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Apoptotic Effect of Bortezomib on Pancreatic Islet Cells in STZ-induced Diabetic Rats

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Abstract

Background: This study aimed to investigate the possible apoptotic role of bortezomib (BMZ) on pancreatic islets of streptozotocin (STZ)-induced diabetic rats.

Methods: Sprague-Dawley rats were divided into groups that were administered BMZ alone or in combination with STZ. To evaluate the effect of BMZ on the development of diabetes, blood glucose levels were measured regularly in the animals. Islet cell viability was determined by staining the islets with fluorescein diacetate and propidium iodide. Expression of the Bcl-2 and bax genes was determined in islet cells by quantitative real-time polymerase chain reaction.

Results: Administering STZ-induced hyperglycemia in the rats reduced the viability of islet cells and the bcl-2/bax ratio. In the group administered BMZ alone, the bcl-2/bax gene expression rate in islets increased significantly compared to the control group. BMZ co-administered with STZ significantly increased islet cell viability and the bcl-2/bax ratio compared to the diabetic group.

Conclusions: This study demonstrates that BMZ may protect pancreatic islet cells from apoptosis by increasing islet viability and upregulating the bcl-2/bax gene expression ratio, even though it failed to protect against the destructive effect of STZ.

Keywords: apoptosis, bortezomib, pancreatic islets, rats, type 1 diabetes

INTRODUCTION

Type 1 diabetes is an autoimmune disease characterized by selective and progressive destruction of insulin-secreting beta cells of the pancreas.[1] The nuclear factor kappa B (NF-κB) signaling pathway plays a role in the development of diabetes.[2,3] NF-κB is a transcriptional factor that transcriptionally activates the genes required for important biological processes, such as cell proliferation, apoptosis, survival, and inflammation.[4,5]

Most studies that have shown the role of NF-κB activation in pancreatic beta cell death indicate that NF-κB has a predominant pro-apoptotic function.[2,6] However, activation of NF-κB is generally considered to be anti-apoptotic and pro-oncogenic in many tumor types. Therefore, blocking NF-κB activation is a successful target therapy for cancer treatment.[4,5] Blocking NF-κB activation directly with chemical inhibitors or transgenic animal models results in resistance to STZ-induced development of diabetes, decreased inflammation, and increased survival of islet grafts in transplantation experiments.[6,7]

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Bortezomib (BMZ) is a proteasome inhibitor approved by the Food and Drug Administration for use in the treatment of multiple myeloma and mantle cell lymphoma. BMZ is a potent inhibitor of the NF-κB pathway.[8] It inhibits the activation of NF-κB by blocking the proteasomal degradation of IκB and inducing apoptosis in many tumor cell types in preclinical studies.[8,9] However, the exact mechanism of how BMZ affects pancreatic islet cell apoptosis remains unclear. A recent study showed that BMZ regulates the immune system in autoimmune diabetes and reported that administering BMZ to pre-diabetic non-obese mice prevents the development of diabetes in 80% of the mice, while it did not show any therapeutic effect when administered alone to overtly diabetic mice.[10] In addition, BMZ reduced islet rejection in another study where BMZ was systemically administered to STZ-induced diabetic mice before islet transplantation.[6] In both studies, it was suggested that BMZ prevented the development of diabetes due to its anti-inflammatory properties, but the apoptotic process in the islets has not been investigated. Other proteasome inhibitors induce apoptosis in mouse and rat beta insulinoma cell lines,[11,12] but the proteasome inhibitors containing MG-132, celastrol and epoxomicin, lactacystin, and N-Acetyl-Leu-Leu-Nle-CHO (ALLN) have quite contradictory results such as reducing, increasing, or no effect on rat islet viability.[11,13,14] The purpose of this study was to investigate the apoptotic

effects of BMZ on rat pancreatic islet cells in STZ-induced diabetes using *in vivo* and *in vitro* experiments.

METHODS

Ethical approval

All procedures were conducted following the accepted principles for the care and use of laboratory animals and were approved by the Local Ethical Committee of Laboratory Animals at Diskapi YB Education and Research Hospital, Ankara, Turkey (Protocol no: 2016/05).

Animals

Female Sprague-Dawley rats (average weight, 200–250 g; age, 4–6 months old) were used to investigate the effect of BMZ on the development of type 1 diabetes. All animals were housed in a special pathogen-free and temperature-controlled room (21 ± 2 °C) with a 12 h light/12 h dark cycle. The rats were fed a standard pelleted diet and water *ad libitum*. All animal experiments were performed following the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) and approved (2016/05) by the Ethical Committee of Laboratory Animals at YB Teaching and Research Hospital, Ankara, Turkey.

STZ-induced diabetic rat model

The STZ-induced diabetes rat model was used to determine the apoptotic effect of BZM on pancreatic islets. The rats were randomly selected and divided into four groups: 1) Control, 2) STZ group, 3) BZM group, and 4) STZ + BZM group (n = 10). Diabetes was induced in the rats by a single intraperitoneal injection of STZ (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 45 mg/kg in freshly prepared citrate buffer (50 mM sodium citrate, pH 4.5).[15] The control group was administered citrate buffer alone. BMZ (0.2 mg/kg, Milenium Pharmaceutical, Cambridge, MA, USA) was injected intraperitoneally 3 times (days 1, 4, and 8) in all related groups as described previously.[16] The first BMZ dose was administered to the STZ+BZM group 2 h before the STZ injection (day 1). Then, the other two BMZ doses were injected as in the BZM group (days 4 and 8). All rats were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine on day 10, and the pancreas was removed.

Blood glucose levels and body weight were measured before the drugs were administered and every other day after the injections. Blood glucose levels were detected in the tail vein blood of all rats using the Accu-check blood glucose meter (Bayer Corp., Whippany, NJ, USA). Rats with blood glucose levels > 250 mg/dL were defined as diabetic.

Isolation and purity of the islets

The islets were isolated as described previously.[17] Briefly, the pancreas was inflated by injecting 7 ml of cold collagenase type V solution (1 mg/ml) into the bile ducts of the rats. The pancreas was removed, and the islets were separated by Ficoll density centrifugation. After isolation, the islets were hand-picked under a stereomicroscope. The number and purity of the islets were determined by counting after diphenylthionarbazon (DTZ; Sigma-Aldrich) staining in which islets were dyed scarlet at 37 °C for 10 min. After adding 50 mM BMZ to the culture medium of the BZM and STZ + BZM groups, all islets were cultured at 37 °C in humidified air and 5% CO_2 in RPMI-1649 medium supplemented with 1% L-glutamine, 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), and a 1% antibiotic mixture (100 units/mL of penicillin and 100 μg/mL of streptomycin; GIBCO, Grand Island, NY, USA) overnight in 25 cm^2 flasks.

Islet viability

The viability of 3–5 islets collected from each rat was analyzed by dual fluorescent staining consisting of fluorescein diacetate (FDA) and propidium iodide (PI) (Sigma-Aldrich).[18] The percentage of viable islets was obtained from the ratio of living cells stained green with FDA to dead cells stained red with PI. Stained islet cells were visualized with a fluorescent microscope (Olympus CX-41: Tokyo, Japan), and the mean viability values were determined using the MatLab color analysis program (MathWorks Inc., Natick, MA, USA).

Real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cultured islets of each rat using the PureLink RNA Mini kit (Ambion Corp. Naugatuck, CT USA), following the manufacturer's instructions. RNA concentration and purity were determined with the Thermo NanoDrop2000 (Thermo-Fisher Scientific, Waltham, MA, USA), and sample integrity was assessed by gel electrophoresis. A 1 µg portion of total RNA was reverse-transcribed using the Applied Biosystems High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The cDNA was diluted 1:10 in RNase-free water. The real-time PCR reaction was performed using the cDNA samples and the TaqMan Gene expression assay kit (Applied Biosystems). The assay ID numbers for the primer pairs and probes were bcl-2 (Rn 99999125_m1), bax (Rn 01480161-g1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Rn 99999916-s1). Each assay was run in triplicate on the ABI-7500 Fast RT-PCT system. The GAPDH gene was used as the endogenous control. Relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method.[19]

Data analysis

Results are presented as mean ± SD or SE per group. Differences between two groups were compared by the independent Student's *t*-test and differences between several groups were detected using one-way variance analysis followed by Tukey's method for multiple

comparisons (SPSS version 20.0; SPSS Inc., Chicago, IL, USA). A $p < 0.05$ was considered significant.

RESULTS

The effect of BMZ on weight and blood glucose in the STZ-induced diabetic rats

To investigate the role of BMZ in the development of STZ-induced diabetes, we generated four different experimental groups of non-obese rats. Their body weights were measured before each injection. All of the animals had similar initial body weights. As shown in Table 1, no significant changes in body weight were observed during the experimental period.

Diabetes was induced by a single STZ injection. The rats were diabetic 3 days following the STZ injection, as indicated by an elevated blood glucose level (349.0 ± 24.8 mg glucose/dL, 10 days), compared with the control group (96.2 ± 2.52) ($p < 0.05$). Blood glucose levels did not change significantly in the BMZ group compared with the control group (98.2 ± 7.1), and they were almost identical to the control. As shown in Figure 1, blood glucose levels increased significantly in the STZ+BZM group (407.6 ± 11.0) compared to the control and BZM groups ($p < 0.05$).

TABLE 1. Body weight changes

Group	First body weight (g)	Final body weight (g)
Control	212.00 ± 5.85	219.00 ± 9.20
STZ group	214.60 ± 12.97	205.60 ± 10.62
BZM group	215.40 ± 19.24	220.40 ± 13.11
STZ+BZM group	209.80 ± 13.06	192.20 ± 4.42

Data are mean ± SE of 10 rats per group. No significant difference was observed between the first and final body weights of the animals.

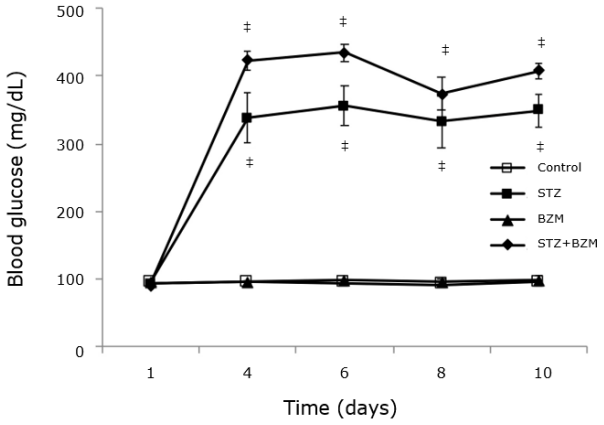

FIGURE 1. Blood glucose levels on different days. Values are mean ± SE of 10 rats per group. The increases in the blood glucose levels of the STZ and STZ+BZM groups were all significant compared with the control and BZM groups after day 4.

The effect of BMZ on islet cell viability

The islets were isolated to investigate the effect of BMZ on islet cell viability. The purity of the islets was determined by DTZ staining. Islet viability was determined by a MatLab analysis following FDA/PI staining.

Average islet viability in the STZ group (63.55 ± 3.90%) was significantly lower than that in the control and BZM groups, respectively (83.13 ± 7.12%; 80.21± 5.60%) ($p < 0.05$) (Figure 2 and Figure 3). BZM alone was not cytotoxic to the islets. In contrast, BZM protected the islets from apoptosis by STZ when STZ and BZM were co-administered to the rats (77.94 ± 10.99%) ($p < 0.05$).

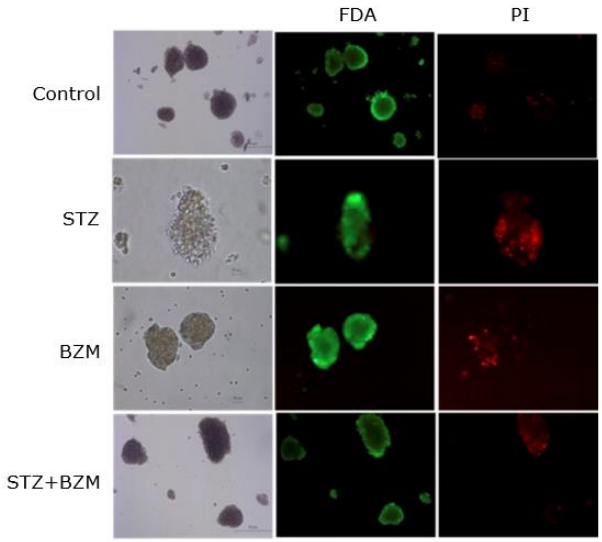

FIGURE 2. Fluorescent microscopic images of living islet cells stained green with fluorescein diacetate (FDA) and dead islet cells stained red with propidium iodide (PI). Images were analyzed with the MatLab program to determine the percentage of cell viability. At least 3 islet samples were used for each rat.

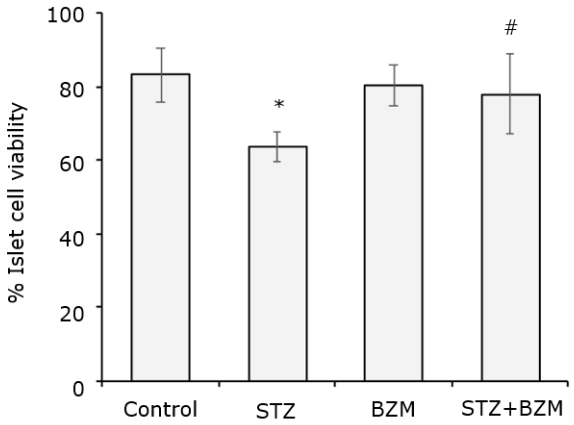

FIGURE 3. Islet cell viability values are expressed according to the results of the MatLab analysis after FDA/PI staining of the islets. The percentages of viable

cells in the control, BZM, and STZ+BZM groups were significantly higher than that in the STZ group ($p < 0.05$). Data are mean ± SD. * $p < 0.05$ vs. control, BZM and STZ+BZM groups; # $p < 0.05$ vs. STZ group.

The effect of BMZ on bax and bcl-2 gene expression levels

The effects of BMZ on bax and bcl-2 gene expression levels in pancreatic islet cells were determined by RT-PCR. As shown in Figure 4a, expression of the pro-apoptotic bax gene increased significantly in STZ-induced diabetic rats, as expected ($p < 0.05$). A decrease in the bax expression level was detected in the STZ + BZM group, compared to the STZ group, indicating that BZM protected the cells from apoptosis by STZ. Furthermore, the expression level of the anti-apoptotic bcl-2 gene was highest in the STZ+BZM group. This increase was significant vs. the control and the STZ and BZM groups ($p < 0.05$). Because apoptosis is mediated by the balance between the bcl-2 and bax gene expression levels, the bcl-2/bax ratio determines cell survival or death. Figure 4b shows that the mitochondrial bcl-2/bax gene expression ratio increased significantly in the BZM group compared to the control group ($p < 0.05$), indicating that BZM promoted an anti-apoptotic effect in the islets. The ability of BMZ to increase the bcl-2/bax ratio in the STZ+BZM group compared to the STZ group ($p < 0.05$) indicates that it protected the islets from apoptosis by STZ.

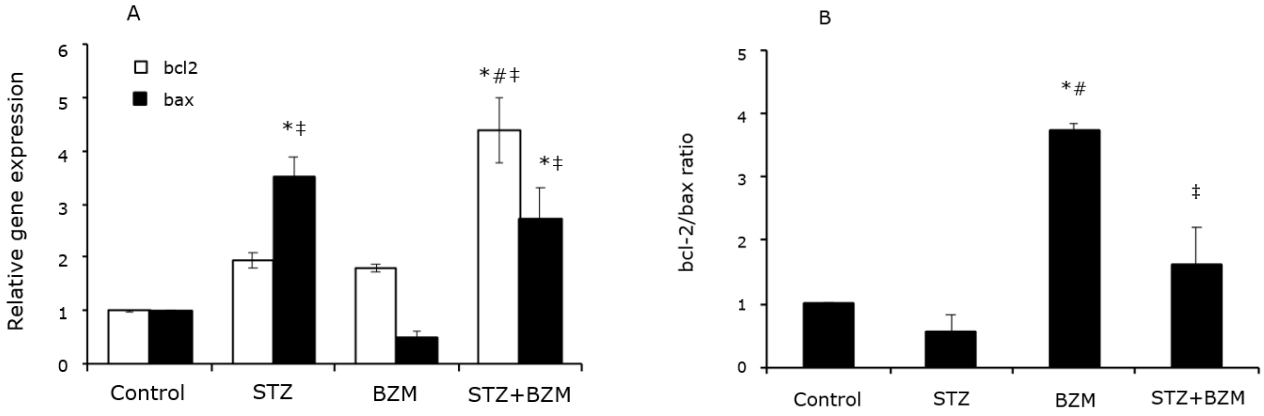

FIGURE 4. Gene expression levels. Bax and bcl-2 gene expression levels in the pancreatic islets were determined by qRT-PCR (A). The bcl-2/bax gene expression ratio (B). Data are mean ± SD. of three separate experiments. * $p < 0.05$ vs. control group; # $p < 0.05$ vs. STZ group; ‡ $p < 0.05$ vs. BZM group.

DISCUSSION

BMZ is a reversible proteasome inhibitor that selectively inhibits the chymotrypsin-like activity of the proteasome and is currently clinically used to treat multiple myeloma and mantle cell lymphoma.[9,20] One of the BMZ anticancer mechanisms is suppression of the NF-κB signaling pathway.[8] Many studies have shown that activation of NF-κB plays a key role in beta cell destruction and progression to diabetes,[2,3] in contrast to the anti-apoptotic function in cancer cells.[4,5] This study was conducted to examine the apoptotic role of BMZ in pancreatic islets from STZ-induced diabetic rats.

STZ is a toxic glucose and N-acetyl glucosamine analog that selectively destroys pancreatic beta cells. Thus, it is widely used to induce type 1 diabetes in experimental animals.[15,21,22] STZ is transported to the pancreatic beta cells via the low-affinity GLUT 2 glucose transporter, which preferentially facilitates the uptake of STZ, and causes DNA alkylation and eventual death of beta cells.[21,22] STZ (single high dose; 35–65 mg/kg rat) administration depletes the pancreatic beta cells, resulting in elevated fasting blood glucose levels within 72 h after administration.[21–23] In this study, we observed a significant increase in blood glucose levels in diabetic rats compared with the control rats 3 days after STZ administration. Moreover, we determined a significant decrease in viability in isolated islets 10 days after administering the STZ. These data confirm that STZ destroyed the pancreatic beta cells by increasing blood glucose levels and decreasing islet cell viability.

Apoptotic cell death is a fundamental mechanism involved in the loss of pancreatic beta cells.[1] The biochemical properties of apoptotic cell death include internucleosomal cleavage of DNA, phosphatidylserine externalization, and proteolytic cleavage of several intracellular substrates. Apoptosis is a tightly regulated process, and the fate of cells depends on the balance between pro-apoptotic and anti-apoptotic proteins. The Bcl-2 family of proteins is a critical regulator of apoptotic cell death. Pro-apoptotic bax gene expression promotes cell death, whereas anti-apoptotic bcl-2 results in cell survival. The Bcl-2/bax ratio determines cell death or survival. Low levels of Bcl-2 and/or high levels of Bax permeabilize the mitochondria after the apoptotic

stimulus. Caspase 3 becomes active with the release of cytochrome *c* from the intermembrane space into the cytoplasm followed by the formation of the apoptosome. Caspase 3 activates the DNase/DNA fragmentation factor to breakdown nuclear DNA, resulting in DNA fragmentation and cell death.[24] In this study, increased expression of bax and bcl-2 was observed in the diabetic rats administered STZ alone. However, because the increase in bax expression was higher than bcl-2 expression and significantly increased compared to the control, the decrease in the bcl-2/bax ratio promoted apoptotic cell death in the rats. This result is compatible with previous studies showing overexpression of the pro-apoptotic bax gene and low expression of the anti-apoptotic bcl-2 gene in pancreatic beta cells exposed to STZ.[25,26]

The main objective of this study was to examine the apoptotic effect of BMZ on pancreatic islets in STZ-induced diabetic rats. BMZ administration alone did not change the fasting blood glucose level or islet viability, compared to the control group. In our study, BMZ was used at a dose of 0.2 mg/kg, which is well tolerated[10,16,27] without any apparent toxicity. Previous studies have reported that many of the side effects of BMZ, such as gastrointestinal toxicity, neuropathy, and anemia, are mild and manageable. BMZ has not been reported to affect blood glucose, except for the indication that it should be used with caution in diabetic patients. The unchanged blood glucose levels observed in the BMZ-administered rats were compatible with the fact that BMZ has no effect on blood glucose in non-diabetic patients.[27] BMZ induces apoptosis in many tumor cells by decreasing cell viability; thus, it is currently used to treat multiple myeloma.[8] However, the effect of BMZ on the viability of pancreatic islet cells is not well known. We observed that BMZ did not change the viability of pancreatic islet cells. This result is consistent with a study showing that BMZ does not change the viability of the MIN6 beta cell line even at a high concentration.[28] Previous studies have reported that other proteasome inhibitors induce severe apoptosis in mouse and rat beta insulinoma cell lines.[11,12] However, the effect of proteasome inhibitors on rat islet viability has been contradictory, as a decrease in viability was observed in rat islets cultured with MG-132,[13] and an increase in viability was observed with non-toxic concentrations of celastrol and epoxomicin,[14] but lactacystin and ALLN exerted no visible adverse effects on rat islet cell viability[11,13] and even partially protected against the toxic effect of pro-inflammatory cytokines such as interleukin-1 and interferon.[11] Interestingly, we observed that administering BMZ alone upregulated bcl-2 and downregulated bax, although the differences were not significant when compared with the control. However, these changes in gene expression led to a significant increase in the bcl-2/bax ratio, indicating that BMZ administration alone may be anti-apoptotic in pancreatic islets, even if it is not reflected in total islet viability. This result seems contrary to the ability of BMZ to induce apoptosis, which is widely used in cancer therapy. However, many studies have shown that BMZ is more selective in tumor cells than normal cells[29,30] and more effective in hematological cancers than solid tumors[29] indicating that BMZ may cause different effects depending on the tissue and cell types and the stress conditions.

In this study, we observed that BMZ co-administered with STZ significantly increased the bcl-2/bax gene expression ratio and islet viability compared to those in the control rats, but the same antiapoptotic effect was not reflected in the blood glucose levels. These results indicate that BMZ does not completely protect beta cells from the selective destructive effect of STZ, and possibly provides partial protection by causing an antiapoptotic effect in islet cells. A recent study has directly shown that BMZ prevents the development of diabetes when administered to pre-diabetic NOD mice.[10] NOD mice are a model of autoimmune diabetes in which no toxin is administered. Therefore, administering BMZ to NOD mice in that study prevented hyperglycemia unlike in our study. In addition, another study demonstrated that systemic BMZ administration of STZ-induced diabetic mice delays the rejection of allogeneic islet grafts, suggesting that BMZ prevents the development of diabetes by delaying beta cell loss.[6] These results may contribute to the protective effect of BMZ, which we observed at the total islet viability and gene expression levels. However, this antiapoptotic protective effect did not prevent the loss of beta cells enough to block the development of diabetes. Blood glucose levels are only affected by beta cell loss, whereas islet viability is the total viability value of all cell types including alpha and delta cells in the pancreatic islets. For example, it has been reported that even STZ administration, which selectively destroys beta cells, increases the survival percentage of alpha and delta cells in pancreatic islets.[23] The finding that BMZ causes a similar proliferative effect by increasing total islet cell viability and bcl-2/bax gene expression rates may explain why we did not see an improvement in blood glucose levels. This condition can also be considered one of several reasons to explain the conflicting results of other proteasome inhibitors on rat islet viability.[11–14] Therefore, it would be more accurate to determine individual cell viability for each cell type rather than total islet cell viability in future diabetes research.

CONCLUSIONS

In conclusion, this study provides evidence that BMZ induced an antiapoptotic effect by increasing islet cell viability and the bcl-2/bax ratio in rat pancreatic islets, although it was inadequate to prevent the loss of beta cells and the development of STZ-induced diabetes. The antiapoptotic mechanism of BMZ on pancreatic islet cells

needs to be further elucidated for each cell type in the pancreas.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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