Identification of Small Molecules That Protect Pancreatic β Cells against Endoplasmic Reticulum Stress-Induced Cell Death

Kim Tran,† Yu Li,† Hongliang Duan,† Daleep Arora,† Hui-Ying Lim,§ and Weidong Wang†,†

†Immunobiology and Cancer Research Program and §Free Radical Biology and Aging Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, United States

Supporting Information

ABSTRACT: Endoplasmic reticulum (ER) stress plays an important role in the decline in pancreatic β cell function and mass observed in type 2 diabetes. Here, we developed a novel β cell-based high-throughput screening assay to identify small molecules that protect β cells against ER stress-induced cell death. Mouse βTC6 cells were treated with the ER stressor tunicamycin to induce ER stress, and cell death was measured as a reduction in cellular ATP. A collection of 17600 compounds was screened for molecules that promote β cell survival. Of the approximately 80 positive hits, two selected compounds were able to increase the survival of human primary β cells and rodent β cell lines subjected to ER stressors including palmitate, a free fatty acid of pathological relevance to diabetes. These compounds also restored ER stress-impaired glucose-stimulated insulin secretion responses. We show that the compounds promote β cell survival by reducing the expression of key genes of the unfolded protein response and apoptosis, thus alleviating ER stress. Identification of small molecules that prevent ER stress-induced β cell dysfunction and death may provide a new modality for the treatment of diabetes.

Type 2 diabetes (T2D) is associated with pancreatic β cell dysfunction and death,† and increasing evidence indicates that endoplasmic reticulum (ER) stress is a major underlying cause of this decline.‡ ER stress has also been implicated in type 1 diabetes and monogenic diabetes.† Thus, compounds that prevent ER stress-induced β cell death hold promise as potential therapeutic agents for diabetes.

Accumulation of misfolded or unfolded proteins in the ER induces activation of the unfolded protein response (UPR). This process is initiated by three ER membrane-associated proteins that act as unfolded protein sensors; IRE1α, PERK, and ATF6, which each set in motion a series of events aimed at restoring ER homeostasis. These unique features of the ER, and as such, β cells are particularly susceptible to changes in ER homeostasis. These unique features of β cells may in part explain why compounds that protect many cell types from ER stress fail to protect β cells.10,12,13

In this study, we sought to identify novel small molecules that protect pancreatic β cells from ER stress-induced dysfunction and death. To this end, we established a HTS assay in which a β cell line is subjected to chronic ER stress with tunicamycin (Tm), which inhibits N-linked glycosylation and causes the accumulation of misfolded proteins.16 We tested the ability of 17600 diverse compounds to promote β cell survival in this assay. Several hits were identified, validated, and further investigated by characterization of their cytotoxicity, z-scan analysis, and sub-cellular localization. Finally, we performed a cell-based high-throughput screening assay to identify small molecules that promote β cell survival. Of the approximately 80 positive hits, two selected compounds were able to increase the survival of human primary β cells and rodent β cell lines subjected to ER stressors including palmitate, a free fatty acid of pathological relevance to diabetes. These compounds also restored ER stress-impaired glucose-stimulated insulin secretion responses. We show that the compounds promote β cell survival by reducing the expression of key genes of the unfolded protein response and apoptosis, thus alleviating ER stress. Identification of small molecules that prevent ER stress-induced β cell dysfunction and death may provide a new modality for the treatment of diabetes.
examining their effects on multiple β cell lines and primary human β cells treated with various chemical and pathophysiological ER stressors. These compounds not only promoted β cell survival but also restored the glucose-stimulated insulin secretion (GSIS) response in the presence of Tm. Finally, we demonstrate that these compounds protect β cells by inhibiting the expression of ER stress-associated and proapoptotic genes through distinct mechanisms. These results suggest that small molecule inhibitors of ER stress-induced β cell death may have therapeutic potential for diabetes.

RESULTS AND DISCUSSION

A Chronic β Cell ER Stress Assay for High-Throughput Screening. In T2D, β cells are under chronic ER stress induced by glucotoxicity, lipotoxicity, and amyloid accumulation due to obesity and insulin resistance. To identify compounds that protect β cells under conditions that mimic chronic ER stress, we developed a cell-based HTS assay in which the mouse insulinoma β cell line βTC6 is treated with Tm for 72 h, which induces characteristics of chronic ER stress. The cell viability is quantified using a luminescent ATP assay amenable to HTS. We first established the optimal dose of Tm for reduction of intracellular ATP levels as an indication of ER stress-induced β cell death. Tm at 0.35 μg/mL reduced cellular ATP levels by 50% compared with DMSO treatment (Figure 1A). This Tm concentration was therefore selected for our primary screen because it provides a sufficiently large window to observe inhibition of cell death by any protective compounds in the screening assay. The final assay was robust and highly reproducible, with a coefficient of variation (% CV) of 7.83% and a Z’ factor of 0.74, using cells incubated with Tm and the vehicle DMSO as the positive control.

Identification of Compounds That Protect β Cells against ER Stress. We screened approximately 17600 compounds from several libraries, including the Microsource Spectrum Diverse Set (2320 bioactive compounds), NIH Clinical Collection (NCC; 840 bioactive compounds), and Maybridge Hitfinder collection (14400 compounds). Compounds were considered hits if they increased ATP levels >3 standard deviations compared with control wells containing Tm + DMSO.

Figure 1. High-throughput screen for compounds that protect β cells against ER stress-induced death. (A) Cellular ATP levels (CellTiter-Glo luciferase activity) of mouse βTC6 cells treated with 0.1% DMSO (control) or varying concentrations of tunicamycin (Tm) for 72 h. Results are the mean ± SD of four replicate wells and representative of three independent experiments. (B) Identification of hit compounds. For the DMSO control and test compound wells, the corrected mean ± SD luminescence signal of 24 replicate wells was calculated. Standard score was calculated as (raw measurement of a compound − mean)/SD of the plate. Compounds were considered hits if they increased ATP levels >3 standard deviations compared with control wells containing Tm + DMSO. (C) Chemical structures of the seven hit compounds.
of control cells treated with Tm and DMSO. We identified 85 hits using this criterion (Figure 1B). Among the most potent hits in the Microsource and NCC collections were the antibiotics telithromycin, demeclocycline, and spectinomycin, as well as the cyclooxygenase-2 inhibitor deracoxib (Figure 1C). Hits from the Maybridge collection included compounds KM10103, RH01687, and RH01386 (Figure 1C). Each of these compounds was tested in more extensive dose-response assays with 2-fold dilutions between 70 μM and 270 nM and confirmed to increase ATP levels in βTC6 cells treated with Tm (Figure 2A). These compounds were therefore selected for further study.

To rule out the possibility that the observed increases in cellular ATP levels were due to an increase in βTC6 cell proliferation, we incubated the cells for 7 days in the presence of the compounds alone. We observed no differences in the proliferation of cells treated with the compounds compared with control DMSO-treated cells, indicating that the increase in cellular ATP levels under ER stress reflects rescue of Tm-induced cell death rather than increased cell proliferation (Supporting Information, Figure S1). To confirm this, we measured the effects of the seven compounds on the activity of caspase-3, a downstream effector of the apoptotic pathway. As expected, caspase-3 activity in βTC6 cells was markedly increased by Tm treatment, but all seven compounds dose-dependently inhibited the activity (Figure 2B). We conclude that these hits protect βTC6 cells from Tm-induced cell death.

**Hit Compounds Inhibit ER Stress-Induced Death of Primary Human β Cells.** The primary screen and confirmatory assays were performed with the βTC6 mouse β cell line. Therefore, we next asked whether the hit compounds have similar effects on additional β cell lines and, most importantly, on primary human β cells. Indeed, Tm-induced death of mouse (MIN6) and rat (INS-1) β cell lines was inhibited by the majority of compounds, although there were some differences between the cell lines in their sensitivity to some compounds (Figure 3A,B, respectively). Next, we examined the compounds’ effects on primary human islets and observed that telithromycin, spectinomycin, KM10103, RH01386, and RH01687 all significantly inhibited Tm-induced cell death, as indicated by the marked reduction of terminal deoxynucleotidyl transferase.

![Figure 2](https://example.com/figure2.png)
dUTP nick end labeling (TUNEL) staining (a marker for cell death) in compound-treated insulin+ β cells compared with DMSO-treated counterparts (Figure 3C and Supporting Information, Figure S2). These results indicate that the majority of the hit compounds are active in protecting rodent β cell lines and human primary β cells against ER stress.

Figure 3. Hit compounds protect primary human β cells and rodent β cell lines against ER stress-induced death. (A, B) Cellular ATP levels of mouse MIN6 (A) and rat INS-1 (B) β cells treated with 0.5 and 0.17 μg/mL Tm, respectively, and the indicated compounds for 72 h. Results are the mean of four replicate wells (with SD not shown for graphical simplicity) and representative of three independent experiments. (C) TUNEL staining in primary human islets. Primary human islets were treated with 0.75 μg/mL Tm and 20 μM of the indicated compounds for 72 h before TUNEL staining. Anti-insulin antibody was used to mark insulin+ β cells, and DAPI was used as a nuclear marker. Tm treatment induced TUNEL staining, which was mitigated or abolished by hit compound treatment.

Hit Compounds Protect against the Effects of Pathophysiological ER Stressors. ER stress can be induced by a number of stimuli acting through distinct molecular mechanisms. We therefore investigated the cytoprotective effects of the compounds when βTC6 cells were treated with thapsigargin (Tg) and brefeldin A (BFA), two commonly used...
Figure 4. Protective effect of hit compounds on β cell death induced by thapsigargin, brefeldin A, and palmitate. (A–C) Cellular ATP levels of βTC6 cells treated with the indicated compounds in the presence of 0.05 μM thapsigargin (Tg, A), 0.2 μg/mL brefeldin A (BFA; B), or 0.7 mM sodium palmitate (SP)/BSA conjugate (C) for 72 h. Results are the mean of four replicate wells (with SD not shown for graphical simplicity) and representative of three independent experiments. (D) TUNEL staining in primary human islets. Primary human islets were treated with 20 μM of the indicated compounds and 0.75 mM SP/BSA for 72 h before TUNEL staining. Anti-insulin antibody was used to mark insulin+ β cells, and DAPI was used as a nuclear marker. SP treatment induced TUNEL staining, which was mitigated or abolished by hit compound treatment.
agents to induce ER stress. Tg induces ER stress by inhibiting sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which disrupts intraluminal Ca^{2+} homeostasis in the ER and causes accumulation of unfolded proteins. BFA inhibits a key guanine nucleotide exchange factor essential for the transport of proteins from the ER to the Golgi. We observed that βTC6 cell death induced by Tg was inhibited by all selected hit compounds except deracoxib (Figure 4A), whereas BFA-induced death was inhibited significantly by RH01687 and RH01386 as well as by other hit compounds to a lesser extent (Figure 4B). Thus, the compounds were effective in reducing cell death induced by the three ER stressors, but the results suggest that the compounds show some cell type and stressor specificity.

We then investigated whether the hit compounds can protect βTC6 cells against a pathologically relevant ER stressor, the long-chain saturated free fatty acid (FFA) palmitate. Free fatty acids are thought to be important physiological mediators of β cell dysfunction and death in T2D, and palmitate has previously been shown to induce ER stress-mediated death of β cells. We found that palmitate-induced death of βTC6 cells was reduced by treatment with telithromycin, spectinomycin, KM10103, RH01687, and RH01386 (Figure 4C). Likewise, death of primary human β cells induced by palmitate was rescued by telithromycin, RH01687, and RH01386 (Figure 4D).

Taken together, these observations demonstrate that the hit compounds can reduce ER stress-related cell death of rat and murine β cell lines and primary human β cells induced by four ER stressors: Tm, Tg, BFA, and palmitate. Notably, the compounds show varying degrees of protection depending on both the β cell type and the ER stressor. This might be explained in part by the differing sites of action of the ER stressors. For example, in addition to inducing ER stress, Tg induces autophagy and BFA disrupts Golgi function. Nevertheless, compounds RH01687 and RH01386 effectively reduced death of rodent β cell lines and primary human β cells induced by all four ER stressors tested, including the pathologically relevant stressor palmitate. Interestingly, RH01687 and RH01386 are structurally similar, both having core nitrogen-containing aromatic rings linked with the 2-nitro-phenylthio group (Figure 1C). We selected RH01687 and telithromycin (as a representative of antibiotics) for further mechanistic analyses.

Cytoprotective Compounds Preserve the Insulin-Secreting Function of β Cells. Secretory cells are particularly vulnerable to agents that disrupt protein translation, folding, and modification. As a result, ER stress directly impairs β cell function, including insulin biosynthesis and GSIS. We next examined whether our hit compounds could re-establish GSIS in β cells treated with Tm. Incubation of INS-1 cells in 25 mM glucose-containing medium increased insulin secretion approximately 2-fold compared with baseline secretion in 2.8 mM glucose medium (Figure 5A). Tm treatment not only abolished insulin secretion stimulated by high glucose concentrations but also reduced basal levels of insulin secretion, consistent with disruption of ER homeostasis (Figure 5A). Addition of RH01687 or telithromycin significantly increased GSIS in the Tm-treated cells and also increased basal levels of secretion, although the latter did not reach the level of statistical significance. We also examined the GSIS response of primary human islets and found that RH01687 and telithromycin were also able to significantly restore the Tm-inhibited GSIS response of these cells (Figure 5B). Thus, RH01687 and telithromycin not only protect β cells against ER stress-induced cell death but also preserve β cell function.

Figure 5. Hit compounds preserve glucose-stimulated insulin secretion in β cells subjected to ER stress. (A) Insulin secretion by INS-1 cells incubated with 2.8 mM or 25 mM glucose in the presence of 0.35 μg/mL Tm and the indicated compounds. Secreted insulin was measured by ELISA after 72 h incubation, and the values were normalized to total cellular protein. (B) Insulin secretion by human islets (50 of equal size) incubated with 2.8 mM glucose or 20 mM glucose in the presence of 0.75 μg/mL Tm and the indicated compounds. Secreted insulin was measured by ELISA after 72 h incubation, and the values were normalized to total islet protein. For both panels A and B, data are shown as glucose stimulation insulin secretion index (= mean of secreted insulin from quadruple wells incubated with 25 mM (for INS-1) or 20 mM (for human islets) glucose/mean of secreted insulin from quadruple wells incubated with 2.8 mM glucose), in which the baseline insulin secretion at 2.8 mM glucose was normalized as 1, and were representative of four independent experiments. *P < 0.05, **P < 0.001 by Student’s t-test compared with control cells treated DMSO + Tm.

Cytoprotective Compounds Protect β Cells by Alleviating ER Stress and Inhibiting Proapoptotic Gene Expression. We next investigated the molecular mechanisms by which the hit compounds exert their protective effects. First we wanted to determine whether our hit compounds protect β cell survival by resolving or alleviating ER stress. ER stress induces the UPR signaling pathways of IRE1α, PERK, and ATF6α, which trigger translational and transcriptional changes aimed at re-establishing ER homeostasis. Failure to resolve or adequately control ER stress can result in UPR-triggered apoptosis. The C/EBP-homologous protein (CHOP) is a transcription factor that is activated during ER stress-triggered apoptosis; it is induced under ER stress mainly by the PERK pathway, although IRE1α and ATF6α also contribute, and is generally used as an ER stress marker of apoptosis. As expected,
Tm significantly induces the expression of CHOP as shown in Figure 6A,B. To determine whether CHOP expression is affected by the hit compounds, we used HEK293 cells stably transfected with a CHOP promoter/Luciferase reporter construct that faithfully reflects endogenous CHOP gene expression.26 Tm treatment of these cells induced the luciferase reporter by ∼3-fold, but treatment with RH01687 or telithromycin significantly inhibited CHOP expression (Figure 6A). To confirm this in βTC6 cells, we analyzed CHOP mRNA expression in βTC6 cells treated with 0.35 μg/mL Tm and the indicated compounds for 24 h. mRNA levels were normalized to Gapdh mRNA and are expressed as the fold increase in mRNA compared with cells treated with DMSO alone. Results are the mean ± SD of n = 3 wells and representative of four independent experiments. *P < 0.05 by Student’s t-test compared with Tm-treated cells. These results indicate that the hit compounds protect β cells by alleviating ER stress.

We then investigated whether our hit compounds affect the expression levels of proapoptotic genes. Under unresolved ER stress, multiple factors, CHOP, ATF4, P53, and JNK, have been reported to be induced to participate in the induction of proapoptotic BH3-only proteins such as Bim, Bad, Noxa, and Puma.27,28 Each BH3-only protein is activated by ER stress in a distinct manner. For example, Bim is activated by CHOP and JNK, whereas Puma and Noxa are activated by ER stress-mediated p53 up-regulation. These BH3-only proteins subsequently stimulate the multidomain proapoptotic proteins Bax or Bak to form homo-oligomers in the outer mitochondrial membrane, leading to caspase-mediated cell death. We examined expression of Bim, Bad, and Bax mRNA in βTC6 cells. Treatment with Tm for 24 h significantly increased the expression of each gene (Figure 6C), but interestingly telithromycin and RH01687 both significantly suppressed Tm-induced increase of Bax mRNA but not of Bim or Bad mRNA (Figure 6C). These results suggest that, although both compounds alleviate ER stress to protect β cells, they appear to do so by targeting different UPR pathways (Figure 6C).

We further asked whether the combination of these two compounds might have an additive effect in protecting βTC6 cells from Tm-induced cell death. Indeed, cells treated with Tm and both telithromycin and RH01687 at varying concentrations showed significantly increased survival compared with cells treated with the same concentrations of either compound alone (Figure 6D). These results indicate that the two compounds inhibit ER stress-induced βTC6 death in an additive manner, consistent with their distinct effects on Bim and Bax expression.

Figure 6. Hit compounds inhibit ER stress-induced expression of CHOP and proapoptotic genes. (A) HEK293 cells stably transfected with a CHOP promoter/Luciferase reporter construct were treated with 1 μg/mL Tm and 20 μM of the indicated compounds for 24 h before luciferase activity was measured. Results are the mean ± SD of n = 8 wells and representative of four independent experiments. *P < 0.05 by Student’s t-test compared with control cells treated with DMSO + Tm. (B, C) qRT-PCR analysis of mRNA levels of CHOP (B) or the proapoptotic genes Bim, Bad, and Bax (C) in βTC6 cells treated with 0.35 μg/mL Tm and the indicated compounds for 24 h. mRNA levels were normalized to Gapdh mRNA and are expressed as the fold increase in mRNA compared with cells treated with DMSO alone. Results are the mean ± SD of n = 3 wells and representative of four independent experiments. *P < 0.05 by Student’s t-test compared with Tm-treated cells. (D) Additive effect of telithromycin and RH01687 on Tm-induced βTC6 cell death. βTC6 cells were treated with 0.35 μg/mL Tm and the indicated concentrations of telithromycin or RH01687, either alone or in combination, for 72 h before measurement of cellular ATP levels. *P < 0.05 by Student’s t-test compared with the same concentrations of telithromycin alone, and #P < 0.05 by Student’s t-test compared with the same concentration of RH01687 alone.
Telithromycin Inhibits ATF4- and CHOP-Induced Protein Synthesis Increase under ER Stress. Several of our hit compounds including telithromycin belong to a class of bacteriostatic antibiotics that primarily block prokaryotic protein synthesis by targeting ribosomes. However, some bacteriostatic antibiotics are just as effective in inhibiting eukaryotic protein synthesis.28 We hypothesize that these antibiotic hits in our screen could protect β cells against ER stress-induced cell death by inhibiting protein translation. Increase in protein synthesis was recently reported to be one mechanism of ER stress-induced cell death.30–32 Under ER stress, phosphorylation of eIF2α by PERK reduces overall protein translation to re-establish ER homeostasis; however, it also preferentially promotes the translation of several mRNAs containing S′-upstream open reading frames such as ATF4, which subsequently induces the expression of CHOP. ATF4 and CHOP act together to activate the expression of genes involved in protein synthesis to restore general mRNA translation. The ATF4- and CHOP-mediated restoration of protein synthesis promotes cell survival after ER homeostasis is re-established when ER stress is transient. However, under severe or prolonged ER stress, in which the initial protein synthesis reduction fails to restore ER homeostasis, the ATF4- and CHOP-mediated protein synthesis increase leads to ATP depletion, oxidative stress, and cell death.30–32

To determine whether telithromycin suppresses the ATF4- and CHOP-mediated increased in β cells, we measured the rate of protein synthesis of βTC6 cells under ER stress with and without telithromycin. Consistent with recent reports,30,31 Tm treatment led to an initial steep decline in protein synthesis, but it underwent a gradual recovery (Figure 7, lanes 1, 2, 4, 6, 8, 10, and 12). We observed that telithromycin attenuated the recovery of protein synthesis in Tm-treated βTC6 cells (Figure 7, lanes 3, 5, 7, 9, 11, and 13). These results suggest that telithromycin protects β cell survival against ER stress likely by inhibiting ATF4- and CHOP-mediated protein synthesis increase.

Discussion. In this study, we have described a novel HTS assay for the identification of small molecule inhibitors of ER stress-induced apoptosis in pancreatic β cells. Several compounds identified were able to protect primary human β cells from cell death induced by various ER stressors, including the diabetes-relevant free fatty acid palmitate. Notably, the compounds appeared to protect against ER stress by inhibiting the expression of key genes known to be involved in UPR-stimulated apoptosis.

Previous HTS efforts have identified compounds that modulate the UPR pathway in cell-free biochemical assays33–39 and that protect non-β cells against ER stress-induced death.10,11 We found that most of those compounds (e.g., salubrinal, STF083010, quercetin) were ineffective in protecting β cells from cell death induced by various ER stressors, including the diabetes-relevant free fatty acid palmitate. Notably, the compounds appeared to protect against ER stress by inhibiting the expression of key genes known to be involved in UPR-stimulated apoptosis.

Figure 7. Telithromycin inhibits ATF4- and CHOP-induced protein synthesis increase under ER stress. Newly synthesized proteins were pulse-labeled with 5 μg/mL puromycin for 10 min in βTC6 cells treated with Tm in the presence or absence of telithromycin (20 μM) at specified time points. Puromycin-bound newly synthesized proteins were detected by immunoblotting with antipuromycin antibody. The intensity of the total signal of each lane was measured with Image-J software and plotted under the image of the immunoblot with the relative intensity of the signal for the sample in the absence of Tm as 1. The data shown is a representative of three independent experiments.
antibiotics. These and our findings may suggest a relationship between ER stress/protein synthesis and cell survival/growth.

Our study suggests that identification of compounds able to protect β cells against ER stress and elucidation of their mechanisms of actions may not only lead to the development of therapeutics for diabetes but also uncover novel players and mechanisms that are unique to the UPR and ER stress response in β cells.

■ METHODS

Cell Culture and Reagents. The mouse insulinoma cell lines βTC6 (ATCC) and MIN6 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1× GlutaMax, 1× nonessential amino acids, and 1 mM sodium pyruvate. INS-1 rat insulinoma cells were maintained in RPMI medium supplemented with 10% FBS, 1× GlutaMax, 1× nonessential amino acids, and 1 mM sodium pyruvate. Human islets were obtained from the Integrated Islet Distribution Program (Duarte, CA) in accordance with Oklahoma Medical Research Foundation internal review board (IRB) and ethical guidelines for the use of human tissue. Standard viability was 80–90% and purity was >80%. Islets were maintained in CMRL medium supplemented with 10% FBS. All cells were grown at 37 °C in a humidified 5% CO₂ atmosphere. Tunicamycin (Tm), brefeldin A (BFA), and thapsigargin (Tg) were from Sigma. CellTiter-Glo and Caspase-Glo 3/7 reagents were from Promega. Salubrinal, guanabenz acetate, STF083013, and kaempferol were from Tocris, quercetin and apigenin were from Cayman, and PP1 Analog II was from Calbiochem.

Compound Libraries. Three compound libraries were screened: 2320 compounds from Microsource Spectrum Diverse Set (Microsource Discovery Systems), 840 from the NIH Clinical Collection (NIH), and 14400 from Maybridge Hitfinder (MayBridge Corporation). All compounds were stored in DMSO at 10 mM. Compounds from the Microsource Spectrum and NIH Clinical Collection (NCC) were formatted in an 8-point 2-fold titration fashion, with high concentration at 10 mM.

High-Throughput Screening Assay. βTC6 cells were resuspended in DMEM/15% FBS and plated at 5 × 10³ cells/(40 μL well) into white clear bottom 384-well plates (Greiner) using an automated liquid handler (Thermo Fisher Scientific). After 24 h incubation at 37 °C, library compounds were added to the wells at a final concentration of 10 μM using a pin-transfer robot (PerkinElmer). Tm in DMEM/15% FBS was then added at a final concentration of 0.35 μg/mL. Control wells contained βTC6 cells + Tm + 0.1% DMSO. After 72 h, the medium was removed and 20 μL of CellTiter-Glo reagent was added. Luminescence was measured 10 min later using an Envision plate reader (PerkinElmer). The Microsource Spectrum library was scored at single point (10 μM), while Microsource and NCC libraries were screened at eight 2-fold serial dilutions with highest concentration of 10 μM.

Screening Data Analysis. Hit selection was based on “standard scores” (Figure 1B). The mean and standard deviation (SD) of luminescence for each compound was determined, and the standard score for each compound was then calculated as (raw measurement of a compound − mean)/SD of the plate. Compounds that increased ATP levels >3 standard deviations compared with control wells (standard score >3) were considered hits. Compounds identified from the primary screen were cherry picked into new 384-well plates in ten 2-fold serial concentration dilutions for validation experiments. The signal/noise ratio of the assay was determined from the mean and SD of the sample and background of the plate as previously described.41 The Z score of the assay was calculated from the means and SDs between DMSO-treated and Tm-treated wells, as previously described.12

ATP and Caspase-3 Assays. Assays were performed as described for the HTS screening with the following exceptions. All cells were incubated in 384-well plates at 3 × 10³ cells/(40 μL well) except human islet β cells, which were added at 10³ cells/well. Final concentrations of test compounds above 10 μM were obtained by repeated addition from the same stock plate of 10 mM compound. Final concentrations of Tm were 0.35 μg/mL for βTC6, 0.5 μg/mL for MIN6, 0.17 μg/mL for INS1, and 0.75 μg/mL for primary human islet cells. Final concentrations of Tg and BFA were 0.05 μM and 0.2 μg/mL, respectively. For assays in which ER stress was induced by palmitate, a stock solution of 5 mM sodium palmitate (SP) in 5% BSA was prepared as previously reported.33 The medium was changed to DMEM/1% FBS/1% BSA (final concentration, taking into account the SP/BSA addition), and SP was added to a final concentration of 0.7 mM. After 72 h incubation at 37 °C, the medium was removed, and cells were incubated with 20 μL of CellTiter-Glo (for ATP levels) or Caspase-Glo 3/7 (for caspase-3 activity) reagents. Luminescence was measured after 10 min (CellTiter-Glo) or 2 h (Caspase-Glo 3/7).

CHOP Reporter Assay. HEK293 cells stably transfected with a CHOP promoter/luciferase reporter were plated at 7 × 10³ cells/well in a 384-well plate and incubated for 16 h. Test compounds were then added, followed by Tm at 1 μg/mL. Luciferase activity was measured with a Bright-Glo kit (Promega) 24 h later.

Glucose-Stimulated Insulin Secretion. INS-1 or primary human islet cells were plated at 1.5 × 10³/well in 96-well plates and incubated overnight. The following day, test compounds and 0.35 or 0.7 μg/mL Tm were added for a further 72 h. Cells were washed and incubated for 2 h in freshly prepared KRBB buffer (115 mM NaCl, 5 mM KCl, 24 mM NaHCO₃, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 2% w/v BSA, pH 7.4) containing 2.8 mM glucose. Cells were then incubated for an additional hour in KRBB buffer containing 2.8, 25 (for INS-1 cells), or 20 mM (for human islets) glucose. The supernatants were collected, and secreted insulin was measured using insulin ELISA kits (for mouse insulin from Millipore and for human insulin from LifeTech). Cells were lysed with RIPa buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors, and total cellular protein was determined with Bradford protein assay. All secreted insulin levels were corrected for total protein. Data were shown as glucose stimulation insulin secretion index = [mean of secreted insulin from quadraple wells incubated with 25 mM (for INS-1) or 20 mM (for human islets)]/ [mean of secreted insulin from quadraple wells incubated with 2.8 mM (for INS-1)].

Quantitative Real-Time PCR. RNA was isolated from βTC6 cells using TRIzol (Life Technologies) and reverse transcribed using oligo d(T) primers (New England Biosystems) and SuperScript III reverse transcription kit (Applied Biosystems). qPCR was performed using SYBR Green mix (Applied Biosystems) with an Applied Biosystems 7500 real-time PCR system. Mouse-specific primers were as follows: Bax, 5′-ACCAAGAAGCTGGCGATGT-3′ and 5′-CACTCTCACATTCTCTCT-3′; Bad, 5′-GGATGACGGCTGTTGTTG-3′ and 5′-TCCCAACACAGGTGGATAAGT-3′; Bim, 5′-CGGCAGTCTCAGAGGAGGACC-3′ and 5′-CATTGTGCAAAA-CACCTCCTCT-3′; Chop, 5′-TTTCACTACTCTT-GACCCTGCCTGTC-3′ and 5′-CTTGGTAACCTCCAGGGAG-3′; C-caspase 3, 5′-CACGTCAGAGTGGGTGCCA-3′ and 5′-CCCTGAGAACACCCGACAGAAGT-3′; and 5′-TGGGAAAGTGGAGTTCCTGT-3′. Relative mRNA levels were normalized against the housekeeping gene Gapdh using the comparative CT method.

Immunofluorescent and TUNEL Staining. Human islets were briefly washed with PBS and fixed with 4% paraformaldehyde for 30 min at RT. Fixed cells were blocked in 5% normal donkey serum in PBS for 1 h. Polyclonal guinea pig anti-insulin (A0564, Dako, 1:500 dilution) was used as primary antibody. Donkey Cy3 anti-guinea pig IgG was used as secondary antibody. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed to detect apoptosis with In Situ Cell Death Detection Kit-Fliorescein (Cat. No. 11684795910, Roche), according to the manufacturer’s instruction. DAPI was used for nuclear counter-staining. Images were taken with Olympus FV1000 confocal microscopy.

Measurement of Protein Synthesis with Puromycin Labeling Assay. βTC6 cells (5 × 10⁴) treated with Tm in the presence or absence of telithromycin at specified time points were pulsed with 5 μg/mL puromycin for 10 min to label newly synthesized proteins.42 Cells were washed 3 times with ice-cold PBS followed by lysis with RIPA buffer supplemented with protease inhibitors, cleared by centrifugation, and analyzed by Western blotting with antipuromycin antibody (Millipore, MABE343, clone 12D10, 1:20000 dilution).
**Statistical Analysis.** Data are presented as means ± SD. Comparisons were performed by two-tailed paired Student’s t-test. A P value < 0.05 was considered statistically significant.

**ASSOCIATED CONTENT**

**Supporting Information**
Data showing that hit compounds do not increase β cell ATP levels by inducing cell proliferation, hit compounds protect human β cells against ER stress-induced death, and known modulators of ER stress do not protect β cells against ER stress. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

**Corresponding Author**
*E-mail: weidong-wang@omrf.org.

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The authors declare no competing financial interest.

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