 7,8-diethyl-10-(1'-D-ribityl)isoalloxazine (diethyl riboflavin) (IV) made available an especially potent antagonist of riboflavin in the rat. It was used by Aposhian and Lambooy (1) to retard significantly the growth of Walker rat Carcinoma 256 in Sprague-Dawley rats.

Some years later, Lane and co-workers (12) cited a private communication from H. G. Petering that the latter had observed partial to complete regression of established Murphy-Sturm lymphosarcoma and Walker rat carcinoma 256 in the use of 7,8-dimethyl-10-(2'-acetoxethyl)isoalloxazine. This compound was modified to 7,8-dimethyl-10-(2'-hemisuccinoyethyl)isoalloxazine and in this form was used by Lane and associates (13) to inhibit the growth of lymphosarcoma in rats.

In all cases utilizing riboflavin antagonists, with the exception of diethyl riboflavin, the quantities of material used varied from 0.5 to 7.4 mg/rat/day. Diethyl riboflavin was administered in daily doses of 32 μg. While it is true that in terms of the growth response of the riboflavin-deficient rat, diethyl riboflavin is the most potent antagonist of riboflavin to be described (inhibition index = 6) (2, 7), the apparent effectiveness of the material suggested that the inhibition of the tumor by the compound was at least in part due to a specific antitumor activity. Part of this report will show that this is not the case and that the only influence exerted by the compound on the tumor was the result of the simultaneous deprivation of riboflavin.

\[ \text{Inhibition index} = \frac{\text{μg analog at 0.5 maximum growth}}{0.3 \text{ μg of riboflavin}} \times \frac{\text{molecular weight of riboflavin}}{\text{molecular weight of homolog}} \]

1 This study was supported in part by grants from the National Cancer Institute: C-1677, CY-2940 and CA-07379-01. Part of this report was presented before the 8th Midwestern Biochemical Conference, Columbia, Mo., Nov. 5-6, 1965.

Received for publication November 3, 1965.
Diethyl riboflavin (IV) possesses some of the properties of riboflavin (I) with respect to its utilization by Lactobacillus casei ATCC No. 7469 (12) and Bacillus lactis acidii (2) throughout the range of limiting concentrations. Its riboflavin antagonism is shown in rats when large quantities are given, but it actually stimulates the growth of the riboflavin-deficient rat when intermediate quantities are given (2). This growth stimulus we now believe to be a manifestation of the material functioning as a coenzyme for some of the flavoprotein enzymes. The rat is able to phosphorylate this homolog of riboflavin (3). As will be pointed out in this paper, this potential to serve in place of riboflavin is revealed by the compound's ability to favor growth of the Walker rat carcinoma 256.

The administration of large quantities of diethyl riboflavin brings about the rapid induction of riboflavin deficiency in the rat. For example, if 2 mg are administered on each of 2 days, 8 days later the rats are riboflavin deficient (7). Yet, it is not practicable to give such quantities to the tumor-bearing rat because the inhibitory action of the compound in the host's tissues is so severe that the inevitable result is the death of the animal.

The fact that diethyl riboflavin can be utilized by some mammalian enzymes but not by others indicated that a biochemical selectivity based on structural specificity is involved. For this reason, it became of great interest to us to synthesize the 2 compounds, 7-ethyl-8-methyl-10-(1'-o-ribityl)isoalloxazine (7-ethyl-8-methylflavin) (II) and 7-methyl-8-ethyl-10-(1'-o-ribityl)isoalloxazine (7-methyl-8-ethylflavin) (III), which might be thought of as “half-steps” between riboflavin and diethyl riboflavin. These syntheses have been accomplished (8), and the biologic activities of these materials are such that they are almost capable of replacing riboflavin in the metabolism of the rat (10). They are unable, however, to support reproduction in the rat. For example, if 2 mg are administered on each of 2 days, 8 days later the rats are riboflavin deficient (7). Yet, it is not practicable to give such quantities to the tumor-bearing rat because the inhibitory action of the compound in the host's tissues is so severe that the inevitable result is the death of the animal.

The growth of Walker rat carcinoma 256 brings about the rapid induction of riboflavin deficiency in the rat. For example, if 2 mg are administered on each of 2 days, 8 days later the rats are riboflavin deficient (7). Yet, it is not practicable to give such quantities to the tumor-bearing rat because the inhibitory action of the compound in the host's tissues is so severe that the inevitable result is the death of the animal.

The fact that diethyl riboflavin can be utilized by some mammalian enzymes but not by others indicated that a biochemical selectivity based on structural specificity is involved. For this reason, it became of great interest to us to synthesize the 2 compounds, 7-ethyl-8-methyl-10-(1'-o-ribityl)isoalloxazine (7-ethyl-8-methylflavin) (II) and 7-methyl-8-ethyl-10-(1'-o-ribityl)isoalloxazine (7-methyl-8-ethylflavin) (III), which might be thought of as “half-steps” between riboflavin and diethyl riboflavin. These syntheses have been accomplished (8), and the biologic activities of these materials are such that they are almost capable of replacing riboflavin in the metabolism of the rat (10). They are unable, however, to support reproduction in the rat (9), and we can now report that 1 of these flavins, 7-ethyl-8-methylflavin (II), appears to have a specific inhibitory activity for Walker carcinoma 256 in the Sprague-Dawley rat. It appears to be a unique situation, since the needs of the host's tissues for flavin are satisfied by this homolog of riboflavin while the needs for Walker carcinoma 256 in the Sprague-Dawley rat. It appears to be a unique situation, since the needs of the host’s tissues for flavin are satisfied by this homolog of riboflavin while the needs for the tumor tissues are apparently not equally well satisfied. It is worthy of special comment that the material is not functioning as an inhibitor of riboflavin but as a replacement for riboflavin. It was administered mixed in a riboflavin-deficient diet at a concentration of 11.1 μg/gm of food. This quantity had been found to be equivalent to 5 μg of riboflavin/gm of food in terms of rate of growth and efficiency of food utilization (10).

Materials and Methods

Study I. Riboflavin versus Riboflavin Deficiency

Thirty male Sprague-Dawley rats weighing from 40 to 45 gm were fed a riboflavin-deficient diet described in 1 of our previous reports (1). After the animals had consumed this diet for 14 days, they were arranged into 10 pairs on the bases of body weight and the rate of gain of body weight; those not suitably matched or showing unusual weight changes were discarded. At this time, slice fragments of the Walker rat carcinoma 256 were implanted aseptically by trocar into the subcutaneous tissue of the right inguinal region of the rats. Both groups of pairs continued to receive the deficient diet, but 1 animal of each pair (Group R) was given by stomach tube 30 μg of riboflavin in 0.5 ml of 6% gum acacia solution each day immediately before being fed. Each of the other members of the pairs (Group D) was given the vehicle alone under the same regimen. The animals of each pair were pair-fed, the R member of each pair having its food limited to the quantity consumed by the D member, who was allowed to eat ad libitum during the time food was available. At the 1st sign of ulceration in any rat (no significant amount of fluid was lost), it and its pair-mate were killed and their tumors removed and weighed, and the tumorless body was also weighed.

Studies II, III, and IV. Riboflavin versus Riboflavin-deficient versus Diethyl Riboflavin

These studies were the same in general details of diet and preparation as those described above for Study I. In each of these studies, however, 64 rats were prepared and 16 trios of animals matched, the remaining 16 animals being designated as the deficient group (Group D). The animals in Group D were allowed to eat ad libitum, and their food consumption was not recorded nor was it related to that consumed by the other groups. In Study II, at the time of implantation, each animal (1 of a
Yoon Soo Kim, Mary M. Aposhian, and J. P. Lambooy

Chart 1. Volumes of the tumors growing in animals being fed a diet supplemented with riboflavin or 1 of the flavins shown. Day 0 is day of implantation. Day 21, 6 of 12 animals remaining in the riboflavin group; 8 of 12 remaining in the other 2 groups. All points on the 7-Et curve are significantly different from the corresponding points on the Rb curve from Day 6 to Day 21 (Day 6, $P = 0.007$; Day 9, $P = 0.002$; Day 12, $P = 0.001$; Day 15, $P = 0.001$; Day 18, $P = 0.009$; Day 21, $P = 0.002$). ME, methyl; ET, ethyl.

trio) in Group A received 3 µg of riboflavin, each animal (2nd of a trio) in Group B received 33 µg of riboflavin, and each animal (3rd of a trio) in Group C received 32 µg of diethyl riboflavin plus 3 µg of riboflavin each day as described above. The quantity of food consumed by the members of a trio in Groups B and C was matched to that consumed by the member of the trio in Group A. Studies III and IV were exact repetitions of Study II except that Group C received 100 µg of diethyl riboflavin and 3 µg of riboflavin each day.

Study V. Riboflavin versus 7-Ethyl-5-methylflavin versus 7-Methyl-8-ethylflavin

Forty-five rats weighing from 47 to 60 gm were distributed in a random manner into 3 groups: Group R [average weight, $54 ± 1$ (an estimate of the S.E. of the mean) gm], Group 7-Et (average weight, $54 ± 1$ gm), and Group 8-Et (average weight, $53 ± 1$ gm). The animals were fed ad libitum the riboflavin-deficient diet referred to above for Study I, except for certain changes in composition as described recently (11). The diet fed to Group R was supplemented with 5 µg of riboflavin/gm, that fed to Group 7-Et was supplemented with 11 µg of 7-ethyl-5-methylflavin/gm, and that fed to Group 8-Et was supplemented with 14.3 µg of 7-methyl-8-ethylflavin/gm. After 28 days on their respective diets, the animals of Groups R, 7-Et, and 8-Et had average weights of 204 ± 5 gm, 194 ± 5 gm, and 201 ± 6 gm, respectively. At this time, 3 rats were selected at random from each group as “nontumor” controls; the average weights of those from Groups R, 7-Et, and 8-Et were $196 ± 2$ gm, $191 ± 1$ gm, and $193 ± 1$ gm, respectively. All other animals were given a fragment implant of tumor. When the tumors became palpable (6th day), measurements of their size were begun. These measurements consisted of the major diameter (d) and the average minor diameter (d') in cm, and they were made at 3-day intervals for 15 days. The volumes of the tumors were calculated from these measurements. Each animal was killed at the 1st sign of ulceration of the tumor, and the tumor was removed, measured, weighed, and the tumorless body weighed. From these terminal values, the calculated volume and the weight of the tumor, its specific gravity was determined. No significant difference was found to exist among the specific gravities of the 3 groups, so all values were used to obtain the average value of $0.89 ± 0.03$ gm/ml. This value made it possible to make a reasonably good approximation of the body weight (total weight — tumor weight) of the animal at any time during the growth of the tumor.

Results

The results of Studies I–IV, in which riboflavin deficiency, riboflavin adequacy, and diethyl riboflavin were compared, are

The equivalence of these quantities of the 3 flavins was demonstrated in Reference 10.
Riboflavin Homologs and Walker 256

CHART 2. The body weights of the tumor-bearing animals. These weights were determined from the weights of the animal plus the tumor less the weight of the tumor calculated from the tumor volume times the average tumor specific gravity. Ultimately, the continued growth of the tumors in the remaining animals of the 7-Et group cause a continuation of the downward slope shown between Day 18 and Day 21. ME, methyl; ET, ethyl.

CHART 3. The ulceration times for the animals in the 3 groups. Note that 1 animal in the 7-ET group showed complete regression of a well-established tumor. RB, riboflavin; 8-ET, 7-methyl-8-ethylflavin; 7-ET, 7-ethyl-8-methylflavin.

Riboflavin is shown by the absence of a decreased tumor growth when the quantity of homolog was tripled. That the tumor appears to satisfy some of its flavin needs by the use of diethyl riboflavin is suggested by the better tumor growth when the homolog is given than when no flavin is given. The beneficial influence of the homolog on the host tissue has already been reported (1), and it is sufficient to say that this observation has been confirmed.

The results of Study V in which riboflavin, 7-ethyl-8-methylflavin, and 7-methyl-8-ethylflavin were compared are summarized in Charts 1-3. It is obvious from inspection of Chart 1 that riboflavin and 7-methyl-8-ethylflavin possess approximately equal activity for the growth of the tumor, while 7-ethyl-8-methylflavin is far less active. The average volumes of the tumors at Day 21 for the 3 groups were as follows: Rb = 41 ± 6 ml, 7-Et = 19 ± 3 ml, and 8-Et = 40 ± 7 ml. There is no difference between the Rb and 8-Et groups, but the difference between the Rb and 7-Et groups is large and represents a 54% inhibition of tumor growth.

Chart 2 shows that while riboflavin and 7-methyl-8-ethylflavin stimulate the rapid growth of the tumor, this growth is in some measure at the expense of the body tissues. Opposed to this normal course of events, the reduced stimulation of tumor growth by 7-ethyl-8-methylflavin is not equally harmful to the host's tissues; these are maintained in spite of the drain imposed by the growing tumor.

Chart 3 is a diagrammatic presentation of the ulceration times for the 3 groups. The Rb group and the 8-Et group are similar; the 7-Et group is different. One animal in Group 7-Et developed a well-established tumor (d = 2.0 cm; d' = 1.4 cm) by the 15th day. This tumor then regressed and did not reappear.

Discussion

7-Ethyl-8-methylflavin and 7-methyl-8-ethylflavin represent a new kind of antimetabolite. They do not exert their biologic activities by virtue of competition with riboflavin but simply by being used in enzyme systems in place of riboflavin. Thus, if
these materials are unable to serve in the biochemical reactions normally catalyzed by riboflavin-containing coenzymes, it must be due to their inability to combine with an apoenzyme, their inability to keep the apoenzyme saturated, or if they do combine with and keep that apoenzyme saturated, the holoenzyme must be inactive. There is no riboflavin present.

There 2 flavins can replace riboflavin in the metabolism of *L. casei* and in the growth, development, and longevity of the Wistar rat (10). Neither homolog is able to support reproduction in the Wistar rat because, apparently, certain critical enzymes function suboptimally during embryogenesis. (A report on the induction of a 100% incidence of a specific birth defect will soon be published from this laboratory.) We are now able to add to the growing list of biologic properties of these flavins that 1 of them, the 7-ethyl-8-methylflavin also fails to permit optimal function of some enzyme or enzymes during growth of the Walker rat carcinoma 256.

In terms of growth of the riboflavin-deficient Wistar rat, and the efficiency of the utilization of food, 7-ethyl-8-methylflavin has 47% and 7-methyl-8-ethylflavin has 36% of the activity of riboflavin. When the quantities of the flavin added to the diets are made equivalent in terms of these percentages of activity, the growth of rats given 1 or another of the 3 flavins permits equal growth. We know from this study that this is true for the Sprague-Dawley rat as well. For growth and efficiency of food utilization, 7-ethyl-8-methylflavin is 30% more potent than 7-methyl-8-ethylflavin, but in terms of the growth of Walker rat carcinoma 256, the former has less than 0.5 the activity of the latter during the period studied.

Acknowledgment

A rat bearing Walker rat carcinoma 256 from which the slice fragments were prepared was generously furnished by Dr. William F. Bale, The University of Rochester.

References

The Influence of Homologs of Riboflavin on the Growth of Walker Rat Carcinoma 256

Yoon Soo Kim, Mary M. Aposhian and J. P. Lambooy


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/26/7_Part_1/1344

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.