

Characterization of a Novel Aldose Reductase Inhibitor, TAT, and Its Effects on Streptozotocin-Induced Diabetic Neuropathy in Rats

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ABSTRACT—TAT {[5-(3-thienyl)tetrazol-1-yl]acetic acid} is a novel aldose reductase (AR) inhibitor. It exhibited highly potent inhibition of partially purified AR from rat lens ($IC_{50}=2.1 \times 10^{-8}$ M), rabbit lens ($IC_{50}=2.3 \times 10^{-8}$ M) and human placenta ($IC_{50}=2.8 \times 10^{-8}$ M). On the other hand, TAT had a weak inhibitory activity against mouse liver aldehyde reductase (ALR) ($IC_{50}=2.4 \times 10^{-6}$ M) and poor inhibitory activity against several adenine nucleotide-requiring enzymes. Against rat lens AR, TAT exhibited an uncompetitive inhibition at a concentration of 1.0×10^{-8} M and a mixed type inhibition at higher concentrations. TAT inhibited sorbitol accumulation in the isolated rat sciatic nerve ($IC_{50}=1.0 \times 10^{-6}$ M), rat lens ($IC_{50}=5.7 \times 10^{-6}$ M), human erythrocytes ($IC_{50}=2.5 \times 10^{-7}$ M), and rabbit erythrocytes ($IC_{50}=2.1 \times 10^{-7}$ M) incubated with high glucose concentrations. The oral administration of TAT (5–100 mg/kg/day) to streptozotocin (STZ)-induced diabetic rats during a 5-day treatment period decreased the sorbitol content in the sciatic nerve, dose-dependently (ED_{50} : 8.8 mg/kg/day for the prevention and 9.0 mg/kg/day for the reversal). Moreover, TAT (2.5–40 mg/kg/day) improved the decreased motor nerve conduction velocity (MNCV) after a 14-day treatment period. There was a significant correlation between MNCV and sciatic nerve sorbitol content. From these results, TAT is expected to be useful for the clinical treatment of diabetic complications.

Keywords: Aldose reductase inhibitor, Epalrestat, Sorbitol, Diabetic neuropathy, Motor nerve conduction velocity

The polyol pathway consists of two enzymes, aldose reductase (AR) (E.C. 1.1.1.21) and sorbitol dehydrogenase (SDH) (E.C. 1.1.1.14). While glucose has a high affinity for hexokinase, the affinity for AR is low. As a result, in the presence of a high glucose concentration, hexokinase becomes saturated and the polyol pathway becomes activated, resulting in the intracellular production of sorbitol and fructose in tissues where this pathway exists. The increased flux of glucose through the polyol pathway is believed to result in the pathogenesis of diabetic cataract (1, 2), retinopathy (3), neuropathy (4) and Groton nephropathy (5). AR inhibitors can reduce tissue sorbitol levels in diabetic animals by inhibiting the conversion of glucose to sorbitol. To date, a number of AR inhibitors such as sorbinil (Pfizer, Groton, CT, USA) (6), tolrestat (Ayerst, Princeton, NJ, USA) (7), epalrestat (Ono Pharm., Osaka) (8), AL-1576 (Alcon, Fort Worth, TX, USA) (9), statil (ICI Pharm., Macclesfield, UK) (10) FR-74366 (Fujisawa Pharm., Osaka) (11), AD-5467 (Takeda Pharm., Osaka) (12) and zopolrestat (Pfizer) (13) have

been found to improve some diabetic complications in animal experiments and have been developed to the point of clinical evaluation.

In this report, we describe the characterization of a novel AR inhibitor, TAT {[5-(3-thienyl)tetrazol-1-yl]acetic acid} (Fig. 1) and its effects on diabetic neuropathy in rats.

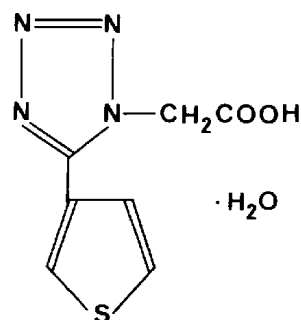


Fig. 1. Chemical structure of TAT, [5-(3-thienyl)tetrazol-1-yl]-acetic acid.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Clea Japan, Tokyo, 6- to 8-week-old), male C57BL/6J mice (Clea Japan, 10-week-old), and male albino rabbits (Japan Laboratory Animals, Tokyo, about 3.0 kg) were used.

Drugs

TAT (Fig. 1), epalrestat and statil were synthesized in the Research Department of Wakamoto Pharmaceutical Co.

For the *in vivo* experiments, TAT was suspended in 0.5% carboxymethyl cellulose and used.

In vitro studies

Preparation of AR: Rat lens AR and rabbit lens AR were prepared as follows: Lenses were homogenized in a Teflon homogenizer with 3 volumes of cold 135 mM sodium phosphate buffer (pH 7.0) and then centrifuged at $63,000 \times g$ for 30 min. The supernatant was dialyzed overnight against 0.05 M sodium chloride and centrifuged at $11,000 \times g$ for 20 min. The supernatant fraction was stored at -80°C until used. Human placenta AR was partially purified using a slight modification of the method of Maragoudakis et al. (14).

Other enzymes: Mouse liver aldehyde reductase (ALR) was prepared as follows: Mouse livers were homogenized in a Teflon homogenizer with 4 volumes of cold 10 mM Tris-HCl buffer (pH 7.3) containing 5 mM 2-mercaptoethanol and centrifuged at $63,000 \times g$ for 30 min. The supernatant was fractionated with 0–70% $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was dissolved in and dialyzed against the same buffer. The dialysate was stored at -80°C until used.

Hexokinase from yeast, lactate dehydrogenase from pig heart, and glutamate dehydrogenase from beef liver were purchased from Oriental Yeast Co. (Tokyo). G-6-P dehydrogenase from yeast and sorbitol dehydrogenase from sheep liver were purchased from Sigma (St. Louis, MO, USA).

Determination of AR and ALR activities and effects of inhibitors: AR activity was assayed spectrophotometrically by a Spectrophotometer UV-240 (Shimadzu, Kyoto). For this assay, we measured the decrease in the concentration of NADPH at 340 nm and 30°C . The reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.2), 0.4 M lithium sulfate, 10 mM DL-glyceraldehyde, 0.15 mM NADPH and the enzyme in a total volume of 1.0 ml. The reference blank contained all of the above compounds except DL-glyceraldehyde. The reaction was initiated by addition of the substrate, and the initial reaction rate was measured for 5 min. The effects of inhibitors on

the enzyme activity were determined by adding them to the reaction mixture. All compounds were dissolved in dimethylsulfoxide. Usually a 10 mM solution was prepared and diluted to the desired concentrations with saline.

Mouse liver ALR activity was assayed by the same method as used for AR activity, except for the substrate (10 mM D-glucuronic acid instead of DL-glyceraldehyde) and the absence of lithium sulfate. The concentration of inhibitor giving 50% inhibition of enzyme activity (IC_{50}) was estimated from the least-squares regression line of the log dose-response curve.

Sorbitol accumulation in intact cells and isolated tissues: The effect of TAT on sorbitol accumulation in tissues was determined according to the method described by Terashima et al. (8). Lenses and sciatic nerves were quickly removed from normal anesthetized rats. The removed lenses were incubated in 5 ml of medium equilibrated with 95% air and 5% CO_2 at 37°C for 4 hr. The medium consisted of 117.6 mM NaCl, 3.78 mM KCl, 0.54 mM MgSO_4 , 0.27 mM NaH_2PO_4 , 0.225 mM KH_2PO_4 , 26.1 mM NaHCO_3 , 0.9 mM KHCO_3 , 1.25 mM CaCl_2 , 50 mM glucose and 0 to 10^{-5} M TAT.

The removed sciatic nerves were incubated in 5 ml of medium equilibrated with 95% air and 5% CO_2 at 37°C for 6 hr. The medium was Krebs-Ringer bicarbonate buffer containing 50 mM glucose and 0 to 10^{-5} M TAT.

Red cells from heparinized rabbit and healthy human blood were washed two times with cold saline, and 1 ml of washed packed cells was incubated in 3 ml of medium equilibrated with 95% air and 5% CO_2 at 37°C for 3 hr. The medium was Krebs-Ringer bicarbonate buffer containing 28 mM glucose and 0 to 10^{-5} M TAT.

Sorbitol levels were measured by enzymatic assay using sorbitol dehydrogenase (Sigma)

In vivo studies

Rats, fasted overnight, were made diabetic by streptozotocin (STZ) injection (60 mg/kg body weight, *i.v.*; Sigma). The drug was dissolved in 3 mM citrate buffer (pH 4.5) immediately before injection. Five days after STZ injection, diabetic rats (plasma glucose, >300 mg/dl) were selected.

Three experiments were designed as follows: Experiment 1 and Experiment 2 were performed to estimate the effect of TAT on the prevention and reversal of sorbitol accumulation. In experiment 1, from the day of STZ injection, TAT was orally administered once daily at 5, 10, 20 and 40 mg/kg for 5 days. In experiment 2, 18 days after STZ injection, TAT was orally administered once daily at 5, 10, 20, 40 and 100 mg/kg for 5 days.

To determine the sorbitol content, the sciatic nerves were removed 3 hr after the final dosing with TAT,

weighed immediately and then frozen at -40°C .

Experiment 3 was undertaken to evaluate the reversal effect of TAT on motor nerve conduction velocity (MNCV). Fourteen days after STZ injection, TAT was orally administered once daily at 2.5, 10 and 40 mg/kg for 14 days. MNCV was measured according to the method of Miyoshi and Goto (15), before STZ injection and at the start point of treatment and one day after the final administration, respectively. After the measurement of MNCV, the rats were sacrificed under ether anesthesia, and the sciatic nerves were removed, weighed immediately, and frozen at -40°C until determination of sorbitol content.

Inhibition% was calculated as follows:

$$\text{Inhibition \%} = \{[D] - [T]\} / \{[D] - [N]\} \times 100$$

where [D] and [N] are the sorbitol contents of untreated diabetic and normal rats, respectively, and [T] is the sorbitol content of diabetic rats treated with TAT.

For determination of glucose level, the sample was drawn into a heparinized tube. Plasma glucose was determined with a glucose analyzer (Enzyme Electrode Analyzer AS200, Toyo Jozo, Tokyo).

Statistical methods

Results are expressed as the mean \pm S.D. The significance of differences were analyzed using Student's *t*-test and Dunnett's multiple comparison test.

RESULTS

Inhibition of aldose reductases and other enzymes

The inhibitory effects of TAT, epalrestat and statil on AR and ALR from various sources are summarized in Table 1. The IC_{50} values of TAT for rat lens, rabbit lens and human placenta AR were 2.1×10^{-8} , 2.3×10^{-8} and 2.8×10^{-8} M, respectively. TAT appeared to be a highly potent inhibitor of AR from these sources. It is noteworthy that TAT is more inhibitory against human placenta AR than epalrestat and statil.

Table 1. Inhibition of aldose and aldehyde reductases by aldose reductase inhibitors

ARI	IC_{50} (μM)			
	aldose reductase			aldehyde reductase
	RL	RaL	HP	ML
TAT	0.021	0.023	0.028	2.4
Epalrestat	0.022	0.039	0.051	1.3
Statil	0.014	0.030	0.052	1.5

ARI: aldose reductase inhibitor, RL: rat lens, RaL: rabbit lens, HP: human placenta, ML: mouse liver.

Table 2. Inhibition of various enzymes by TAT

Enzyme	Inhibition at 50 μM (%)
Hexokinase ^{a)}	9
G-6-P dehydrogenase ^{b)}	2
Lactate dehydrogenase ^{c)}	0
Sorbitol dehydrogenase ^{d)}	0
Glutamate dehydrogenase ^{e)}	7

^{a)}yeast, ^{b)}yeast, ^{c)}pig heart, ^{d)}sheep liver, ^{e)}beef liver.

The IC_{50} value of TAT for mouse liver ALR was 2.4×10^{-6} M. TAT is less inhibitory than epalrestat and statil. The inhibitory effect of TAT on a number of adenine nucleotide-requiring enzymes (hexokinase, G-6-P dehydrogenase, lactate dehydrogenase, sorbitol dehydrogenase and glutamate dehydrogenase) were investigated. The results are summarized in Table 2. These enzymes were unaffected or only slightly inhibited by 50 μM TAT.

Kinetic studies

An enzyme kinetic study of TAT was conducted with rat lens AR. The Lineweaver-Burke plot shows that TAT was an uncompetitive inhibitor at low concentration of 1.0×10^{-8} M and a mixed type inhibitor at higher concentrations (Fig. 2).

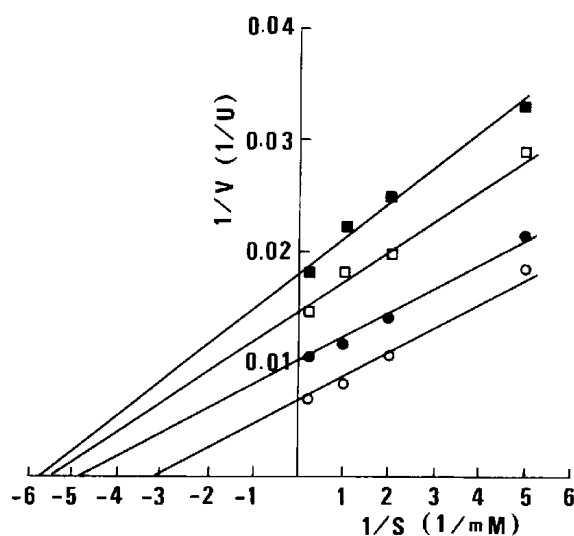


Fig. 2. Effect of TAT on the Lineweaver-Burke plot of rat lens aldose reductase activity with glyceraldehyde as the substrate. The ordinate represents the reciprocal of initial velocity expressed as the change in optical density per 5 min. The abscissa represents the reciprocal of glyceraldehyde concentrations ranging between 0.2 and 10 mM. \bullet , 1×10^{-8} M; \square , 2×10^{-8} M; \blacksquare , 3×10^{-8} M TAT; and \circ , uninhibited control.

Inhibition of sorbitol accumulation in cultured tissues

The ability of TAT to inhibit sorbitol accumulation was examined in isolated rat sciatic nerve and lens and washed human and rabbit erythrocytes. The results of these studies are summarized in Table 3. TAT inhibited sorbitol accumulation concentration-dependently with IC_{50} values of 2.5×10^{-7} M for human erythrocytes and 2.1×10^{-7} M for rabbit erythrocytes, respectively. The IC_{50} values of TAT for rat sciatic nerve and lens were 1.0×10^{-6} M and 5.7×10^{-6} M, respectively.

Inhibition of polyol accumulation in sciatic nerve

To investigate the inhibitory effect of TAT on sorbitol accumulation in the sciatic nerve of diabetic rats, two experiments were designed. TAT was orally administered to diabetic rats for 5 days, immediately or 18 days after STZ injection. As shown in Table 4, the diabetic rats had significantly higher glucose level at the time of sacrifice than normal rats. TAT had no effect on blood glucose level.

In untreated diabetic rats of two experiments (Tables 5 and 6), sorbitol was markedly accumulated in the sciatic

nerve. Administration of TAT prevented the accumulation of sorbitol dose-dependently. The ED_{50} values for the prevention and the reversal were 8.8 and 9.0 mg/kg, respectively.

Effect on MNCV in diabetic rats

In experiment 3 (Table 7), 14 days after STZ injection, each MNCV of untreated diabetic rats showed a significant reduction compared with that of normal rats. Treatment of each diabetic rat with TAT at 40 mg/kg for the following 14 days recovered dose-dependently the decreased MNCV to approximately the normal level.

A statistically significant improvement in MNCV was observed at 10 and 40 mg/kg. There were no significant differences in MNCV between normal rats and TAT-administered rats at 10 and 40 mg/kg. The correlation between MNCV and sciatic nerve sorbitol content is plotted in Fig. 3. There was a significant correlation ($r = -0.402$, $P < 0.01$) between MNCV and sciatic nerve sorbitol content.

Table 3. Inhibitory effect of TAT on sorbitol accumulation in intact cells and isolated tissues

TAT (M)	Erythrocytes (nmol/g Hb)		Sciatic nerve (nmol/g wet wt.) (n=3)	Lens (μ mol/g wet wt.) (n=6)
	human (n=4)	rabbit (n=2)		
Non incubation	28.2 \pm 3.8	19.2 \pm 5.0	32.3 \pm 28.2	0.03 \pm 0.05
0	106.1 \pm 2.7	48.3 \pm 3.0	401.3 \pm 70.8	1.11 \pm 0.23
10^{-7}	78.4 \pm 4.2**	36.9 \pm 3.3*	322.0 \pm 70.6	1.38 \pm 0.19
10^{-6}	49.4 \pm 3.8**	27.0 \pm 3.4**	252.0 \pm 46.7*	0.70 \pm 0.20**
10^{-5}	38.1 \pm 1.4**	21.7 \pm 1.1**	72.7 \pm 49.8**	0.56 \pm 0.17**

Erythrocytes were incubated in 28 mM glucose for 3 hr. Rat sciatic nerves and lenses were incubated in 50 mM glucose for 6 hr and 4 hr, respectively. Values represent the mean \pm S.D. of 2–6 experiments. * $P < 0.05$, ** $P < 0.01$ vs. non-TAT-treated group.

Table 4. Characteristics of animals in each group at the end of each experiment

Animal group	Dose (mg/kg/day)	Prevention		Reversal	
		body weight (g)	plasma glucose (mg/dl)	body weight (g)	plasma glucose (mg/dl)
Normal		309 \pm 14	133 \pm 15	419 \pm 13	74 \pm 8
Diabetic		271 \pm 9 [#]	438 \pm 41 [#]	286 \pm 20 [#]	444 \pm 46 [#]
Diabetic + TAT	5	265 \pm 15	407 \pm 13	280 \pm 23	414 \pm 60
	10	259 \pm 10	411 \pm 19	280 \pm 31b	434 \pm 51
	20	280 \pm 9	432 \pm 33	280 \pm 17	460 \pm 63
	40	265 \pm 20	490 \pm 85	297 \pm 36	404 \pm 50
	100	—	—	294 \pm 28	440 \pm 58

Values represent the mean \pm S.D. of 6 rats. [#] $P < 0.01$ vs. normal group. No significant difference between the untreated diabetic and TAT-treated groups.

Table 5. Effect of TAT on sorbitol accumulation in the sciatic nerve of 5-day diabetic rats (prevention study)

Animal group	Dose (mg/kg/day)	Sciatic nerve sorbitol (nmol/g wet wt.)	Inhibition (%)	ED ₅₀ (mg/kg/day)
Normal		52 ± 9		
Diabetic		901 ± 238 [#]		
Diabetic + TAT	5	693 ± 170*	24	
	10	368 ± 72**	63	8.8
	20	213 ± 42**	81	
	40	106 ± 18**	94	

The treatment was started immediately after streptozotocin injection, and TAT was orally administered once daily for 5 days. Values represent the mean ± S.D. of 6 rats. [#]P < 0.01 vs. normal group. *P < 0.05 **P < 0.01 vs. untreated diabetic group.

Table 6. Effect of TAT on sorbitol accumulation in the sciatic nerve of 23-day diabetic rats (reversal study)

Animal group	Dose (mg/kg/day)	Sciatic nerve sorbitol (nmol/g wet wt.)	Inhibition (%)	ED ₅₀ (mg/kg/day)
Normal		91 ± 16		
Diabetic		1785 ± 322 [#]		
Diabetic + TAT	5	1296 ± 337*	29	
	10	879 ± 252*	53	9.0
	20	463 ± 180*	78	
	40	223 ± 48*	92	
	100	67 ± 44*	101	

The treatment was started 18 days after streptozotocin injection, and TAT was orally administered once daily for 5 days. Values represent the mean ± S.D. of 6 rats. [#]P < 0.01 vs. normal group. *P < 0.01 vs. untreated diabetic group.

Table 7. Effect of TAT on motor nerve conduction velocity (MNCV) and sciatic nerve sorbitol of diabetic rats

Animal group	Dose (mg/kg/day)	MNCV (m/s)			Sciatic nerve sorbitol (nmol/g wet wt.)
		Time after STZ injection			
		0	14	28 (day)	
Normal		25.3 ± 1.7	28.5 ± 1.1	31.3 ± 2.0	182 ± 55
Diabetic		—	26.7 ± 1.4 [#]	26.4 ± 1.8 [#]	1391 ± 580 [#]
Diabetic + TAT	2.5	—	—	28.0 ± 2.0	1138 ± 212
	10	—	—	29.2 ± 2.8*	661 ± 184**
	40	—	—	30.5 ± 2.7**	340 ± 121**

The treatment was started 14 days after streptozotocin (STZ) injection, and TAT was orally administered once daily for 14 days. Values represent the mean ± S.D. of 9–11 rats. [#]P < 0.01 vs. normal group. *P < 0.05, **P < 0.01 vs. untreated diabetic group.

DISCUSSION

TAT is a novel AR inhibitor, a carboxylic acid derivative and is structurally different from the previously reported inhibitors. In this report, we described the pharmacological characterization of TAT in vitro and in vivo.

Depending on the structure of the inhibitor employed,

the ARs from different species have shown significant differences in their susceptibility to inhibition. Table 1 showed that the inhibitory activity of statil for rat lens AR was much higher than that of TAT and epalrestat, whereas that of TAT for rabbit lens and human placenta AR was much higher than that of epalrestat and statil. Mouse liver ALR was less sensitive to TAT than epalrestat and statil.

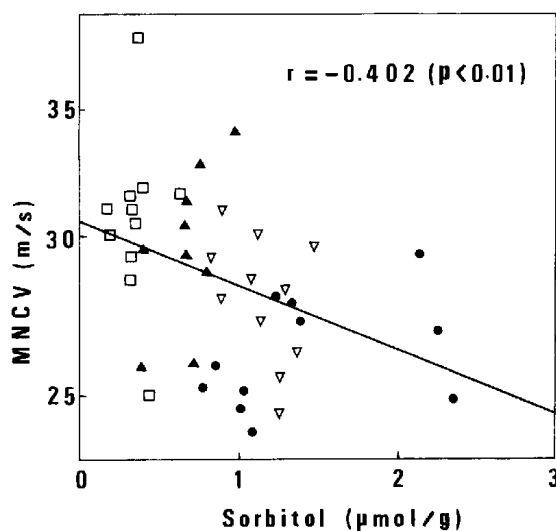


Fig. 3. Correlation between motor nerve conduction velocity (MNCV) and sciatic nerve sorbitol content in untreated diabetic rats (●); diabetic rats treated with TAT at 2.5 mg/kg (▽), 10 mg/kg (▲) and 40 mg/kg (□). Total number of data points is 42.

In kinetic studies, TAT at a concentration of 1×10^{-8} M showed uncompetitive inhibition, whereas increasing the concentration of inhibitor produced mixed type inhibition. TAT exhibited the same kinetic pattern of inhibition as epalrestat (8).

We have demonstrated that TAT inhibited sorbitol accumulation of various isolated tissues incubated in the presence of high concentration of glucose. The IC_{50} values of TAT for rat sciatic nerve, rat lens, human erythrocytes and rabbit erythrocytes were 1.0×10^{-6} , 5.7×10^{-6} , 2.5×10^{-7} and 2.1×10^{-7} M, respectively. In our experiment, IC_{50} values of statil and epalrestat for human erythrocytes were 2.8×10^{-7} and 1.3×10^{-6} M, respectively (data not shown). The inhibitory activity of TAT for human erythrocytes was the same as that of statil, but was stronger than that of epalrestat.

To demonstrate the efficacy of TAT against diabetic neuropathy, we investigated the in vivo effect on the polyol level in the sciatic nerve and MNCV. In both the prevention (experiment 1) and reversal (experiment 2) studies, treatment of diabetic rats with TAT for 5 days reduced the elevated sorbitol in sciatic nerves, dose-dependently. The ED_{50} values were 8.8 and 9.0 mg/kg/day, respectively. In our experiment, the ED_{50} value of statil for the sciatic nerve in the same reversal study was 6.4 mg/kg/day (data not shown). The inhibitory effect of TAT against sorbitol accumulation in the sciatic nerve was approximately 1.4-fold less than that of statil. In experiment 3, treatment of diabetic rats with TAT for 14 days reduced the elevated sorbitol and ameliorated the reduced MNCV,

dose-dependently. As reported previously (16), there was a significant correlation between MNCV and sciatic nerve sorbitol content.

Our results indicate that TAT is effective for improving STZ-induced diabetic neuropathy in rats and is expected to be useful for the clinical treatment of diabetic complications.

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