

An immunomodulating protein, Ling Zhi-8 (LZ-8) prevents insulinitis in non-obese diabetic mice

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Summary. Ling Zhi-8 (LZ-8), a novel and recently discovered immunomodulatory protein having *in vivo* immunosuppressive activity, was tested for *in vivo* effect against Type 1 (insulin-dependent) diabetes mellitus in the non-obese diabetic mouse, the disease having immunologically mediated aetiology in this animal. LZ-8 had mitogenic activity *in vitro* towards spleen cells of the non-obese diabetic mice as previously shown towards those of DBA/2 mice. Intraperitoneal administration of LZ-8 twice weekly into the mice (10.3–12.6 mg/kg body weight) from 4 weeks of age prevented insulinitis and an almost normal number of insulin producing cells were observed. Extreme insulinitis and reduction of the number of insulin producing cells were observed in the pancreata of the untreated non-obese diabetic mouse.

No cumulative incidence of diabetes mellitus was observed in the LZ-8 treated group, while cumulative incidences of 70% and 60% were observed in an untreated group followed up to 42 weeks of age when the incidence of diabetes was defined as a plasma glucose level of greater than 11 mmol/l and as a urine glucose level of greater than 2 + , respectively. T cell subset population analysis was performed to further investigate the action of LZ-8 on the non-obese diabetic mouse which revealed that LZ-8 treatment increased in L3T4⁺/Lyt-2⁺ ratio.

Key words: LZ-8, immunomodulator, NOD mouse, Type 1 (insulin-dependent) diabetes, insulinitis, autoimmunity, T cell subset.

We have previously reported the isolation and characterization of a novel protein, named Ling Zhi-8 (LZ-8), which has an $M_r = 13,100$ to $17,100$, a low carbohydrate content ($< 1.3\%$), and an isoelectric point of 4.4, from the mycelial extract of *Ganoderma lucidum* [1]. The complete amino acid sequence was determined and indicated that LZ-8 consists of a non-covalent homodimer of N-terminal blocked polypeptides bearing primary and predicted secondary structural similarities with immunoglobulin heavy chain [2]. *In vitro*, LZ-8 can haemagglutinate sheep, but not human, erythrocytes and has blastogenic activity towards mouse spleen cells; examination of its *in vivo* activity revealed an ability to inhibit systemic anaphylaxis and reduce the Arthus reaction [1]. Thus, LZ-8 seems to be a new mitogen and immunomodulator with therapeutic potential, whose mechanism of action needs clarification.

To further characterize LZ-8, we turned to the non-obese diabetic (NOD) mouse, an animal model of human Type 1 (insulin-dependent) diabetes of immunologically mediated aetiology. Aspects of disease in this model have been well studied, including a sex difference in incidence (80% cumulative incidence in females, $< 20\%$ in males, after 30 weeks of age) and various symptoms (i.e., hyperglycaemia, glucosuria) followed by deterioration (i.e., weight loss) before death [3]. Histologically, lymphocytic

infiltration into the pancreatic islets (insulinitis) begins in most animals after 5 weeks of age, leading to destruction of Beta cells and overt diabetes [4]. Various studies have implicated T cells [5–13], B cells [14], natural killer cells [15] and macrophages [16, 17] as having roles in disease development. Immunological anomalies in NOD mice have also been described, including impaired cell-mediated immunity [18], interleukin-2 metabolism [15], and a unique class II MHC [19, 20].

The well defined immunological parameters of diabetes in the NOD mouse makes it a good choice for *in vivo* characterization of the immunomodulatory properties of LZ-8. The demonstrated suppressive activity of LZ-8 towards other immune reactions *in vivo* suggested that it might prevent insulinitis in the NOD mouse.

Materials and methods

Animals

Four-week-old female NOD (unique I-A and no expression of I-E; total 43 mice), and DBA/2 mice (H-2^d; total three mice, specific pathogen-free grade) were purchased from Clea Japan, Inc. (Tokyo, Japan). All mice were housed under specific pathogen-free conditions before and during use in the experiments.

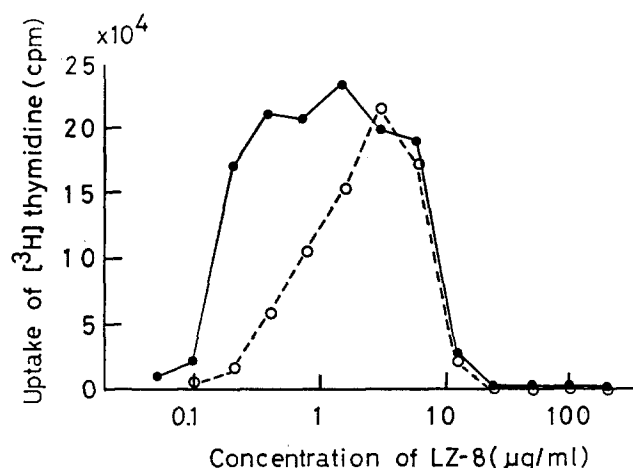


Fig. 1. Effect of LZ-8 on proliferative response of NOD and DBA/2 mice splenic cells. Splenic lymphocyte fractions from non-diabetic NOD (●) and DBA/2 (○) mice were cultured as described under "Materials and methods" at the indicated concentrations of LZ-8. Each point is the average of triplicate measurements

Purification of LZ-8

LZ-8 was purified from *G. lucidum* mycelia by gel filtration followed by ion-exchange chromatography as previously described and LZ-8 of greater than 97% purity, judged by Tricine-SDS polyacrylamide slab gel electrophoresis and Coomassie brilliant blue R staining [1], was used throughout this study.

Proliferative response of spleen cells to LZ-8

Three NOD (5-week-old, non-diabetic) or DBA/2 mice were killed by cervical dislocation and the spleens were removed using sterile technique. Pooled spleen cell preparations were obtained after passing the spleens through a wire screen (150 mesh), and erythrocytes in the suspensions were haemolysed with 0.16 mol/l NH_4Cl , 0.17 mol/l Tris-HCl, pH 7.7. After washing the cells with 10 ml of RPMI 1640 and centrifugation at $150 \times g$ for 10 min, the cells were resuspended in 10 ml of RPMI 1640 supplemented with 10% fetal calf serum 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 200 mmol/l L-glutamate and 10 mmol/l HEPES, pH 7.3 as described by Strong et al. [21]. Cells (0.1 ml, 5×10^5 cells/ml) were seeded onto a 96-well microtitre plate (Falcon 3072) and various concentrations of LZ-8 in 0.1 ml PBS were added. After incubation of the cells at 37°C under 5% CO_2 in air for 42 h, 10 μl of [^3H]thymidine (0.25 μCi , Amersham Japan, Tokyo, Japan) was added and the cells were incubated for a further 6 h under the same conditions. The cells were harvested onto a glass filter which was then dried and transferred to an Omnivial (Wheaton, New Jersey, USA). Toluene base scintillator (2 ml) was added to the vials, which were then counted in a Beta-matic II Liquid Scintillation Counter (Kontron, Zurich, Switzerland).

Table 1. The effects of LZ-8 administration on NOD mice up to 19 weeks of age

Treatment	Average \pm SD			
	Body Weight (g)	Pancreas Weight (mg/g BW)	Spleen Weight (mg/g BW)	Plasma glucose (mmol/l)
None	24.1 \pm 2.1	7.6 \pm 0.2	2.7 \pm 0.4	23.1 \pm 12.4
LZ-8	25.1 \pm 0.2 NS	8.4 \pm 0.8 NS	4.2 \pm 0.6 ^b	7.7 \pm 0.4 ^a

Five control untreated and five LZ-8 treated (from 4 weeks of age, 140 μg twice weekly doses) NOD mice were killed at 19 weeks of age and the above measurements were taken. Statistical comparison of average values with those of untreated group, (NS); non-significant, ^a $p < 0.05$, ^b $p < 0.01$ by Student's *t*-test. BW = body weight

Administration

LZ-8 (700 $\mu\text{g}/\text{ml}$) in phosphate buffered saline was administered in 0.2 ml doses throughout the experiments. Since a constant amount of LZ-8 was given to the animals which grew during the course of the experiments, the amount given per body weight became decreased during the preventive experiments in Figure 3 and Table 1 from 12.6 and 10.3 mg/kg, respectively, to 5.5 mg/kg at 42 weeks of age for the LZ-8 treated group in Figure 3. In these experiments, treatment began from 4 weeks of age and was continued twice weekly by intraperitoneal injection. The mice in the control group were untreated. Urine-glucose was measured by Pretest kit (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), and plasma-glucose in blood obtained from tail vein was measured with Glucose B-Test (Wako Pure Chemical Industries, Ltd.), twice monthly. Incidence of diabetes was defined twice monthly by both estimations of a plasma-glucose level of greater than 11 mmol/l and of an urine-glucose level of greater than 2+.

Histology and immunohistochemistry

Paraffin sections (4–6 μm) of NOD mouse pancreata were prepared from the 10 mice described in Table 1, and for histological analysis were stained with haematoxylin and eosin. Insulitis was estimated 10 islets in a mouse. For immunohistochemical analysis, the avidin-biotin-peroxidase complex (ABC) method of Hsu et al. [22] was used. The pancreas sections were pre-treated with 0.3% H_2O_2 in methanol followed by normal swine serum. The sections were then incubated with guinea pig anti-porcine insulin antibody (IgG) for 1 h followed by biotinylated rabbit anti-guinea pig IgG antibody (IgG) (Lipshaw, Detroit, Mich., USA) for 30 min and then stained with an ABC kit (Vector Laboratories, Burlingame, Calif., USA) as described previously [23].

Analysis of T cell subsets

Mesenteric lymph node cells from 11-week-old untreated and LZ-8 treated (5 each) and 42-week-old untreated and LZ-8 treated NOD mice (3 each) were obtained after passing the nodes through a wire screen (150 mesh). Aliquots of cells (1×10^6 cells) were incubated with 1 μg of FITC-conjugated anti-Thy 1.2 (30-H12), anti-Lyt-1 (53-7.3), anti-Lyt-2 (53-6.7), or 0.2 μg of phycoerythrin-conjugated anti-L3T4 (GK 1.5) (all from Becton-Dickinson, Mountain View, Calif., USA) at 4°C for 30 min. Then, after fixation with 6% formalin, the cells (1×10^4 cells for each monoclonal antibody) were analysed by flow cytometry on a FACStar (Becton-Dickinson). In the case of the 11-week-old animals, five individual cell preparations were made and analysed, whereas for the 42-week-old animals, the lymph nodes were pooled into one cell preparation. Five individual spleen preparations were also made and analysed as described above for the 11-week-old animals.

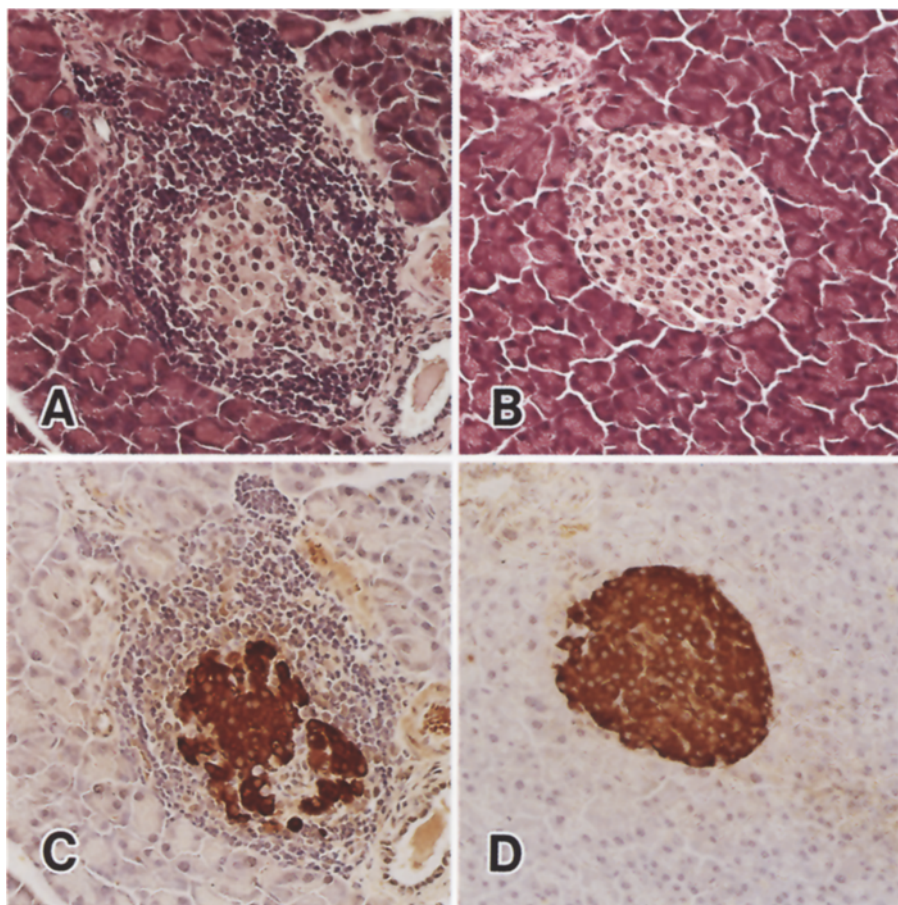


Fig. 2A–D. Histological and immunohistochemical examination of pancreatic islets of NOD mice with or without LZ-8 treatment. Pancreata of an untreated NOD mouse and that of an LZ-8 treated 19 weeks of age NOD mouse were stained with haematoxylin and eosin (**A, B**) or by avidin-biotin peroxidase complex method after treatment with anti-insulin antibody (**C, D**)

Statistical analysis

Statistical analysis was performed by Student's *t*-test.

Results

During LZ-8 purification, one-way biological specific activity was monitored by proliferative response assay using DBA/2 mouse spleen cells [1]. This study describes the *in vivo* effects of LZ-8 on diabetes in the NOD mouse but first, the biological effect of LZ-8 on NOD mouse spleen cells *in vitro* was examined by the same assay; using cells from 5-week old animals, maximum [³H]thymidine uptake occurred at 1.56 μ /ml and uptake decreased sharply at or above concentrations of 12.5 μ /ml (Fig. 1). The response of NOD mouse spleen cells to LZ-8 concentrations of less than 1.0 μ /ml was greater than the response of DBA/2 mouse spleen cells to similar concentrations of LZ-8 (Fig. 1). Intraperitoneal administration of LZ-8 twice weekly into female NOD mice from 4 to 19 weeks of age prevented insulinitis and typical pancreatic histological and immunohistochemical preparations are shown in Figure 2. The untreated mouse which had near-diabetic plasma glucose, showed massive lymphocytic infiltration (Fig. 2A), whereas the LZ-8 treated mouse which was non-diabetic, appeared almost normal, with only slight infiltration (Fig. 2B). Using anti-insulin antibody to detect pancreatic Beta cells, the entire islet

was stained in the LZ-8 treated mouse (Fig. 2D), but only limited areas stained in the untreated mouse (Fig. 2C).

LZ-8 is a novel protein and side effects of its administration *in vivo* have not yet been described. Some effects of LZ-8 treatment in NOD mice are shown in Table 1; pancreatic histology, plasma and urine glucose levels, as well as whole body, pancreatic, and spleen weights were compared between five untreated and five LZ-8 treated mice at 19 weeks of age. LZ-8 treated mice had non-diabetic plasma glucose levels while the average was tripled in untreated mice, with three out of five mice having both extremely elevated plasma (21.5–38.0 mmol/l) and urine (3+) glucose levels ($p < 0.05$). Histological examination revealed only slight insulinitis in 80% of LZ-8 treated mice whereas in most cases, untreated mice had severe insulinitis with or without decrease in islet number. Body and pancreatic weights were not significantly changed by LZ-8 treatment, although the averages were higher for LZ-8 treated animals. Thus, the major side effect of LZ-8 treatment was an increase in spleen weight. This was not accompanied by lymphadenopathy and microscopic structure of spleens from both the untreated and LZ-8 treated mice were basically similar, except for higher numbers of lymphatic nodules and a larger germinal centre in spleens of LZ-8 treated mice (data not shown). There were no other noticeable side effects judging from the behaviour of the mice during the experiments or visual examination of spleen, liver, pancreas, kidney, lung, or intestine. Acute toxicity of LZ-8 at 10 mg/kg ad-

Table 2. T cell subset analysis of NOD mice after LZ-8 treatment up to 11 and 42 weeks of age

Age in weeks (Organ)	Treatment	Cells stained (%)				
		Thy 1.2	Lyt-1	Lyt-2	L3T4	L3T4/Lyt-2
11 (LN)	None	75.5 ± 3.0	84.1 ± 1.8	22.7 ± 1.1	59.9 ± 1.0	2.64 ± 0.14
	LZ-8	68.1 ± 4.1 ^a	77.0 ± 4.3 ^a	18.0 ± 2.3 ^b	53.6 ± 2.8 ^b	3.01 ± 0.30
11 (Spleen)	None	42.4 ± 5.8	62.7 ± 1.4	15.7 ± 0.7	35.2 ± 1.8	2.25 ± 0.11
	LZ-8	39.1 ± 7.4	54.9 ± 7.1	12.2 ± 2.2 ^a	33.5 ± 2.6	2.81 ± 0.35 ^a
42 (LN)	None	73.9	73.5	28.8	47.9	1.66
	LZ-8	72.8	76.1	23.3	50.0	2.15

Cell preparation was made and analysed as described in "Materials and methods". ^a $p < 0.05$, ^b $p < 0.01$ by Student's *t*-test. LN = lymph node

ministered i.v. into Institute of Cancer Research USA (ICR) mice was low as body weight dropped slightly one day later but was normal thereafter (data not shown).

The ability of LZ-8 to prevent diabetes incidence was examined and intraperitoneal administration of LZ-8 twice weekly into female NOD mice from 4 weeks of age completely prevented incidence until 42 weeks of age (Fig. 3). At the same time, in the control untreated mice, 50% cumulative incidence was observed at 26 and 32 weeks of age, 70% and 60% at 40 weeks when incidence was defined as a plasma glucose level of greater than 11 mmol/l and as a urine glucose level greater than 2+, respectively. We could detect anti-LZ-8 IgG in the sera of LZ-8 treated mice after 28 and 38 weeks of age in the experiment of Figure 3 (data not shown). However, there was no anaphylactic reaction or other noticeable side effects judging from the behaviour of the mice during experiments.

To examine the in vivo action of LZ-8 in more detail, T cell subset population analysis was performed (Table 2). LZ-8 treatment leads to significant ($p < 0.05$ and $p < 0.01$) decreases after 11 weeks in all subpopulations measured from lymph nodes. Analysis of another secondary lymphoid organ, the spleen, provided similar findings in that LZ-8 treatment lead to decreases in all subpopulations and an increase in L3T4⁺/Lyt-2⁺ ratio after 11 weeks (Table 2). By 42 weeks, differences in

Thy 1.2⁺, Lyt-1⁺, and L3T4⁺ subpopulations became diminished, mostly due to decreases in untreated subpopulations. We also analysed T cell subsets of lymph node cells obtained from a diabetic NOD mouse (42-weeks-old) and the results were; 86.7%, Thy 1.2⁺; 80.6%, Lyt-1⁺; 31.6%, Lyt-2⁺; 48.7%, L3T4⁺; and the ratio of L3T4⁺/Lyt-2⁺ was 1.54. Although these values were obtained from only one mouse, the subpopulations of Lyt-2⁺, L3T4⁺, and the ratio of L3T4⁺/Lyt-2⁺ were close to 42-week-old non-diabetic untreated NOD mice (Table 2). The overall effect of LZ-8 treatment seemed to be an increase in L3T4⁺/Lyt-2⁺ ratio which was evident after both 11 and 42 weeks. Coincidentally, LZ-8 treatment had no effect on the T cell subset population in ICR mouse lymph nodes or spleen at the same times (data not shown).

Discussion

This study describes the effect of LZ-8 on insulinitis in NOD mice, based on its previously shown in vivo immunomodulatory activities. Under the described treatment conditions (twice weekly, intraperitoneal injections, 140 µg per dose), mice received approximately 10 mg/kg body weight of LZ-8 at the beginning of treatment, although this became reduced to 5.5 mg/kg due to weight increase during the experiment. This treatment plan was clearly preventive for insulinitis in the NOD mice and seemed to be effective immunogenically and mitogenically in other studies. For instance, LZ-8 prevented anaphylaxis in CFW mice at dosages of between 6.9 and 7.4 mg/kg body weight [1]. Also, LZ-8 administered to DBA/2 mice at 10 mg/kg body weight resulted in stimulated [³H]thymidine uptake by spleen cells from these mice (unpublished data). The in vitro activity of LZ-8 is clearly dose dependent (Fig. 1, [1]), therefore in vivo dose dependency studies need to be done. Nevertheless, the present treatment plan was effective for prevention of insulinitis although it may not have been optimal for administration effect. The only side effect of treatment seemed to be increased spleen weight. LZ-8 also seemed to have low toxicity.

The underlying mechanism of the in vivo effects of LZ-8 on insulinitis in the NOD mouse is unknown but it may be interesting to compare it with two other immunomodulatory drugs, cyclosporine and OK 432. All are able to prevent diabetes and decrease severity of insulinitis if treatment is started before insulinitis occurs (i.e., at about 4 weeks of age) [15, 24]. However, the charac-

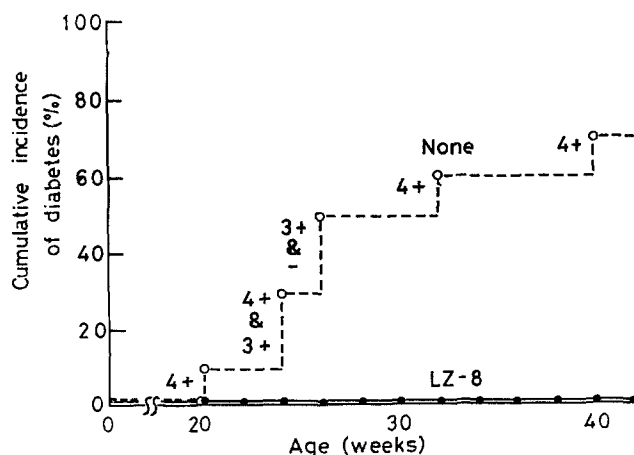


Fig. 3. Effect of LZ-8 administration on incidence of diabetes mellitus in NOD mice. Ten control untreated and ten LZ-8 treated NOD mice were observed for onset of diabetes as described in "Materials and methods". Symbols (-, 3+, 4+) are urine glucose levels of mice diagnosed as diabetic with plasma glucose level greater than 11 mmol/l

teristics of cyclosporine and OK 432 are very different. Cyclosporine, a fungal cyclic polypeptide, is a potent inhibitor of IL-2 production and T cell activation in vitro and suppressor of organ allograft in vivo [25], while OK 432, a streptococcal preparation, increases IL-1 and IL-2 production and activates macrophages and killer T cells in vitro [15] and is an in vivo immunopotentiator used for cancer chemotherapy [26, 27]. On the other hand, LZ-8 is mitogenic towards mouse spleen cells as well as unfractionated human peripheral blood lymphocytes containing 10% macrophages and stimulates the production of IL-1 and IL-2 as well as other lymphokines in vitro (unpublished data), although it is preventive in vivo against immune responses such as systemic anaphylaxis and the Arthus reaction [1]. Our recent experiments revealed that LZ-8 could significantly suppress pokeweed mitogen-induced or antigen-specific IgG production by human peripheral blood lymphocytes in vitro (unpublished data). These results point out that suppressor T cells are generated by the LZ-8 stimulation and the mechanism of suppression resembles the generation of suppressor effector T cells by Con A stimulation [28, 29]. It appears that in the NOD mouse, the occurrence of diabetes seems to be mainly determined by a balance between autoimmunity and suppression and that immunotherapies such as those described above and others [30] can be effective in altering the course of disease and may be useful in the treatment of human disease. While the precise mechanism of LZ-8 action in vivo was not determined, it did alter subset distribution of T cells, which are known to play a role in disease. Our unpublished results also suggest an activity towards macrophages. Changes in T lymphocyte subsets have also been noted in human diabetes mellitus [31]. Being mitogenic and stimulatory towards lymphokine production in vitro, LZ-8 may also have some further, yet unknown action to explain its immunomodulatory activity in vivo. LZ-8 resembles lectins in biological activity of lymphocyte activation in vitro, although it is not a lectin per se [1], and it bears structural similarity to immunoglobulin domains [2]. These points, along with the existence of suppressive [32] or suppressor cell-inducing lymphokines [33] and the yet unchecked possibility that LZ-8 affects their production, may provide keys to understanding its mechanism of action. It is hoped that in the future, LZ-8 will also be clinically useful as well as providing insight into the diabetic disease process, but further investigation is necessary.

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