

THE INFLUENCE OF A NEW RUTIN DERIVATIVE ON HOMOCYSTEINE, CHOLESTEROL AND TOTAL ANTIOXIDATIVE STATUS IN EXPERIMENTAL DIABETES IN RAT

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Abstract

Diabetes is a widely spread metabolic disease that involves disturbances in the glucose, lipid and mineral levels, as well as in redox reactions. One of the major risk factors in diabetes is the cardiovascular disease and its complications. Several biochemical parameters such as hypercholesterolemia, hyperhomocysteinemia and the reactive species are considered aggravating factors for the diabetes. The first therapeutic option in diabetes is to lower glucose levels and most often to decrease these major risk factors. The study investigated the influence of a new rutin derivative (coded L3) on the previously mentioned risk factors in experimentally-induced diabetes in rats. L3 contains a pyrimidine group attached to the rutin molecule. We envisaged two pharmacological activities for this compound: the effect of lipid level decrease and an anti-oxidative activity. Diabetes was induced by a single dose of 110 mg/kg bw of alloxan. The influence of L3 over the diabetic status was determined measuring the levels of glucose, cholesterol, homocysteine and total antioxidant status (TAS) initially and after one month of previous alloxan and L3 administration. The obtained data suggest that the new rutin derivative, coded L3, presents anti-oxidative properties and decreases cholesterol and homocysteine levels, thus conferring protection against certain major risk factors in diabetes.

Rezumat

Diabetul zaharat este o boală metabolică larg răspândită, care implică modificări ale nivelurilor de glucoză, lipide și minerale. Unul din factorii de risc major în boala diabetică îl constituie bolile cardiovasculare și complicațiile lor. Mai mulți parametri biochimici, cum sunt hipercolesterolemia, hiperhomocisteinemia și speciile reactive de oxigen sunt considerați factori agravanți ai diabetului. Primele opțiuni terapeutice sunt scăderea nivelurilor de glucoză și mai ales diminuarea acestor factori de risc. Studiul investighează influența unui nou derivat de rutozid (notat L3) asupra acestor factori de risc, în diabetul experimental la șobolani. Pentru derivatul L3 (care conține în structură un rest pirimidinic) s-au urmărit două efecte: hipolipemiant și antioxidant. Diabetul a fost indus

prin administrarea unei singure doze de aloxan (110 mg/kg corp). Influența derivatului L3 asupra statusului diabetic a fost determinată prin măsurarea nivelelor de glucoză, colesterol, homocisteină și statusul antioxidant total la momentul zero și după o lună de la administrare. Datele obținute sugerează că derivatul L3 prezintă proprietăți antioxidante și scade nivelele de colesterol și homocisteină, conferind astfel protecție împotriva factorilor de risc major din diabet.

Keywords: rutin, flavonoids, experimental diabetes, hyperhomocysteinemia.

Introduction

Diabetes is a widely spread metabolic disease and for this reason it is also referred to as “diabetic outbreak” [32, 36]. The severity of this disease consists not only in its high morbidity but also in the fact that the current life style determines the onset of this illness at even earlier ages than in the past [5]. Diabetes involves disturbances in the glucose, lipid and mineral levels, as well as in the redox reactions [21, 31]. One of the major risks factors in diabetes is the cardiovascular disease and its complications. Recently, hyperhomocysteinemia was also considered a risk factor in diabetes such as hypercholesterolemia. Clinical studies revealed that even small increases in homocysteine levels also increased significantly the risks for cardiovascular events [4, 10]. For the diabetic patient, hyperhomocysteinemia adds to an already impaired health and the vital risk increases exponentially [26, 33]. Hyperhomocysteinemia is a main cause in endothelial disorders, responsible for the disturbance of the endothelial functions including vasodilatation and anti-thrombosis. The reactive species are contributing to the mechanism through which homocysteine triggers these effects [25]. The reactive oxygen and nitrogen species cause the oxidation of many functional circulating compounds [29] and trigger the formation and release of species involved in cellular signaling [9]. Therefore hyperhomocysteinemia, added in a diabetic state, becomes an aggravating factor [3, 27]. Type 1 diabetes needs insulin replacement therapy to sustain life. Individuals with type 2 diabetes may not require insulin to survive, but 30% or more benefit from insulin therapy to control blood glucose. The traditional treatment choice for type 2 diabetes is represented by sulfonylureas and biguanides. Novel classes of rapid-acting insulin secretagogues (the meglitinides and D-phenylalanine derivatives) are alternatives to the short-acting sulfonylureas. All these different classes of drugs are mainly designed to keep glucose levels under control.

Rutin, also named rutoside, quercetin-3-rutinoside and sophorin, is a citrus flavonoid glycoside with pharmacologic activity, found in buckwheat, the leaves and petioles of *Rheum* species and asparagus [15]. Rutin inhibits platelet aggregation [12, 19] and prevents atherosclerotic lesions in mice [7],

rats [22], hamsters [2] and rabbits [13]. Rutin has also an antioxidant activity [18]. Recent studies pointed out that rutin reduces lipid levels [8, 11, 14, 34, 35]. Since hypercholesterolemia, hyperhomocysteinemia and oxidative status are aggravating factors in diabetes, our study aimed the investigation of the influence of a rutin derivative (coded L3) on the previously mentioned parameters in a model of experimental diabetes induced by alloxan administration, resulting in the damage of the pancreatic beta cells. The new rutin derivative has a rosuvastatin-like structure and is expected to have significant hypolipidemic effects. Consequently, we envisaged two pharmacological activities for this compound: the beneficial effect of lipid level decrease and an anti-oxidative activity.

Materials and Methods

The rutin derivative L3 was synthesized using a previously described general method, by reaction of rutin, 1, 3-dichloro-2-propanol and barbituric acid [17]. The structure of the rutin derivative is presented in Figure 1:

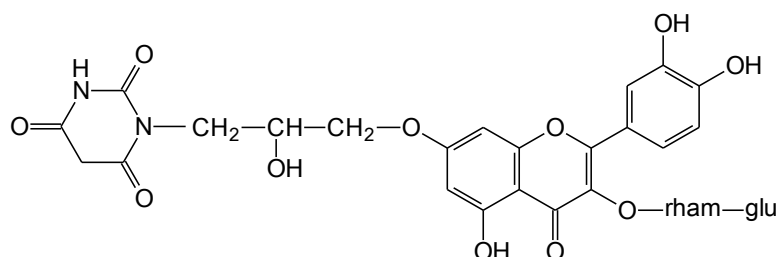


Figure 1
Chemical structure of rutin derivative L3

The melting point was measured using an Electrothermal Mel-Temp melting point apparatus and it is uncorrected. The IR spectrum was recorded on a FT/IR "Jasco 670 Plus" spectrometer. The ^1H -NMR spectrum was recorded on a Bruker AC-300F, 300MHz instrument using DMSO- d_6 as solvent. The elemental analysis was performed on an "Exeter Analytical" CE-440 elemental analyzer.

The pharmacological screening was performed on three groups of 10 adult Wistar male rats, weighing 150-200g. The animals from all groups received standard food that contained 0.5mg/kg body weight folic acid and 10 mg/kg body weight vitamin B12 and free access to water. All procedures were carried out in accordance with the Directive 86/609/EEC (24th

November 1986), regarding the protection of animals used for experimental and other scientific purposes.

Experimental diabetes was induced by intraperitoneal (i.p.) administration of a single dose of 110 mg/kg b.w. of alloxan [1]. Three groups of animals were defined as follows: a control group that received no treatment; a second group was administered alloxan 110mg/kg b. w., single dose i.p.; a third group received alloxan 110mg/kg b. w., single dose i.p. and L3 10.96 mg/ kg b.w. as single daily dose by oral route, for a period of 30 days. The dose for L3 derivative was calculated as 1/20 from 50% lethal dose (DL50). Samples of blood were taken from the retro-orbitary plexus/sinus at nine o'clock in the morning.

In order to investigate the influence of L3 in the diabetic status we have determined the concentration of the following parameters before and a month after alloxan administration: blood glucose, cholesterol, homocysteine concentration and total antioxidant status (TAS).

TAS in plasma, glucose and cholesterol concentrations were measured using a Radox kit for manual use. Total plasma homocysteine was determined by the immunoassay technique using an Axis Homocysteine EIA kit.

The statistical analysis for all determined parameters was realized by comparing groups using the analysis of variance (One-Way ANOVA) followed by the Bonferroni post hoc test; a coefficient $p < 0.05$ was considered to indicate a statistically significant difference between groups.

Results and Discussion

Chemistry

3-[[6-O-(6-deoxy- α -L-manopyranosyl)- β -D-glucopyranosyl] - oxy] - 2 -(3,4-dihydroxyphenyl)-5-hydroxy-7-(oxy-(β -hydroxy-propyl)-N-(2,4,6-trioxypyrimidinyl)-4H - 1 -benzopyran-4-one (**L3**)

Yellow crystalline powder (yield 82.47%), mp = 190-192°C, IR (KBr, cm^{-1}): 3300 (linked OH), 3000 (N-H), 2920 (CH), 1710 (C=O pyrimidine ring), 1660 (C=O aromatic ring), 1600 (aromatic structure), 1500 (aromatic C=C), 1365, 1310, 1210, 1060 (C-O-C), 810 (aromatic substitutes); $^1\text{H-NMR}$ (DMSO- d_6) δ ppm: 11.73 (s, 1H, NHCO), 7.47 (s, 1H, aromatic), 6.72 (d, 1H, aromatic), 6.29 (s, 1H, aromatic), 6.10 (s, 1H, aromatic), 5.42 (s, 1H, H-1 glucosyl), 4.54 (s, 1H, H-1 ramnosyl), 3.88 (s, 2H, CH_2N), 3.44 (s, 2H, CH_2CO); Elemental analysis: Calculated for $\text{C}_{34}\text{H}_{38}\text{N}_2\text{O}_{20}$: C: 51.38; H: 4.82; N: 3.52; Found: C: 51.52; H: 4.71; N: 3.48.

Pharmacological Screening

Glucose levels determined in rat plasma initially and after alloxan administration are presented in Table I:

Table I
Concentrations of glucose in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	Glucose concentration (mg/dL) Mean $\pm$ SD	
	Initial	Final
Control	78.31 $\pm$ 4.32	80.3 $\pm$ 3.25
Alloxan i.p. 110mg/kg b.w. single dose	72.12 $\pm$ 2.29	99.67 $\pm$ 3.34*
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	76.85 $\pm$ 3.98	80.028 $\pm$ 1.79

* The mean difference is significant at the $p = 0.05$ level

Our data show that for the control group, there is no statistical difference between the initial and final glucose concentration.

For animals that received alloxan, blood glucose levels are significantly increased 30 days after alloxan administration when compared with the initial moment, confirming the increase of glycemia that was expected ($p=0.0001$ when compared to the initial measurement).

For animals that received alloxan and the rutoside derivative L3, glucose levels are only slightly higher when compared with the initial moment.

A significant decrease in glucose concentration is found when comparing animals treated and not treated with L3, at the end of the study period ($p=0.0001$ compared to the final measurement).

We cannot assume that L3 has a hypoglycemic activity. We rather believe that the early administration of L3 prevents the onset of hyperglycemia.

The total antioxidant condition determined in rat plasma initially and after alloxan administration is presented in Table II:

Table II
Total antioxidant status in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	TAS (mmol/L plasma) Mean $\pm$ SD	
	Initial	Final
Control	1.61 $\pm$ 0.295	1.55 $\pm$ 0.296
Alloxan i.p. 110mg/kg b.w. single dose	1.48 $\pm$ 0.328	1.02 $\pm$ 0.074*
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	1.55 $\pm$ 0.443	1.45 $\pm$ 0.086

* The mean difference is significant at the $p = 0.05$ level

TAS levels present similar values for the control group at the beginning and at the end of the study. In the group that received only alloxan, hyperglycemia was present, and TAS levels decreased as compared to the initial moment ($p=0.0251$ as compared to the initial measurement).

In the group that received alloxan and the L3 derivative, TAS level also decreased after a month of L3 exposure as compared to the initial measurements, yet not significantly ($p=0.979$).

A significant increase of TAS concentration was observed in the group that received alloxan and the L3 derivative when compared to the group that received only alloxan, at the end of the study ($p=0.0484$ when comparison refers at the final measurement). These data suggest that in the group that received alloxan and the L3 derivative, the oxidative-reducing balance was less affected by either lower glucose levels or by the anti-oxidative activity of L3 derivative.

Homocysteine concentrations determined in rat plasma initially and after alloxan administration are presented in Table III:

Table III

Homocysteine concentrations in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	Hcys ($\mu\text{m/L}$) Mean $\pm$ SD	
	Initial	Final
Control	7.43 ± 1.04	7.28 ± 1.21
Alloxan i.p. 110mg/kg b.w. single dose	8.62 ± 1.47	8.97 ± 1.37
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	7.62 ± 0.61	6.67 ± 1.04

Homocysteine levels in the control group as well as in the group that received only alloxan showed no significant variation between the initial and the final moment of the study. These data suggest that high glucose levels maintained for a 30 days period does not generate an increase in the homocysteine levels.

For the group receiving alloxan and the rutin derivative L3, homocysteine levels decreased when comparing the final and the initial measurements, yet the differences were not statistically significant ($p=0.504$).

A significant decrease in the homocysteine levels was observed when comparing the group receiving alloxan and the rutin derivative L3 to the group that received only alloxan, at the final moment of the experiment. This suggests that L3 derivative manages to somehow decrease the homocysteine level ($p=0.0015$).

The cholesterol concentrations determined in rat plasma initially and after the alloxan administration are presented in Table IV:

Table IV

Cholesterol concentrations in rat plasma determined initially and after 30 days.

Group N = 10 animals/group	Cholesterol (mg/dL) Mean $\pm$ SD	
	Initial	Final
Control	48.29 $\pm$ 6.95	50.91 $\pm$ 5.38
Alloxan i.p. 110mg/kg b.w. single dose	56.03 $\pm$ 6.16	58.07 $\pm$ 8.82
Alloxan i.p. 110mg/kg b.w. single dose; L3 orally 10.96 mg/kg b.w. single daily dose, 30 days	58.26 $\pm$ 8.43	46.6925 $\pm$ 5.09*

* The mean difference is significant at the $p = 0.05$ level

For the control group as well as for the group that received only alloxan, the initial cholesterol concentrations are similar to those obtained at the end of the experiment. These data show that high glucose levels does not necessarily trigger the rise of cholesterol levels.

For the group that received alloxan and the L3 derivative, cholesterol levels present a significant decrease after a month of L3 exposure when compared with the initial measurements ($p=0.0103$ when compared to initial measurement). A significant decrease in cholesterol concentration was also observed when comparing the group that received alloxan and the L3 derivative to the group that received only alloxan at the end of the experiment ($p=0.012$ when compared to the final determination).

Hyperglycemia induced by alloxan is a type I diabetes, resulting from the damage of the pancreatic beta cells. High levels of glucose were present for a 30 days period in the conditions of alloxan administration. Even if hyperglycemic levels were not high, alloxan administration triggers a situation that mimics type I diabetes [6, 20, 30]. The alloxan model was chosen mainly for two reasons. The first reason is the ease of administering a single i.p. dose, protecting the animals from unnecessary stress. The second reason is the irreversible damage of the pancreatic beta cells that is induced by alloxan and is similar to the type I diabetes.

Consistent with the classically described model, a significant high levels of glucose were reached, so we can assume that a similar diabetes status was achieved. In the group receiving alloxan, the hyperglycemia triggers, by known mechanisms, the increase of oxidative events, which affects the oxidative-reducing balance. Our results are consistent with data cited by the literature [16].

Our data show that hyperglycemia does not increase substantially the homocysteine and the cholesterol levels. In a diabetic-like condition we experimentally induced, TAS concentration decreases significantly but the

cholesterol and the homocysteine show an almost normal level. These data suggest that after a month of exposure to hyperglycemia, the redox balance is the most affected parameter.

L3 derivative administration determines, in alloxan induced diabetes, a significant decrease in the glucose concentration, the homocysteine and cholesterol levels, and prevents the increase of the oxidative events. In diabetes, cholesterol as well as homocysteine represent aggravating factors and, as a consequence, the decrease in their concentrations determines a protective effect on the redox balance.

The L3 derivative was designed for two pharmacological activities: the decreasing effect of cholesterol levels generated by the pyrimidine ring (rosuvastatin like) and a protective effect on vessels, generated by the rutin side-structure. Our data indeed show that L3 derivative determines a significant decrease in the cholesterol levels.

On the other side, in the presence of L3, homocysteine levels significantly decrease. It is known that homocysteine is a cardiovascular risk factor affecting endothelium functions. Our data suggest that L3 administration may have a protective effect on blood vessel, by decreasing homocysteine levels.

Recent studies show that the excess of homocysteine conduces to an auto-oxidative reaction and generates free toxic radicals [23, 24, 28]. When homocysteine levels are low, the free radicals generation is scarce and, as a consequence, TAS levels are less affected. In L3 administration TAS levels decrease only slightly and correlate with the measured low homocysteine levels.

Therefore, our research suggests that L3 administration may exert a protective activity for diabetics' condition, reducing risk factors such as cholesterol, homocysteine and free-radicals.

L3 has a hypoglycemic activity, but we consider that the decrease obtained in glucose levels is mainly due to the early administration of L3 which prevents the onset of the hyperglycemia, rather than the prospect of curing the already installed hyperglycemia in diabetics.

We assume that the decrease in glucose levels is not primarily due to a hypoglycemic activity of L3, but is in fact a consequence of lowering effects of L3 on all aggravating risk factors.

Conclusion

The new rutin derivative taken into study, noted L3, presents anti-oxidative properties and a lowering activity on cholesterol and homocysteine levels in a similar diabetes state. It is also effective in

decreasing glucose levels when administered in the early stages of disease. It may be postulated that this effect is obtained through a decrease of the known risk factors mentioned above.

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