Laboratory and Clinical Studies with the Riboflavin Antagonist, Galactoflavin.* (29158)

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The induction of riboflavin deficiency has been reported to cause regression of tumors in rodents(1-5). Riboflavin restriction alone is unsatisfactory for the rapid and consistent production of an evident deficiency state in man(6-9) and does not lend itself to a clinical therapeutic trial. Several efforts from this laboratory to induce human deficiency rapidly by means of riboflavin antagonists effective in rats have met with failure(10,11). The drugs were rapidly hydrolyzed in humans, but in rats significant amounts of active drug were present in plasma and were excreted unchanged. We have studied various riboflavin antagonists in an attempt to find one which would have anti-tumor activity in rodents and would be metabolized similarly in rodents and in humans. This report describes pharmacological studies with 10-d-dulcityl-7,8-dimethyl-isoalloxazine, galactoflavin, an analog which produced regression of lymphosarcoma in mice(5). Signs suggestive of riboflavin deficiency appeared in 2 of 5 patients.

Methods. Male Sprague-Dawley rats weighing 160-210 g and maintained on stock laboratory chow were fasted for 24 hours and then fed by gavage 50 mg galactoflavin† per kg of body weight. Blood samples were obtained in heparinized syringes via jugular puncture and urine was collected in brown bottles. Water was available *ad libitum* during this 48 hour period.

Five patients with advanced neoplastic diseases were placed on diets calculated to contain less than 0.5 mg of riboflavin per day (12), at least 0.5 g of protein per kg of body weight, and calories equal to the subject's usual intake.‡ A vitamin and mineral supplement was given daily: Vit. A, 5,000 U; Vit. D, 1,000 U; thiamin hydrochloride, 3 mg; nicotinamide, 15 mg; pyridoxine hydrochloride, 2 mg; folic acid, 5 mg; cyanocobalamine, 1 μg; calcium pantothenate, 5 mg; ascorbic acid, 75 mg; d-α-tocopheryl acid succinate, 5 mg; cobaltous chloride $\cdot$ 6 H_2O, 2 mg; ferrous sulfate, 22 mg; cupric sulfate $\cdot$ 5 H_2O, 4 mg; potassium iodide, 0.2 mg; manganous sulfate $\cdot$ 5 H_2O, 1.5 mg; sodium molybdate $\cdot$ 2 H_2O, 0.5 mg; zinc sulfate $\cdot$ 7 H_2O, 5.3 mg; magnesium sulfate $\cdot$ 7H_2O, 61 mg. Hematopoietic, hepatic, and renal functions were studied by the usual clinical laboratry tests. After one week on the riboflavin-restricted diet patients were given single oral doses of galactoflavin initially, then were placed on oral maintenance doses. Blood and urine were analyzed for flavin content(10,11). Urine samples were studied by chromatography on Whatman No. 3 MM paper in 3 solvent systems: descending, butanol-acetic-water (4:1:5); ascending, butanol-pyridine-water (6:4:3); ascending, isoamyl alcohol-0.1 M potassium citrate (1:8). The dry, developed chromatograms were examined in the dark for fluorescent spots with a long wave length (3,600 Å) ultraviolet lamp. Urine pH was adjusted to 7.0 and plasma samples were deproteinized with 5% trichloracetic acid and restored to pH 7.4 prior to chromatography. The effect of galactoflavin on growth of *Lactobacillus casei* ATCC No. 7649 was measured following 24 hours of incubation in riboflavin free assay medium to which increasing amounts of riboflavin were added (11).

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‡ Examples of foods used in the diet are fruits and fruit juices, oatmeal, vegetables other than dried peas and beans, salmon, tuna, cottage and American cheese, eggs, coffee, sugar, hard candy, salt and pepper. Milk was limited to ½ cup a day. Daily calcium intake varied from 300-400 mg.

TABLE I. Plasma Flavin in Rats Following Single Oral Doses of Galactoflavin, 50 mg/kg.

Rat wt	Dose	Hr after drug admin 0	½	1	2	4
(g)	(mg)	(μg flavin per 100 ml plasma)				
170	8.5	5	12	11	13	
170	8.5	5	25	17	19	
190	9.5	5	9	10	13	
190	9.5	5	17	16		19
170	8.5	5	11	13		19
180	9.0	5	17	18		19
180	9.0	5	11	9		14

Results. Bacterial studies: The growth of *L. casei* was half-maximum at a ratio of galactoflavin to riboflavin of 323:1 (weight basis) when concentration of galactoflavin was 5.0 μg/ml (Fig. 1). Bacterial growth was completely inhibited at a galactoflavin-riboflavin ratio of 625:1 at this concentration of galactoflavin. The antagonism was reversed at a ratio of 200:1. Chromatograms of the bioassay medium to which galactoflavin was added and which was then autoclaved, cooled, inoculated with *L. casei* and incubated for 24 hours, revealed a single yellow fluorescent band corresponding in mobility to galactoflavin in each of the 3 solvent systems. The concentration of flavin in these samples, determined from fluorescence measurements, closely approximated the original amounts of galactoflavin in the samples.

Rat studies: The 24 hour urine flavin recovery in 4 rats fed a single dose of galactoflavin of 50 mg/kg ranged from 1.2 to 2.5% of the administered dose (112-226 mg). Urine and plasma from these rats contained a single yellow fluorescent component upon chromatography which had the same mobility as galactoflavin in the various solvent systems. The plasma flavin concentration increased 2 to 5 times above the fasting level within the first 4 hours (Table I). The highest level observed was 25 μg/100 ml of plasma.

Clinical studies: The duration of continuous drug administration varied from 22 to 68 days (average 42 days). One of the 4 patients (C.D.) receiving 1 g of galactoflavin 3 times a day complained of anorexia and nausea after 20 days which subsided on dosage reduction to 0.5 g twice daily. The other 3 patients tolerated 1 g three times daily for 22, 45 and 50 days respectively without adverse gastrointestinal symptoms. A dose of 3 g three times a day nauseated the patient on this program after 6 days. No toxic effects on the liver, kidneys, central nervous system or cardiovascular system were shown in these patients as determined by clinical and laboratory observations.

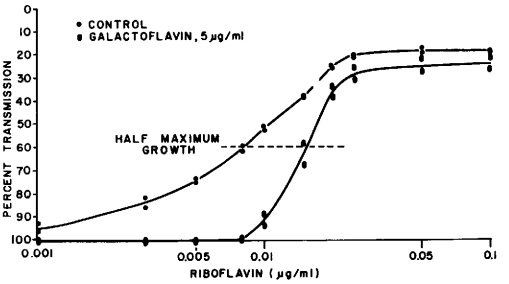

FIG. 1. Inhibition of growth of *Lactobacillus casei* by galactoflavin.

Table II shows the 24 hour urine total flavin following single oral doses of 1 g of galactoflavin to each patient. Chromatograms of urine and plasma samples from each of these patients showed a single fluorescent band which had the same mobility as galactoflavin in the 3 solvent systems previously cited. Table III illustrates the 24 hour urine flavin excretion for 2 patients following increasing single oral doses of galactoflavin.

TABLE II. Urine Flavin Following Single Oral Dose of 1 g of Galactoflavin to Patients.

Patient	Age	Sex	Primary cancer	Wt	Dose	Urine flavin Total	Urine flavin Recovery
	(yr)			(kg)	(mg/kg)	(mg)	(% of oral dose)
R.S.	51	♀	breast	63	16	101	10.1
T.C.	39	♀	lung	52	19	42	4.2
D.E.	58	♂	rectum	64	16	90	9.0
C.D.	55	♀	uterus	33	30	66	6.6
S.G.	47	♀	"	58	17	15	1.5

TABLE III. Urine Flavin Following Increasing Single Doses of Galactoflavin.

Patient	Dose	Hr after drug admin 0-8	8-16	16-24	24 hr urine flavin Total	Recovery
	(g)	(mg)	(mg)	(mg)	(mg)	(% of oral dose)
R.S.	1.0	45.2	45.3	10.2	100.7	10.1
	2.0	59.9	18.9	3.3	82.1	4.1
	3.0	59.2	16.2	3.6	79.0	2.6
S.G.	1.0	33.8	5.2	2.6	41.6	4.2
	2.0	29.7	6.4	1.3	37.4	1.9
	3.0	21.8	11.4	1.8	35.0	1.2

TABLE IV. Effect of Increasing Dose and Frequency of Galactoflavin Administration on Urine Flavin Content of Patient T.C.

Single dose	Frequency	Daily dose	24 hr urine flavin Total	Recovery
(g)		(g)	(mg)	(% of oral dose)
1.0	Once	1.0	66.3	6.6
.5	Every 8 hr	1.5	139.0	9.3
1.0	" " "	3.0	189.0	6.3
2.0	" " "	6.0	181.5	3.0
3.0	" " "	9.0	138.5	1.6

Excretion was maximum in the first 8 hours and approximately 90% of the drug recovered in 24 hours was excreted in the first 16 hours. Minimum quantities were excreted on the second day. Increases in dose from 1 to 3 g did not result in an increase in total urine flavin. Small decreases were actually observed. Table IV depicts the effect of increasing the dose and frequency of galactoflavin administration in the same patient. This produced a 2- to 3-fold increase in the 24 hour flavin excretion at dose levels of 0.5 to 3.0 g every 8 hours when compared to a single dose of 1 g. The percentage of the orally administered dose which appeared in the urine progressively decreased with increasing size of the oral dose. In Table V the details are shown for drug administration and the mean urine flavin content during continuous drug administration to each patient on various dosage schedules. The total dose of galactoflavin varied from 66 to 165 g with a mean of 117.3 g. The mean 24 hour flavin content varied from 31.9 g to 144.7 g in the 5 patients. Although there was marked variation from patient to patient in total flavin excretion, each individual excreted a fairly constant quantity of flavin in the urine for each dosage regimen.

Table VI illustrates plasma flavin concentrations following single oral doses of galacto-

TABLE V. Urine Flavin Content During Continuous Administration of Galactoflavin.

Patient	Dose	Interval between doses	Duration of treatment	Total dose	Daily urine flavin content
	(g)	(hr)	(days)	(g)	(mg)
R.S.	1.0	8	22	66	127 ± 5
T.C.	.5	8	5	7	110 ± 19
	1.0	8	7	21	145 ± 18
	1.5	8	4	18	120 ± 7
	2.0	8	5	30	144 ± 11
	3.0	8	6	54	137 ± 9
			27	130	
D.E.	.5	12	9	9	65 ± 6
	.5	8	14	21	109 ± 2
	1.0	8	45	135	129 ± 3
			68	165	
C.D.	.5	12	22	22	32 ± 2
	1.0	8	20	60	44 ± 2
			44	82	
S.G.	1.0	8	50	150	86 ± 4

C.D. also received galactoflavin on this schedule intermittently for 68 days, for a total of 110 days of drug administration and a cumulative dose of 150 g.

Tabulated daily urine flavin content is mean and standard error for the days drug was administered at each dose.

TABLE VI. Plasma Flavin Concentrations Following Single Oral Doses of Galactoflavin.

Patient	Dose	Hr after drug admin 0	1	2	3	4	24 hr urine flavin
	(g)	(μg flavin/100 ml plasma)					(mg)
R.S.	1.0	1		45	87	24	101
	2.0	0		49	80	23	82
	3.0	1		94	86	26	79
T.C.	1.0	0	49	72	63	24	66
D.E.	1.0	0		25	42	52	90
C.D.	1.0	0	16	21	10		15
S.G.	1.0	0	40	58	58	17	42
	2.0	0	40	73	63	18	37
	3.0	2	40	72	55	13	35

flavin. Twenty-four hour urinary flavin excretion on the day of the study is also shown. Concentration of plasma flavin appeared to peak in 2 to 4 hours, except in patient D.E. These peak levels varied from 20.6 to 94.4 μg per 100 ml of plasma. Similar plasma concentrations were observed following 1, 2 and 3 g doses in the same patient. The patient with the lowest urinary flavin excretion had the lowest plasma concentrations, but no other correlation between plasma levels and urine flavin content was apparent. In patients receiving continuous galactoflavin therapy 3 times a day, flavin concentrations in plasma obtained prior to drug administration in the morning varied from 4.8 to 48.2 μg per 100 ml of plasma. The higher plasma levels were observed in those patients who also showed high plasma concentrations 8 hours after single doses of drug (R.S. and D.E.).

Patients R.S. and C.D. developed sore mouth and throat, cheilosis and angular stomatitis after 14 and 53 days of galactoflavin administration, respectively. The findings abated in R.S. after discontinuation of galactoflavin and substitution of a regular diet, but persisted in C.D. Patient D.E. developed anemia and reticulocytopenia during 53 days of galactoflavin administration. The hemoglobin fell from 13.5 to 4.9 g per 100 ml of blood and the reticulocytes from 0.4-1.2% to 0.1%. The red blood cells were normal in the peripheral blood smears. Galactoflavin was discontinued and a regular diet was instituted. Reticulocytes reached a height of 4.4% in 17 days and hemoglobin concentration rose to 10.4 g in 30 days. Galactoflavin was readministered for 15 additional days without further effect on blood elements.

Discussion. Galactoflavin has been shown by others to be an effective antitumor agent in rodents(5). In the present studies it was a reversible inhibitor of the growth of *Lactobacillus casei*. Another riboflavin analog, the sodium hemisuccinate of ethanolflavin, had been shown to be itself inactive but was hydrolyzed to an active antagonist during the autoclaving step of a bioassay procedure (11). Galactoflavin did not appear to be altered in such fashion. The observed inhibition of bacterial growth could be attributed to the unchanged drug or to some derivative formed within the bacteria. Chromatograms of plasma and urine from patients and from rats fed galactoflavin revealed a single yellow fluorescent component with the same Rf as galactoflavin. Urine flavin recoveries and plasma flavin concentrations were somewhat higher in humans, but a major difference was not observed between rats and humans.

An oral pharmacological trial was undertaken with galactoflavin because its limited solubility precluded adequate parenteral administration. Doses as high as 3 g a day did not produce untoward gastrointestinal effects. Excretion of flavin in the urine was unchanged after a single oral dose of 1, 2 or 3 g in the same patient. When galactoflavin was given at 8 hour intervals, the flavin in urine increased 2 to 3 times above values obtained with the same total single dose. Urine flavin excretion reached a maximum at a dose of 1 g every 8 hours, so that prolonged drug administration was conducted on this schedule. The wide range of urine flavin excretion between patients bore no relation to body weight. Daily flavin excretion was approximately constant for each individual on long term drug administration. Flavin excretion in the urine decreased rapidly upon cessation of drug ingestion.

The erythematous oral mucosa, cheilosis, angular stomatitis, and glossitis which developed in 2 patients appeared characteristic of changes described in diet-induced riboflavin deficiency(8,9). The subsidence of lesions in patient R.S. upon withdrawal of

galactoflavin suggested that the lesions were induced by the drug. Prolonged periods of under-nutrition complicated the clinical course of patient C.D. and the mucosal lesions persisted despite galactoflavin withdrawal. It is probable that a number of factors contributed to the clinical picture in this case.

The anemia and reticulocytopenia observed in patient D.E. were due to depressed bone marrow function. Withdrawal of galactoflavin and substitution of a regular diet resulted in reticulocytosis and a rise in hemoglobin. Galactoflavin appeared to depress erythropoiesis and this finding may have been the result of induced riboflavin deficiency. However, the evidence is inconclusive. Anemia related to riboflavin deficiency has been described in swine(13), dogs(14), and monkeys (15), but proven cases of riboflavin deficiency anemia in humans have not been documented to our knowledge.

Since galactoflavin appeared to be well tolerated and had activity suggestive of a riboflavin antagonist in some of these patients, further studies of the drug have been initiated in combination with diets containing less riboflavin.

Summary. 1. The riboflavin analog, 10-d-dulcityl-7, 8-dimethyl-isoalloxazine (galactoflavin), was absorbed following oral administration to rats and to human patients. Plasma flavin levels and urine flavin excretion were greater in humans than in rodents. 2. Absorbed galactoflavin appeared unchanged in plasma and urine of both species. 3. Maximum absorption of drug was achieved by administration of 1.0 g doses at 8 hour intervals. This dosage was tolerated without undesirable effects for 2 to 5 months by 5 patients. 4. A program of galactoflavin administration in conjunction with a diet calculated to contain less than 0.5 mg riboflavin daily produced clinical signs and symptoms suggestive of riboflavin deficiency in 2 patients and anemia in one patient.

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