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Prevention of Cataract Development in Severely Galactosemic Rats by the Aldose Reductase Inhibitor, Tolrestat (42048)

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Abstract. With a fixed time period of galactose feeding, the rate of appearance of lenticular opacities depended on the severity of galactosemia, while with a fixed amount of galactose fed, the rate was time dependent. The capacity of tolrestat, a structurally novel inhibitor of aldose reductase (AR), to control cataract development was assessed in rats fed 30–50% galactose with the diet for 7 to 277 days. In rats fed 30% galactose for 31 days, the controlling effect of tolrestat was dose dependent, and no cataracts were detected at a dose of 35 mg/kg/day. In rats given tolrestat with the diet for 14 days, then rendered severely galactosemic with a diet containing 50% galactose, and subjected to continued treatment with tolrestat at a dose of 43 mg/kg/day, no changes were detected by slit-lamp microscopy after 207 days. The preventive effect was also dose dependent. In view of the established similarity in the pathogenesis of galactosemic and diabetic cataracts, the results obtained with tolrestat support its potential for controlling cataract development in diabetics.

According to Kinoshita's hypothesis (1, 2), the process of sugar cataract development is initiated by the osmotic effects of intracellular accumulation of polyols formed by the action of aldose reductase (AR). The concept has been substantiated by the use of AR inhibitors to control the development of cataracts in galactosemic (2–6) and diabetic (7–9) rats. The presence of sorbitol in the lens of the human diabetic (10), whether cataractous or not, suggests that the sequence of events postulated by the osmotic hypothesis may also occur in man (11). Adding to the interest in AR inhibitors is the probability that the consequences of increased AR activity in hyperglycemia may also play a role in the development of some other complications of chronic diabetes, e.g., of neuropathy (12–19).

We have recently described tolrestat (Alredase, *N*-[[5-(trifluoromethyl)-6-methoxy-1-naphthalenyl]thioxomethyl]*N*-methylglycine; AY-27, 773) (Fig. 1), a potent inhibitor of AR, which is structurally distinct from other reported AR inhibitors (20, 21). *In vitro,* tolrestat inhibits, in dose-dependent fashion, the AR from bovine lenses, $IC_{50} = 3.5 \times 10^{-8}$ *M*. Upon oral administration to galactosemic and streptozotocin-diabetic rats, tolrestat decreased, in dose-dependent fashion, the accumulation of galactitol and sorbitol in the lens and sciatic nerve. Pharmacokinetic and metabolic disposition studies with [^{14}C]tolrestat in laboratory animals (22) and man (23) demonstrated that tolrestat is well absorbed, undergoes elimination from serum at half-lives of 3.5 hr in rats, 11 hr in dogs, 9 hr in monkeys, and 10 hr in man, and does not accumulate in the body.

We describe herewith studies demonstrating the capacity of tolrestat to prevent the development of cataracts in severely galactosemic rats.

Materials and Methods. The AR inhibitors alrestatin (3) and tolrestat were synthesized in our department of chemistry. In all experiments, male Crl:COBS-CD (SD) rats (Sprague–Dawley, Charles River Breeding Laboratories) were used.

Rate of cataract development. The rate of cataract development as dependent on the amount of galactose ingested and duration of exposure to galactose was investigated in rats weighing 60–80 g, with unlimited access to water and to Purina Laboratory Chow containing galactose in amounts varying from 30 to 50% (w/w). A group fed chow was

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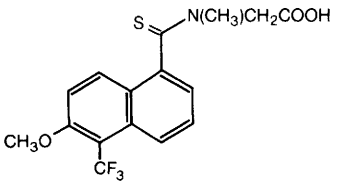

FIG. 1. Structure of tolrestat.

included to record the aspect of normal lenses. There were six animals per group. After 6, 8, 10, 14, and 20 days, a 2% aqueous solution of atropine sulfate was applied to the eyes, the rats were lightly anesthetized with ether or nembutal, and the eyes were examined by slit-lamp microscopy. Using a modification of the classification of Sippel (24), the detectable changes were arbitrarily graded into four stages, as described earlier (25), i.e., Stage 0: lenses similar to those of rats fed chow; Stage I: faint peripheral opacity; Stage II: irregular peripheral opacity and slight involvement of the lens center; Stage III: irregular opacity involving the entire lens; Stage IV: pronounced opacity, readily visible macroscopically as a white spot. The number of lenses which attained a given stage was recorded. In addition, an arbitrary "Opacity Index" (O.I.) was calculated for each group by giving no points to Stage 0, one point to Stage I, two points to Stage II, etc., and by dividing the sum of the points by the total number of lenses per group. The classification of the lenticular changes was carried out by persons who were not completely unaware of the treatment status of the animals, since they had to use the nongalactosemic and the galactosemic nontreated control groups as reference. The chosen stages of lenticular changes are, however, readily distinguishable, thus minimizing the possible effect of bias on the conclusions.

In short-term studies, rats were given access to a diet containing 40% galactose with or without an AR inhibitor, and the O.I. was assessed on Day 7. Alrestatin (0.7% admixed to the diet) was used as reference.

Galactitol accumulation in the lens. For 4 consecutive days, rats weighing about 70 g were given unlimited access to water and Purina Laboratory Chow supplemented with 10 to 50% galactose. On Day 5, about 2 hr after food withdrawal, the rats were killed, the lenses were removed, homogenized in 5% trichloroacetic acid, and the extracts were analyzed for galactitol by a modification of a method for glycerol determination (26).

Control of cataract appearance by tolrestat. Rats weighing about 75 g were given for 31 days free access to water and to Purina Laboratory Chow supplemented with 30% galactose (w/w) and alrestatin (0.7% of diet) or tolrestat (0.01, 0.015, and 0.02% of diet). The actual daily doses were calculated at the end of each experiment from the total food intake during the study and the mean body weight of the animals. There were 10 rats per group. The eyes were examined daily, and the appearance of macroscopically visible cataracts was recorded.

Prevention of cataract development in severely galactosemic rats by tolrestat. To test the role of the rate of uptake of tolrestat, rats were either pretreated with tolrestat, or not, before they were given access to galactose. Rats weighing 75–90 g were randomly divided into four groups with 10 animals per group. For 14 consecutive days, all rats were allowed free access to water and Purina Laboratory Chow, except that the chow given to Group IV contained 0.04% tolrestat. Starting with Day 15, 50% galactose was added to the chow for all rats except Group I, and 0.04% of tolrestat was added to the diets of Groups III and IV. Initially, the dose of tolrestat ingested with the diet was calculated two to three times per week from the averages of the recorded food intake and the mean body weights of the animals; later, such calculations were made every 2 or 3 weeks. At time intervals ranging from 1 to 4 weeks, the eyes of all rats were examined by slit-lamp microscopy; typical galactosemic lenticular changes were classified as described above. Group I served as untreated normal control, and Group II as untreated galactosemic control.

Subsequently, in another experiment, rats weighing 80–90 g were at random divided into seven groups with 10 rats per group. For 14 consecutive days, all rats were given free access to Purina Laboratory Chow and water; Groups III–VII received tolrestat admixed to the chow at five different concentrations, varying from 0.007 to 0.04%. At the end of the 14-day pretreatment period, 50%

galactose was admixed to the diet of all rats except Group I, and Groups III–VII continued to receive tolrestat at the same dose levels as during the pretreatment period. The actual doses of tolrestat were calculated as described above. At 2- to 4-week intervals, the eyes of all rats were examined by slit-lamp microscopy, and the lenticular changes were classified as described above.

Results. *Rate of cataract development monitored by slit-lamp microscopy.* The rate of cataract development depended upon the amount of galactose ingested and upon the duration of feeding on a galactose-containing diet: with a fixed amount of galactose admixed to the diet, the rate of cataract development was time dependent, while with a fixed time period of galactose feeding, the rate of cataract development depended upon the amount of galactose ingested (Table I). As shown in Table II, accumulation of galactitol in the lens of galactosemic rats was linearly related ($r = 0.954$, $P < 0.0008$) to the amount of galactose ingested. The lenticular galactitol levels found at a time when the lenticular changes are still reversible, i.e., on Day 5, in rats fed 25% galactose (45.8 ± 2.4 nmole/mg) and 50% galactose (68.8 ± 2.8 nmole/mg) are consistent with those reported earlier, viz., 38.6 ± 4.4 nmole/mg after 4–6 days on 25% galactose (25) and 73 nmole/mg after 5 days on 50% galactose (27).

TABLE I. RATE OF CATARACT DEVELOPMENT IN GALACTOSE-FED RATS: DEPENDENCE ON GALACTOSE INTAKE AND DURATION OF EXPOSURE

Galactose in diet (%)	Day	Percentage of lenses at Stage			
		I	II	III	IV
30	6	92	8		
	8	67	33		
	10	58	42		
	14		75	25	
	16		50	50	
	20		25	75	
40	6	17	58	25	
	8		42	58	
	10		50	50	
	14		8	75	17
	16			50	50
	20			17	83
50	6		75	25	
	8		42	58	
	10		25	75	
	14			33	67
	16			33	67
	20			25	75

Note. The lenses were examined by slit-lamp microscopy and arbitrarily graded into four stages (25). The number of lenses which attained a given stage is expressed as percentage of the total number of lenses per group. There were six rats per group.

TABLE II. GALACTITOL ACCUMULATION IN THE LENSES OF GALACTOSE-FED RATS: DEPENDENCE ON GALACTOSE INTAKE

Galactose		Galactitol accumulation in lenses (nmole/mg ± SE)[a]
In diet (%)	Ingested (g/kg/day)	
10	13.0	23.4 ± 1.4
15	20.6	42.2 ± 2.7
20	29.5	43.1 ± 2.0
25	34.0	45.8 ± 2.4
30	43.7	55.4 ± 4.9
40	65.0	62.8 ± 1.6
50	71.5	68.8 ± 2.8

[a] There were six rats per group.

The results in Fig. 2 illustrate the usefulness of the O.I. in short-term comparative studies of different AR inhibitors. We have selected a 7-day exposure to a diet containing 40% galactose and resulting in an O.I. >2.0. In three separate experiments with alrestatin, the O.I. varied in controls from 2.10 to 2.35, with no variation in the treated groups (O.I. = 1.0). With the experimental conditions used, tolrestat was at least 36 times more potent than alrestatin (Table III).

Control of cataract appearance by tolrestat. Alrestatin (983 mg/kg/day) and tolrestat (11, 16, and 20 mg/kg/day), given for 31 days admixed to a diet containing 30% galactose, had no effect on the food intake and body weight gain. As presented in Table IV, tolrestat produced a similar retardation in the rate of cataract appearance as alrestatin at about 50 times lower doses. The effect of tolrestat was dose dependent. In a separate experiment carried out under similar experimental conditions, no cataracts were detected in rats ingesting tolrestat at a dose of 35 mg/kg/day.

Prevention of cataract development in severely galactosemic rats by tolrestat. As shown

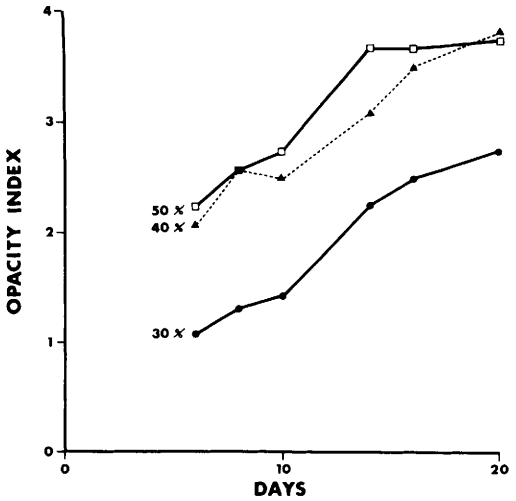

FIG. 2. Opacity Index (O.I.) of the lenses of galactosemic rats as a function of duration of exposure to diets containing 30, 40, or 50% galactose (w/w). The lenses were examined by slit-lamp microscopy and arbitrarily graded into four stages (25). The O.I. was calculated by giving no points to Stage 0, one point to Stage I, two points to Stage II, etc., then dividing the sum of the points by the total number of lenses per group. There were six rats per group.

TABLE IV. EFFECT OF AR INHIBITORS ON THE APPEARANCE OF VISIBLE CATARACTS IN RATS FED 30% GALACTOSE

	Percentage of lenses with visible cataracts				
			Tolrestat (mg/kg/day)		
Day of treatment	Controls	Alrestatin (mg/kg/day) 983	11	16	20
16	0	0	0	0	0
17	0	0	5	0	0
18	0	0	10	0	0
19	5	0	10	0	0
20	10	0	15	5	0
21	25	0	35	5	0
22	40	0	35	5	0
23	45	0	35	5	0
24	45	0	40	10	0
25	65	0	40	15	0
26	70	0	40	15	0
27	70	0	45	15	0
28	70	5	45	15	0
29	80	5	50	15	0
30	85	5	65	15	10
31	90	10	65	15	10

Note. There were 10 rats per group.

in Table V, 14-day pretreatment of rats with tolrestat (0.04% of the diet, corresponding to 58 mg/kg/day), followed by feeding chow containing 50% galactose and 0.04% tolrestat up to 277 days, prevented the appearance of cataracts. Without pretreatment, the development of cataracts was considerably delayed, but not prevented. On occasion, in rats fed galactose and tolrestat, some faint pin dots or shadows were noticeable; when appearing at the periphery, the changes were arbitrarily classified as Stage I and were combined with Stage 0; if occurring toward the center of the lens, the changes were marked with an asterisk. These faint changes are different from those typical of galactosemia and resulting in cataract formation. It is pertinent that, in a separate experiment with normal rats given for 90 days access to Purina Chow containing tolrestat at a dose of 70 mg/kg/day, the appearance of the lenses examined by slit-lamp microscopy was undistinguishable from those of rats fed Purina Chow.

TABLE III. EFFECT OF TOLRESTAT ON CATARACT DEVELOPMENT IN RATS FED 40% GALACTOSE FOR 7 DAYS

AR inhibitor	Dose (mg/kg/day for 7 days)	No. of affected lenses at Stage I	Stage II	Stage III	Opacity index
Control (10)[a]	—	0	10	10	2.5
Alrestatin (9)	1007	16	2	0	1.1
Tolrestat (10)	28	20	0	0	1.0

[a] Number of rats.

As illustrated in Table VI, in rats fed for 207 days on a diet containing 50% galactose, the effect of tolrestat on lenticular opacity development was dose related, the change between no cataract and cataract occurring in about one-half of the severely galactosemic rats treated with tolrestat at daily doses between 28 and 43 mg/kg. It is of interest that the faint changes marked with an asterisk were less frequent with the highest dose of tolrestat used.

Discussion. Although in rat lens the activity of AR is increased by diabetes but not by galactosemia (28), the galactose cataract is the easiest of the sugar cataracts to produce

TABLE V. CONTROL BY TOLRESTAT OF CATARACT DEVELOPMENT IN GALACTOSEMIC RATS: EFFECT OF PRETREATMENT

Group[a]	Day	Tolrestat (mg/kg day)	Percentage of lenses at Stage				
			O + I	*	II	III	IV
No pretreatment	111	63	10	0	30	10	50
	126	62	30	10	0	10	50
	146	61	15	35	0	0	50
	179	58	15	35	0	0	50
	206	61	15	35	0	0	50
	277	57	20	30	0	0	50
Pretreatment	111	56	85	15	0	0	0
	126	58	100	0	0	0	0
	146	55	100	0	0	0	0
	179	54	100	0	0	0	0
	206	56	95	5	0	0	0
	277	53	90	10	0	0	0

[a] All rats had free access to Purina Laboratory Chow containing 50% galactose (w/w) and 0.04% tolrestat (w/w). For 14 days before galactose administration, the pretreated group received chow with 0.04% tolrestat and the nonpretreated group, chow only. The average dose of tolrestat ingested in a given time interval was calculated from the average food intake and body weight. There were 10 rats per group. All untreated rats used as control had bilateral macroscopic cataracts after 28 days of access to 50% galactose in diet.

* Faint lenticular changes detected by slit-lamp microscopy as isolated pin dots or shadows in the center of the lens.

in rats and has been widely used as an experimental model since the initial observation of Mitchell (29). Our results show that, with a fixed time period of galactose feeding, the rate of appearance of lenticular opacities in rats depends on the severity of galactosemia, i.e., upon the amount of galactose ingested (30), while, with a fixed amount of galactose admixed to the diet, the rate is time dependent; similar results were thus obtained by feeding rats for 14 days on chow containing 30% galactose or for 6 days on chow containing 50% galactose.

The rate of cataract development in rats fed galactose depends on the amount of galactose absorbed and taken up by the lens and on the rates of AR-catalyzed formation of galactitol and its elimination in the lens. Our results demonstrate that initially, i.e., before the loss of functional integrity of the lens due to osmotic swelling, the accumulation of galactitol was linearly related to the amount of galactose ingested. The effect of an AR inhibitor on cataract formation, on the other hand, depends on its concentration at the site of AR in the lens at the time of galactose presentation. The degree of AR inhibition in the lens therefore depends on the interplay of the pharmacokinetics of the AR inhibitor with those of galactose and will thus vary in dependence of the experimental condition used. It is important to point out that in the 207-day study in rats fed a diet containing 50% galactose and varying doses of tolrestat, the ratio of AR inhibitor to AR substrate, i.e., galactose, varied from 1:6300 with the 10 mg/kg daily dose of tolrestat to 1:1175 with the dose of 57 mg/kg/day.

In the lenses of rats with acute severe galactosemia, the uptake of galactose and its conversion to galactitol appears to be much faster than the uptake of tolrestat. This is suggested by the finding that 14-day pretreatment with tolrestat prevented the appearance of any relevant lenticular changes in rats fed for 277 days on a diet containing 50% galactose and tolrestat at a dose of 58 mg/kg; however, even without pretreatment, cataract formation was greatly delayed. As shown in a separate experiment, the protective effect of tolrestat in severely galactosemic rats was dose dependent, and no relevant lenticular changes were detected by slit-lamp microscopy after feeding for 207 consecutive days a diet containing 50% galactose and 0.03% tolrestat [43 mg (120 μmole)/kg/day]. Pre-

TABLE VI. DOSE-DEPENDENCE OF THE PREVENTIVE EFFECT OF TOLRESTAT ON CATARACT DEVELOPMENT IN GALACTOSEMIC RATS

Tolrestat (mg/kg/day)	Day	Percentage of lenses at Stage				
		O + I	*	II	III	IV
9	28	0	0	0	10	90
10	55	0	0	0	0	100
20	28	30	0	5	35	30
21	55	20	10	0	0	70
19	109	0	10	0	0	90
18	150	0	10	0	0	90
19	207	0	10	0	0	90
28	28	55	0	15	10	20
30	55	20	35	0	0	45
28	109	20	25	0	0	55
27	150	15	30	0	0	55
27	207	20	25	0	0	55
42	28	100	0	0	0	0
44	55	70	30	0	0	0
44	109	70	30	0	0	0
43	150	70	30	0	0	0
43	207	70	30	0	0	0
59[a]	28	100	0	0	0	0
60	55	89	11	0	0	0
60	109	94	6	0	0	0
57	150	100	0	0	0	0
57	207	83	17	0	0	0

Note. All rats had for 14 days, free access to Purina Laboratory Chow containing 0.007, 0.015, 0.02, 0.03, or 0.04% tolrestat (w/w). From Day 15 on, 50% galactose (w/w) was added to the chow, and the treatment continued. The average dose of tolrestat ingested during a given time interval was calculated from the average food intake and body weight. There were 10 rats per group. All untreated galactosemic rats used as control had bilateral macroscopic cataracts after 28 days of access to 50% galactose in the diet.

[a] One rat died during the experiment.

* Faint lenticular changes detected by slit-lamp microscopy as isolated pin dots or shadows in the center of the lens.

vention of macroscopically visible cataracts in rats fed 50% galactose was previously observed with the AR inhibitor sorbinil when it was admixed to the diet at a dose of 60 mg (253 μmole)/kg/day (31). That cataract development in rats fed 50% galactose is prevented by treatment with two structurally distinct inhibitors of AR provides strong evidence that sugar cataract formation, at least in rats, is indeed initiated by AR.

Rats made acutely severely galactosemic by feeding on 50% galactose diet are often used to study the biochemical, functional, and morphological changes involving AR. It is important to take into account, however, the profound difference in the rates at which such changes are developed by severe galactosemia, particularly in the lens, as compared to those produced by hyperglycemia in diabetes.

In short-term experiments where macroscopic cataracts are as yet not developed, we have used slit-lamp microscopy to assess the lenticular changes and have arbitrarily calculated an "Opacity Index" to monitor the progression of the changes. Rats weighing about 70 g and fed for 7 days chow containing 40% galactose proved to be useful for preliminary evaluation of AR inhibitors.

Results obtained in human lenses by Chylack (11) suggest that diabetes may confer upon the lens an increased susceptibility to damage via osmotic stress from enhanced AR-catalyzed production of sorbitol and its decreased elimination. The increased activity of AR in the diabetic lens may thus account for the increased frequency with which cataracts mature in diabetic patients (32). It is pertinent that in human diabetic lenses incubated with glucose, the AR inhibitor alrestatin decreased the elevated sorbitol levels to those found in nondiabetic lenses (11). The results reported here show tolrestat to be about 50 times more potent than alrestatin in controlling the rate of lenticular opacity development. Because of its considerably greater AR inhibitory potency, tolrestat may have a much greater potential for controlling cataract development in diabetics. Interestingly, in a recent overview (33), potent AR inhibitors are regarded as the only potential anticataract agents currently in development that may prove effective in man.

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