Notice of Retraction

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This article has been retracted by the Editorial Committee of The Pharmaceutical Society of Japan because it contains scientific misconduct. Although the data published in this article were generated in part by the first author, the authors violated authorship and sponsorship protocol.

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Protective effects of bazedoxifene paired with conjugated estrogens on pancreatic β-cell dysfunction

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Summary

Bazedoxifene (BZA) is a novel selective estrogen receptor modulator that exhibits estrogen agonist activity in the bone but estrogen antagonist activity in the breast and uterus. We recently demonstrated that BZA mimics estrogenic actions in reversing metabolic abnormalities while protecting the endometrium from estrogen stimulation in ovariectomized (OVX) mice. However, it is unknown whether BZA, alone or paired with estrogens, can protect pancreatic $\beta$-cell function or survival. Thus, the aim of this study was to evaluate the preventive effects of estrogens [$17\beta$-estradiol or conjugated estrogens (CE)], BZA and their combinations against $\beta$-cell failure. Cytokine-mediated caspase-3 activation of MIN6 $\beta$-cells was significantly inhibited by combined treatment with CE and BZA, but not after exposure to estrogens or BZA alone. BZA did not stimulate transcriptional activation of estrogen response element (ERE) but did inhibit estrogens-induced ERE activation in MIN6 cells. Furthermore, in vivo administration of BZA, alone or paired with CE, reduced hyperglycemia and diabetic incidence and improved glucose tolerance, without causing uterine hypertrophy in streptozotocin-treated OVX mice. Serum insulin/glucose ratio significantly increased in the BZA/CE combination group compared to the OVX vehicle, although there were no changes in pancreatic insulin content. Thus, BZA could enhance the protective effects of estrogen on $\beta$-cell survival, potentially mediated via an ERE-independent mechanism, suggesting a novel therapeutic application of tissue-selective estrogen complex with BZA.

Key words: Bazedoxifene; Conjugated estrogens; $\beta$-cell survival; Diabetes; Menopause
Introduction

Selective estrogen receptor modulator (SERM) is a synthetic non-steroidal agent that exhibits estrogen receptor (ER) agonistic or antagonistic activities depending on the target tissue. Tissue-selective estrogen complex (TSEC), which pairs a SERM with one or more estrogens, has been clinically and non-clinically demonstrated to be a promising therapeutic option for postmenopausal symptoms.\textsuperscript{1,2) A therapeutic formulation containing bazedoxifene (BZA) and conjugated equine estrogens (CE) [Duavee, Pfizer Inc.] is the first TSEC approved by the US FDA (3 October 2013) for the treatment of moderate-to-severe vasomotor symptoms associated with menopause and prevention of postmenopausal osteoporosis. BZA/CE combination provides the desirable effects of estrogens for management of menopausal hot flashes and bone loss while simultaneously protecting the breast and endometrium from estrogen stimulation without the need for progestin.\textsuperscript{3) }

Since estrogen plays a significant role in the control of energy homeostasis and glucose metabolism,\textsuperscript{4) we previously investigated the effects of BZA/CE in preventing postmenopausal metabolic dysfunction in ovariectomized (OVX) mice.\textsuperscript{5) Our data revealed that BZA maintained and/or enhanced the effects of CE in preventing adiposity, systemic inflammation, insulin resistance, and glucose intolerance without causing endometrial hyperplasia in OVX mice fed a high-fat diet (HFD). Additionally, Barrera et al.\textsuperscript{6) reported similar results in relation to the effects of BZA/CE on obesity and type 2 diabetes in OVX mice. Metabolic benefits of BZA alone were lost in ER-\textalpha knocking out mice, indicating that BZA exhibits estrogen-mimetic action in metabolic regulation.\textsuperscript{5) In a series of TSEC studies on postmenopausal diabetes, we investigated the combination effects of BZA and estrogens [CE or 17\beta-estradiol (E2)] on the prevention of pancreatic \textbeta-cell failure by using MIN6 cells and streptozotocin (STZ)-exposed mice.
**Materials and methods**

**Cell culture**

Mouse MIN6 β-cells were kindly provided by Dr. Donald Steiner at University of Chicago. Cells (passage 20–30) were maintained at 37°C and 5% CO2 in DMEM supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate.

**Caspase assay**

To assess apoptosis, caspase 3/7 activity was determined using a Caspase-Glo 3/7 assay kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, MIN6 cells were cultured in 96-well plates and incubated with E2 (10⁻⁸ M), CE (10⁻⁸ M), BZA (10⁻⁷ M), E2 + BZA, CE + BZA or PBS for 24 h. Cell apoptosis was induced by treatment with a mixture of proinflammatory cytokines (IL-1, TNF-α, IFNγ) for 18 h in presence of hormone drugs. After incubation with Caspase-Glo reagent for 60 min, luminescence was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). Caspase 3/7 activity was expressed in relative luminescence units corrected for the total protein concentration.

**Glucose-stimulated insulin secretion**

Glucose-stimulated insulin secretion (GSIS) from MIN6 cells was determined using a static incubation protocol. Cells were cultured in 24-well plates until ~80% confluence and pretreated with hormone drugs for 24 h, as described for the caspase assay. Cells were washed with PBS and preincubated in glucose-free KRB buffer for 30 min. The medium was then changed to KRB buffer with 2.8 mM glucose, collected after 40 min, centrifuged, and used for insulin ELISA (Millipore Co., Billerica, MA). Cells were washed with glucose-free
KRB and incubated in the KRB with 16.7 mM glucose for 40 min. Medium was centrifuged and used for insulin ELISA. The results were normalized with protein concentrations.

**Plasmid transfection and luciferase assay**

MIN6 cells were cultured in 24-well plates and incubated with the mixture of 2 μL Lipofectamine 2000 (Invitrogen) and 0.8 μg of a reporter construct containing an estrogen response element (ERE) for 6 h. Cells were treated with hormone drugs for 24 h, as described for the caspase assay. For measurements of luciferase activity, cells were lysed with the Cell Culture Lysis Reagent (Promega). After centrifugation, 2–6 μg of the total protein were used in a Luciferase Assay System (Promega). Values are reported as relative luciferase units corrected for the total protein concentration.

**Animals and surgery**

Female 8-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were housed with a 12-h light-dark cycle. The mice were subjected to either a bilateral OVX or a sham operation under anesthesia with 1.2% avertin solution (i.p.). After a recovery period of 2 weeks, mice were divided into 6 treatment groups as follows: 1) sham + vehicle, 2) sham + STZ + vehicle, 3) OVX + STZ + vehicle, 4) OVX + STZ + CE, 5) OVX + STZ + BZA, 6) OVX + STZ + CE + BZA. Mice were treated with 50 mg/kg STZ (dissolved in 50 mM citrate buffer, pH 4.5) or with citrate buffer alone intraperitoneally for 5 consecutive days. All compounds were administered orally to mice once daily for 5 weeks with vehicle (saline, 2% Tween 80, 0.5% methylcellulose), CE 2.5 mg/kg, BZA 3 mg/kg or CE 2.5 mg/kg + BZA 3 mg/kg in the vehicle solution. The administered dosages of CE and BZA were chosen to ensure optimal maintenance of the estrogen action in the absence of uterine growth. All mice received phytoestrogen-free HFD (TD04059, 52% Kcal from anhydrous milk fat,
Harlan Teklad, Madison, WI, USA) and water *ad libitum* during the experimental period. At the end of the study, mice were euthanized by an overdose of avertin, and blood was collected by cardiac puncture. All animal work was performed in compliance with the Institutional Animal Care and Use Committee at the Northwestern University.

**Glucose and insulin measurements**

Random-fed blood glucose was measured from blood obtained from the tail vein using an OneTouch Ultra 2 glucose meter (LifeScan, Inc., Milpitas, CA). Hyperglycemia was defined as a non-fasting blood glucose level $\geq 200$ mg/dL. Cumulative incidence of diabetes was calculated as a percentage of hyperglycemic mice at each time point. Oral glucose tolerance test (OGTT) was performed at 3 weeks after STZ treatment. Mice were fasted overnight (16 h), and a glucose load (2 g/kg) was administered orally. Blood glucose and plasma insulin levels were measured from the tail vein at 15, 30, 60 and 90 min after administration of glucose. The area-under the curve (AUC) for glucose and insulin was calculated for each group of animals during OGTT. Following euthanasia, plasma was separated by centrifugation at 3000 $g$ for 20 min at 4°C and used for the determination of insulin levels using an ELISA kit (Millipore).

The pancreas was isolated, homogenized in acidified ethanol, extracted overnight at 4°C, and centrifuged. The insulin content of the supernatant was determined using an ELISA kit (Millipore) and expressed in ng/mg pancreas.

**Statistical analyses**

Data were analyzed by one-way ANOVA using SAS software for Windows release 9.2 (SAS Institute Inc., Cary, NC, USA) on the W32_VSHOME platform. To test for differences in uterine weights among the treatment groups, analysis of covariance (ANCOVA) with final
body mass as a covariate was used. Homogeneity of regression assumptions of the ANCOVA model were tested and met in each analysis. Differences in cumulative incidence of diabetes were determined by the log-rank test. The Least Squares Means option using a Tukey–Kramer adjustment was used for multiple comparisons among the treatment groups. Data were presented as the mean ± SEM. P values < 0.05 were considered statistically significant.

Results

Effects of TSEC with BZA on caspase activity, insulin secretion, and ERE activity in MIN6 cells

Estrogens protect pancreatic β-cell survival through ERα and ERβ via ERE-independent mechanism.9) To determine whether BZA affects estrogen action on β-cell survival and function, we measured caspase-3 activation (an indicator of apoptosis) and GSIS in MIN6 cells (Fig. 1A and B). Treatments with E2, CE, and BZA showed the reduced caspase-3 activation, but these effects were not statistically significant. Importantly, BZA enhanced the estrogen’s effects, resulting in the significant suppression of caspase-3 activation by CE + BZA treatment. We also observed that all hormone treatments increased insulin secretion in MIN6 cells in response to high glucose stimulation, although these changes did not reach statistical significance.

We next examined transcriptional activation of ERE in MIN6 cells to elucidate whether BZA acts via ERE-dependent pathway (Fig. 1C). As expected, treatments with E2 and CE significantly increased ERE activity by 4- and 3-fold, respectively. BZA alone did not affect ERE activity but significantly inhibited E2 or CE-induced ERE activation in MIN6 cells. These results indicate that, in combination with estrogens, BZA may protect β-cell survival via ERE-independent mechanisms.
Effects of TSEC with BZA on β–cell failure and uterine stimulation in OVX mice with STZ-induced diabetes

Since the US FDA approved the combination of CE with BZA as a first TSEC drug, we continued our focus on the ability of CE to protect against β–cell failure in vivo. Consistent with previous findings in OVX mice fed a HFD, BZA alone prevented body weight increase without uterine stimulation in STZ-treated OVX mice (Fig. 2A and B). The combination of CE and BZA had a greater impact on body weight reduction than each alone while simultaneously protecting uterine from estrogen stimulation. STZ treatment induced a progressive hyperglycemia with a corresponding increase in diabetes incidence, and these changes were more pronounced in OVX vehicle mice than in sham vehicle (Fig. 3A and B). Treatments with BZA, alone or with CE, significantly reduced a rise in the blood glucose level and diabetic incidence on day 35 in OVX + STZ mice, however there was no significant reduction following treatment with CE alone. Moreover, all treatments improved glucose tolerance (Fig. 3C and D), and CE+BZA treatment showed significantly higher insulin levels in response to glucose compared to the OVX vehicle (Fig. 3E and F), indicating that CE+BZA improves glucose-stimulated rapid insulin secretion. The non-fasting insulin/glucose ratio significantly increased in the CE+BZA group compared to the OVX vehicle, but not in CE or BZA groups (Fig. 3G). However, in this study, we did not observe any changes in pancreatic insulin content after hormone treatments in OVX + STZ mice (Fig. 3H).

Discussion

The combination of CE with BZA has been shown to be an appropriate alternative to hormone therapy for the treatment of menopausal symptoms, due to its ability to prevent menopause-associated vasomotor symptoms, bone loss, and sleep problems without
undesirable endometrial and breast stimulations in animal and human studies. Furthermore, we recently demonstrated that BZA, with or without CE, improved energy and glucose homeostasis in OVX mice fed a HFD. BZA/CE combination improved systemic insulin response by enhancing suppression of hepatic glucose production and glucose uptake in peripheral tissues, revealing the preventive role of BZA/CE against type 2 diabetes in menopause. In a series of TSEC studies on diabetes mellitus, the current data showed that BZA/CE combination protects β-cells from cytokine-mediated apoptosis and alleviates hyperglycemia by increasing insulin secretion in a STZ-induced classic animal model of type 1 diabetes without causing uterine hyperplasia. It was demonstrated earlier that E2 treatment protects β-cells from STZ-induced apoptosis and helps to sustain insulin production in an ERα-dependent manner. Although we did not observe significant alterations in pancreatic insulin content following CE treatment in this study, it is important to note that BZA enhanced, rather than reversed, the effect of CE on β-cell protection (Fig. 1A and 3).

In the classic ER signaling pathway, estrogen-activated ERα binds to either an ERE or a non-ERE tethered promoter to initiate gene transcription. Liu et al. reported that E2 protects β-cell survival via ERE-independent, extra-nuclear mechanisms, as well as G protein–coupled ER (GPER)-dependent mechanisms. In our study, BZA did not alter basal ERE activity but significantly inhibited estrogens-induced ERE activation in MIN6 cells. This result indicates that BZA, like E2, may protect β-cell survival, potentially mediated via ERE-independent genomic or non-genomic action. In non-genomic signaling pathway, membrane estrogen-ER complexes activate protein-kinase cascades, leading to phosphorylation of target transcription factors in the cytoplasm. Thus, further studies are needed to understand molecular mechanisms underlying ERE-independent genomic or non-genomic actions of BZA or TSEC in pancreatic β-cells.

Because of undesirable stimulation of estrogen-sensitive tissues by ER activation, the
regulation of the ER signaling pathway in a tissue-specific manner is critical for the
development of an ideal SERM. BZA has been reported to show improved tissue selectivity
compared to other SERMs. Indeed, BZA treatment was associated with significant
increases in bone mineral density in OVX rats as well as inhibition of E2-induced breast
cancer cell proliferation while less stimulating uterine than a typical SERM raloxifene.
Along with previous reports on metabolic regulation by BZA, our current data revealed
beneficial actions of BZA as an estrogen agonist in peripheral tissues such as liver, adipose
tissue, skeletal muscle, and pancreas. These findings suggest that BZA/CE combination
therapy could be used for the prevention of postmenopausal metabolic diseases beyond its
current use as a treatment of postmenopausal osteoporosis and vasomotor symptoms. The
present study provides critical information to guide further clinical studies in postmenopausal
women for novel therapeutic applications of BZA that would focus on its anti-diabetic
activity.

Acknowledgments
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Conflict of Interest
The authors declare no conflict of interest.
References and Notes


11) Le May C, Chu K, Hu M, Ortega CS, Simpson ER, Korach KS, Tsai MJ, Mauvais-Jarvis F. Estrogens protect pancreatic beta-cells from apoptosis and prevent insulini-


Figure legends

**Figure 1. Effects of TSEC with BZA on caspase activity, insulin secretion, and ERE activity in MIN6 cells.** MIN6 cells were incubated with vehicle, E2 (10^-8 M), CE (10^-8 M), BZA (10^-7 M), E2 + BZA, or CE + BZA for 24 h. (A) Caspase 3/7 activity in MIN6 cells exposed to a mixture of cytokine (IL-1β 50 U/mL, TNF-α 1,000 U/mL and IFNγ 1,000 U/mL). (B) Glucose-stimulated insulin secretion from MIN6 cells by static incubation. (C) Luciferase activity in MIN6 cells transfected with an ERE reporter construct. Values represent means ± SEM (n = 5). Caspase activity and ERE luciferase activity were expressed as relative luminescence units corrected for the total protein concentration. *Significantly different from the vehicle group treated with cytokines (A) or ERE reporter (C) (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

**Figure 2. Effects of TSEC with BZA on body weight and uterine stimulation in OVX mice with STZ-induced diabetes.** Mice were subjected to sham or OVX surgeries and received the indicated hormone treatments for 5 weeks. (A) Body weights. (B) Uterine weights. Means are adjusted for the final body mass as a covariate using the ANCOVA analysis. Values represent means ± SEM (n = 10). *Significantly different from the OVX + STZ vehicle group (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

**Figure 3. Effects of TSEC with BZA on glucose and insulin homeostasis in OVX mice with STZ-induced diabetes.** Mice were subjected to sham or OVX surgeries and received the indicated hormone treatments for 5 weeks. Diabetes was induced by multiple (for 5 consecutive days) low dose (50 mg/kg) intraperitoneal injection of STZ. (A) Fed blood glucose. (B) Cumulative incidence of diabetes was calculated as a percentage of
hyperglycemic mice (glucose level ≥ 200 mg/dL) at each time point. (C) Glucose concentrations during OGTT, (D) area under the curve (AUC) for glucose for (C), (E) insulin concentrations during OGTT and (F) AUC for insulin for (E). (G) The ratio of non-fasting insulin (pg/mL) and glucose (mg/dL) at day 35 was used as an index of insulin deficiency in mice. (H) Pancreatic insulin content. Values represent means ± SEM (n = 6-10).

*Significantly different from the OVX + STZ vehicle group (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
Figure 1

A

Caspase 3/7 activity (RLU)

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B

Insulin secretion (ng/μg protein)

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C

ERE luciferase activity (RLU)

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Figure 3

A. Blood glucose (mg/dL)

B. Cumulative incidence of diabetes (%)

C. Blood glucose (mg/dL)

D. AUC Glucose (mg/dL x 90 min)

E. Plasma insulin (pg/mL)

F. AUC Insulin (pg/mL x 90 min)

G. Insulin/glucose

H. Pancreatic insulin (ng/mg tissue)