ing a preference for the D-isomer in the formation of monoester do not negate that model because the extreme favoring of D-isomer is only at the first step. Subsequent steps all involve bis 2′, 3′ esters and in the formation of diester, preexisting Ac-L-Phe monoester was shown to react 2.5 times as fast as preexisting Ac-D-Phe monoester[12]. During the formation of diester, the incoming D- and L-isomers reacted about the same, so the preference for D was lost at the formation of diester. However, the results showing that peptidylation can give an L-preference may require some rethinking of such models regarding the origin of protein synthesis. Because we know that evolution eventually devised a method of making only L-based peptides, the origin and evolution must have proceeded along lines which we see can favor the L-isomer given D-ribose nucleotides. The new insight furnished by previous and present data would suggest that intramolecular transfer of incoming amino acids from the adenylate anhydride to adenylate ester[14] and intermolecular transfer of the growing peptide might have been involved at some point. Both of these procedures favor the L-isomer. Furthermore, the favoring of the D-isomer may have been diminished if the reactions were carried out in a hydrophobic milieu perhaps at an oil-water interface as we have previously suggested[23].

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Potential aldose reductase inhibitors: 1,2,4-triazolidine-3,5-diones and 2-(3,4,5-trimethoxybenzoyl)-4,4-diethyl-3,5-isoxazolidinedione

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Abstract. 1,2,4-Triazolidine-3,5-diones and the 3,5-isoxazolidinedione were observed to be potent inhibitors of rat lens aldose reductase activity. In vivo in streptozotocin-diabetic rats, selected agents at 20 mg/kg/day, orally for 21 days reduced significantly the sorbitol levels of rbc, lens and sciatic nerves, suggesting that these derivatives may have some usefulness to treat clinical complications of diabetes mellitus.
Key words. Aldose reductase inhibitors; 1,2,4-triazolidine-3,5-diones; 3,5-isoxazolidinediones; diabetes.

Long-term diabetes leads to complications in other tissues including neuropathy (peripheral nerve dysfunction), nephropathy (intracapillary glomerulo sclerosis), vascular complications (capillary basement membrane thickness, pericyte loss in capillaries, aneurysm), retinopathy, cataracts, and skin and bone disorders[1–4]. In the last decade there has been considerable interest in aldose reductase inhibitors to control aldose reductase dependent polyol accumulation which initiates sugar cataract formation. Accumulation of polyol in the lens

can lead to hyperosmotic effects resulting in influx of fluid and to membrane permeability changes with loss of cellular integrity, swollen lens fibers, increased vacuoles, and increased lens hydration with reductions in the levels of GSH, NADPH, ATP, amino acids and myo-inositol[2]. Ion fluxes and protein synthesis are altered followed by a lens opacification[2]. A number of hydantoin derivatives, e.g. sorbinil (3-6-difluro-spirochroman-4,5′-imidazolidine-2′,2′-dione), as well as tolrestat (N-((5-(triflurometh yl)-6-methoxy-1-naphthalenyl)thioxomethyl)-N-methyl glycine), statil (3,4-bromo-2-fluro-benzyl-4-oxo-3H-phthalazine-ly)acetic acid; epalrestat (E-5-E-2-methyl-3-phenylpropenylidine-rhodanine-3-acetic acid), M-79,175 (2-methyl-6-fluro-spirochroman-4,5′-imidazolidine)-2,4′-dione; CT112 (5-(3-ethoxy-4-n-pentyloxyphenyl) thiazolidine-1,4-dione); AL1576 and ADN138 (8′-chloro-2′,3′-dihydrospiro(pyrrolidine-3′,6′-pyrrolo-(1,2,3-de)[1,4]benzoxazine)-2,5,5′-trione) have been investigated as aldose reductase inhibitors[2,5–8]. The purpose of this study was to investigate the possibility of 1,2,4-triazolidine-3,5-dione derivatives having aldose reductase inhibitor activity.

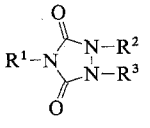
Methods

Source of compounds. A series of 1,2,4-triazolidine-3,5-diones (table 1)[9–12] and 2-(3,4,5-trimethoxybenzoyl)-4,4-diethyl-3,5-isoxazolidinedione[13–15] have previously been synthesized. The chemical and physical characteristics have been reported in these references. Substrates and cofactors for the biochemical assays were purchased from Sigma Chemical Co. The standard, tolrestat (Ay 27773), was a gift from Wyeth-Ayerst.

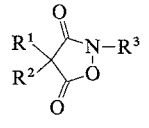
Pharmacology assays. In vitro aldose reductase (alditol:NADP oxidoreductase) activity was determined by the method of Hayman and Kinoshita[16] using DL-glyceraldehyde as a substrate, and the rate of oxidation of NADPH at 22 °C was determined over 5 min. Lenses were obtained from male Sprague Dawley rats (~ 350 g). The lenses were pooled, homogenized in distilled ice cold water (1:1) and centrifuged at 10,000 × g for 15 min. The supernatant was used for the enzyme assay. Drugs were prepared as a 1 mM stock solution in 1% carboxymethylcellulose/water (1% CMC) by homogenation. Drugs were tested at final concentrations of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M. IC_{50} values were calculated as the concentration which afforded 50% inhibition using a semilog plot on concentration of drugs vs percent inhibition.

Steptozotocin diabetes, i.e. induced elevated plasma glucose levels, was induced in rats using the method of Simard-Duquesne[4]. Sprague Dawley rats (~ 160 g) were fasted overnight. A freshly prepared solution of streptozotocin (STZ) (Sigma Chemical Co. in 0.03 M citrate buffer pH 4.5) was injected (0.1 ml) at a dose of 55 mg/kg, i.v. Control rats were injected with vehicle (1% CMC). Food (Ralston Rodent Chow) and water were provided ad libitum for the remainder of the experiment. The animals were maintained in 12-h cycles of light and dark at 22 °C. Forty-eight hours after STZ injection, the animals were bled from the ventral tail vein, and the serum glucose levels were determined (Sigma kit 510). The animals were divided at random into 4 groups each of which was administered one of the following drugs: diabetic-vehicle (1% CMC), diabetic-compound **7** at 20 mg/kg/day, diabetic-compound **9** at 20 mg/kg/day and diabetic-tolrestat at 10 mg/kg/day by oral intubation needle. Drugs were administered for 21 days, orally. The animals were sacrificed and the lenses, sciatic nerves and blood were collected from each animal. Each tissue was homogenized in 1.5 ml of 1 M perchloric acid and analyzed for sorbitol by the fluorometric method of Malone et al.[17]. The tissue samples were centrifuged; then 1 ml supernatant was adjusted to pH 9.0 with KOH and was neutralized, followed by centrifugation. Blood was centrifuged 3000 × g for 8 min, the plasma removed and rbc were reconstituted to original volume in buffered PBS, pH 7.4. A sorbitol standard (10 nM) was prepared. The supernatant (1 ml) or the rbc aliquot (1 ml) was

Table 1. IC_{50} Values of inhibition of rat lens aldose reductase in vitro



Compounds **1–8**



Compound **9**

Compound	R^1	R^2	R^3	Aldose reductase $IC_{50} \times 10^{-8}$ M
1	4-MeO-Ph	H	H	2.18
2	t-Bu	H	H	4.70
3	4-MeO-Ph	CCl_3CO	H	2.82
4	t-Bu	n-$C_5H_{11}CO$	H	1.68
5	4-NO_2-Ph	n-$C_5H_{11}CO$	H	0.93
6	Ph	MeCO	MeCO	4.80
7	Ph	MeCO	H	2.42
8	t-Bu	n-$C_5H_{11}CO$	n-$C_5H_{11}CO$	2.50
9	Et	Et	(3,4,5-MeO)$_3$-PhCO	1.85

Table 2. The effects of derivatives on in vivo sorbitol levels of Sprague-Dawley rats after 21 days administration at 20 mg/kg/day orally

N = 5 groups	Plasma glucose mg/dl ± SD	Sorbitol level Red blood cells nmol/g Hb ± SD	Lens nmol/mg ± SD	Sciatic nerve nmol/mg ± SD
Diabetic vehicle 1% CMC	455 ± 21	155.1 ± 12.4	36.74 ± 6.06	1.309 ± 0.114
Diabetic compound **7**	480 ± 22	100.5 ± 10.0*	14.29 ± 3.78*	0.903 ± 0.09*
Diabetic compound **9**	436 ± 21	86.2 ± 9.2*	16.10 ± 4.01*	0.797 ± 0.089*
Diabetic tolrestat	486 ± 22	36.1 ± 6.0*	16.82 ± 4.10*	0.770 ± 0.087*
Control vehicle 1% CMC	213 ± 15*	54.4 ± 7.3*	0.67 ± 2.58*	0.055 ± 0.074*

*$p \leq 0.001$ (Student's t-test)

added to 0.5 M glycine buffer pH 9.4 containing sorbitol dehydrogenase (Sigma Chemical Co.) (0.45 mg) and 0.14 mg NAD (Sigma Chemical Co.). The reaction was incubated for 30 min at 37 °C. The NADH fluorescence was measured using an Amino Bowman spectrofluorimeter with the excitation at 366 nm and the emission at 452 nm. Samples were performed in triplicate.

Results and discussion

These studies have demonstrated that 1,2,4-triazolidine-3,5-diones and 2-(3,4,5-trimethoxybenzoyl)-4,4-diethyl-3,5-isoxazolidinedione were effective inhibitors of aldose reductase activity in vitro with the IC_{50} values (table 1) using the rat lens being between 0.93 and 4.8×10^{-8} M concentrations. These values are comparable to the IC_{50} values for other aldose reductase inhibitors obtained from bovine lenses, e.g. tolrestat 3.5×10^{-8} M, but they were more active than sorbinil 1.5×10^{-6} M and alrestin 2.7×10^{-6} M. It should be noted that the triazolidinediones and the isoxazolidinedione represent examples of the few agents which have demonstrated potent inhibition of the enzyme activity without possessing carboxylic acid groups on the aromatic rings of the molecule, which supposedly denotes specific activity. The substituted R groups in compounds **1**–**8** suggest that some variability among the structural features leads to improved IC_{50} values over other substituted derivatives. For example, R^2 substitutions of n-$C_5H_{11}CO$ at position −1 in compounds **4** and **5** but not substituted at position −2 (R^3-H) as in compound **8** afforded good results in reducing enzyme activity, and 4-nitrophenyl substitution at position −4 gave excellent activity as in compound **5**. When compounds **7** and **9** were tested in vivo in rats with elevated blood glucose levels induced by streptozotocin two-fold over the control-vehicle 213 mg/dl baseline (table 2), the triazolidine-3,5-dione and the 2-benzoyl-3,5-isoxazolidinedione were active after 21 days of treatment. Compounds **7** and **9** reduced rbc sorbitol levels by 35% and 44%, respectively, which was not as significant as the reduction by tolrestat at 65%. Nevertheless, in the lens and the sciatic nerve, the sorbitol levels were reduced equally effectively based on the analysis of variance test. Obviously, further structure activity relationships need to be performed to determine the most potent agent in each chemical class as an aldose reductase inhibitor which can be used clinically.

Two major structural classes of aldose reductase inhibitors exist, i.e. spirohydantoins and carboxylic acid derivatives[8]. Whereas several of the triazolidinedione derivatives afford slightly acidic compounds because of the presence of hydrogen atoms at positions 1 and/or 2 of the ring, substitutions in both positions resulted in loss of activity, e.g. compound **8**. However, the substitutions at position 1 of the triazolidinediones appeared to be necessary for good activity. The 3,5-isoxazolidinediones[8] which have previously been reported were not active in vivo, nor were they substituted on the nitrogen at position 2. The current compound **9** was substituted with a benzoyl group at this position and was active in vivo in rats in lowering sorbitol levels in the rbc, lens and nerve. This compound is not acidic in nature. Thus it appears that these two chemical classes of agents offer additional potential agents for aldose reductase inhibition which do not fit the structure requirement proposed in the literature.

Eight-week studies have been performed in rats treated with compounds **7** and **9** at 20 mg/kg/day, orally. Based on clinical chemistry assays, hematological assessments, organ weights and tissue morphology no toxicity was demonstrated by these compounds[18].

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Effect of retinoic acid on liver transglutaminase activity and carbon tetrachloride-induced liver damage in mice

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Abstract. Transglutaminase (TGase) activity in the cytosol fraction of the mouse liver increased following intraperitoneal injection of retinoic acid. Retinoic acid inhibited the carbon tetrachloride-induced increase in serum alanine transaminase activity. These findings suggest that TGase is involved in the effect of retinoic acid on carbon tetrachloride-induced liver damage.
Key words. Retinoic acid; transglutaminase; liver damage; carbon tetrachloride; serum alanine transaminase; Ca^{2+} uptake.

Transglutaminase (TGase, EC 2.3.2.13) is a calcium-dependent enzyme that catalyzes the formation of covalent linkages between the gamma-carboxamide group of glutamine residues in some polypeptides and the amino groups of either peptide-bound lysine or primary amines[1-3]. Mammalian liver tissue is very rich in TGase[4,5], but its physiological role is almost unknown. Transglutaminase has been shown to cross-link hepatocellular cytokeratins in rat liver[6]. Cross-linking of cytokeratins is expected to increase the stability of the cytoskeletal network and influence the fluidity of the plasma membrane. Retinoic acid, a differentiation promoter[7], has been found to increase TGase activity in rat liver[8]. Recently, we reported that the degree of decrease in the TGase activity of the cytosol fraction induced by carbon tetrachloride treatment was closely related to an increase in serum alanine aminotransferase (ALAT) activity[9]. These findings suggest a relationship between TGase activity and resistance to hepatotoxins. In the present study we investigated whether an enhancement of TGase activity induced by retinoic acid treatment attenuates carbon tetrachloride-induced liver damage in mice.

Materials and methods

Animals. Male *ddY* mice weighing 18–25 g (SLC, Hamamatsu, Japan) were kept under the following conditions: a 12-h light-dark cycle (light: 09.00 h–21.00 h), temperature 23 ± 1 °C and humidity 55 ± 5%, and free access to food (F2, Funabashi Farms, Funabashi, Japan) and tap water.

Chemicals. [1,4-^{14}C]putrescine dihydrochloride (^{14}C-PUT, 4.4 GBq/mmol) and $^{45}CaCl_2$ (1.26 GBq/mg) were from New England Nuclear, USA. All-trans retinoic acid and N,N-dimethylcasein were from Sigma, USA. Putrescine dihydrochloride was from Nacalai Tesque, Japan. All other reagents were of analytical grade.

Retinoic acid treatment and tissue preparation for measurement of TGase. In the experiment with normal mice, each mouse received 200 μmol/kg of retinoic acid suspended in olive oil intraperitoneally. The liver was removed after perfusion with cold physiological saline at the indicated times under sodium pentobarbital anesthesia (50 mg/kg, i.p.). Mice of the control group were injected with olive oil. In the experiments involving carbon tetrachloride damage to the liver, mice were injected s.c. with olive oil (control liver) or carbon tetrachloride (0.25 g/kg) 8 h after i.p. injection of olive oil or retinoic acid (200 μmol/kg). After 16 h, the liver was removed after perfusion with cold physiological saline under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The liver was homogenized with 9 volumes of ice-cold buffer (0.25 M sucrose, 3 mM Tris, 1 mM EDTA, pH 7.4) by 10 strokes of a loose-fitting Dounce homogenizer. The homogenate was filtered through nylon mesh (No. 100) and subcellular fractionation was performed by the method described previously[9].

Measurement of TGase activity. Transglutaminase activity was measured by the method described previously[9], with the minor modification that the final pH in the assay mixture was 8.0. The enzyme activity was ex-