Published in final edited form as:

Bioorg Med Chem. 2016 June 15; 24(12): 2621–2630. doi:10.1016/j.bmc.2016.03.057.

Identification of 1,2,3-triazole derivatives that protect pancreatic β cells against endoplasmic reticulum stress-mediated dysfunction and death through the inhibition of C/EBP-homologous protein expression

Hongliang Duan[†], Daleep Arora[†], Yu Li^{‡,†}, Hendra Setiadi[†], Depeng Xu[†], Hui-Ying Lim[#], and Weidong Wang^{‡,†,*}

[†]Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, Oklahoma 73104, United States

[#]Aging and Metabolism Program, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, Oklahoma 73104, United States

[‡]Department of Medicine, Division of Endocrinology, Department of Physiology, Harold Hamm Diabetes Center, The University of Oklahoma Health Science Center, 941 Stanton L. Young Blvd., Oklahoma City, Oklahoma 73104, United States

Abstract

The C/EBP-homologous protein (CHOP) acts as a mediator of endoplasmic reticulum (ER) stress-induced pancreatic insulin-producing β cell death, a key element in the pathogenesis of diabetes. Chemicals that inhibit the expression of CHOP might therefore protect β cells from ER stress-induced apoptosis and prevent or ameliorate diabetes. Here, we used high-throughput screening to identify a series of 1,2,3-triazole amide derivatives that inhibit ER stress-induced CHOP-luciferase reporter activity. Our SAR studies indicate that compounds with an *N*,1-diphenyl-5-methyl-1*H*-1,2,3-triazole-4-carboxamide backbone potentially protect β cell against ER stress. Several representative compounds inhibit ER stress-induced up-regulation of CHOP mRNA and protein, without affecting the basal level of CHOP expression. We further show that a 1,2,3-triazole derivative **4e** protects β cell function and survival against ER stress in a CHOP-dependent fashion, as it is inactive in CHOP-deficient β cells. Finally, we show that **4e** significantly lowers blood glucose levels and increases concomitant β cell survival and number in a streptozotocin-induced diabetic mouse model. Identification of small molecule inhibitors of CHOP expression that prevent ER stress-induced β cell dysfunction and death may provide a new modality for the treatment of diabetes.

*Corresponding author. Weidong-wang@ouhsc.edu.

Supporting Information. Supporting figures 1–8.

Notes

The authors declare no competing financial interest.

INTRODUCTION

The dysfunction and death of insulin-producing pancreatic β cells are key elements in the pathogenesis of type 1 and type 2 diabetes.^{1, 2} Increasing evidence indicates that endoplasmic reticulum (ER) stress, a condition in which misfolded proteins accumulate in the ER, plays an important role in the decline in pancreatic β cell function and mass.^{3, 4} Indeed, ER stress alone can initiate and propagate all of the characteristics of β cell failure and death observed in diabetes.⁵ In addition, several causes for β cell dysfunction and death in diabetes, including lipotoxicity, glucotoxicity, oxidative stress, amyloid deposition, and insulin mutations, have been known to be associated with unresolvable chronic ER stress.^{3, 6, 7}

ER stress induces activation of the unfolded protein response (UPR) through three ER membrane proteins IRE1 α , PERK, and ATF6. The UPR is an adaptive mechanism aimed at re-establishing ER homeostasis by altering the translation, folding, and post-translational modification of all secreted and membrane proteins.^{8–11} However, failure to adequately resolve ER stress can result in UPR-triggered apoptosis.^{8–12}

The transcription factor C/EBP-homologous protein (CHOP) is a key mediator of ER stress-induced apoptosis.^{13, 14} Under ER stress, CHOP is activated mainly by the PERK pathway, although IRE1 α and ATF6 α also contribute.^{15, 16} CHOP has been linked to ER stress-mediated β cell apoptosis in the pathogenesis of diabetes.^{5, 14, 17–20} Furthermore, CHOP deficiency is known to delay the onset and ameliorate ongoing symptoms of diabetes by improving β cell function and survival.^{18, 19} Therefore, compounds that inhibit the expression or activity of CHOP could increase β cell function and survival, and prevent the onset or progression of diabetes. However, such compounds have not yet been identified.

In this study, we describe the identification of small molecules that inhibit CHOP expression and protect pancreatic β cells from ER stress-induced dysfunction and death. We used high-throughput screening to identify several 1,2,3-triazole derivatives that inhibit CHOP activity in a reporter cell line, and inhibit ER stress-induced β cell death and impaired glucose-stimulated insulin secretion (GSIS). This group of compounds protects β cells by inhibiting ER stress-induced upregulation of CHOP mRNA and protein. Importantly, we show that the 1,2,3-triazole derivatives significantly lower blood glucose levels and increase β cell survival and number in a streptozotocin-induced diabetic mouse model. Our results suggest that small molecule inhibitors of CHOP expression may have therapeutic potential for diabetes.

RESULTS AND DISCUSSION

Identification of Compounds that Inhibit ER Stress-mediated CHOP Up-regulation via High Throughput Screen

To identify compounds that inhibit the expression of CHOP, we established a HEK293 cell line stably transfected with a *CHOP* promoter:*luciferase* reporter (CHOP-Luc) construct that faithfully reflects endogenous CHOP gene expression.^{21, 22} The CHOP-Luc activity in this cell line was induced ~4-fold by tunicamycin (Tm, 1 μ g/mL), a potent ER stress inducer that causes the accumulation of misfolded proteins due to inhibition of N-linked glycosylation.²³

Using high-throughput screening of ~50,000 structurally diverse small molecules, we identified a number of compounds that inhibited the CHOP-Luc activity of Tm-treated HEK293 cells. A series of four 1,2,3-triazole amide analogs **1a–d** (Table 1) inhibited CHOP-Luc activity in these cells to varying degrees (with IC₅₀s ranging from 0.013~1.5 μ M). We also examined the effect of the 1,2,3-triazole derivatives on endogenous CHOP expression. HEK293 cells exposed to Tm showed a time-dependent increase in CHOP mRNA levels, and by 4 h, levels were ~12-fold higher. Notably, addition of a representative hit compound, **1d**, partially but significantly suppressed the Tm-induced upregulation of CHOP mRNA (Figure 1A).

Given the key role played by CHOP in ER stress-induced apoptosis^{13,14,15} and the observation that CHOP deletion protects β cell function and survival under ER stress^{18, 19}, we investigated whether the 1,2,3-triazole derivatives could protect the rat β cell line INS-1 from the deleterious effects of ER stress. Tm treatment for 72 h reduced the viability of INS-1 cells by ~70% compared with DMSO-treated cells, as measured by intracellular ATP level (Figure 1B). We found that INS-1 cell viability was dose-dependently rescued by co-incubation with **1d** (Figure 1B). As shown in Table 1, all hits of the 1,2,3-triazole derivatives protected INS-1 cells to some degree, with maximal activity ranging from 12% to 33% rescue of cell viability.

Structure-Activity Relationship Studies of 1,2,3-Triazole Derivatives

To identify more potent 1,2,3-triazole derivatives, we performed structure-activity relationship (SAR) analysis on additional analogs of this series and assessed their protective activity on ER-stressed INS-1 cells. First, we found that 4-ethyl substitution in the left phenyl ring (R1) (**3c** and **3d**) greatly improved β cell-protective activity compared to **1a–d**, with maximal activity at 72% and 82%, respectively (Table 2) and similar EC₅₀ ranges. Second, in the presence of the 4-ethyl group in R1, derivatives with 3-fluoro (**4e**) (Figure 1C), 2-methyl (**4a**, **4k** (Supplemental Figure 1), **4l**), and 2-ethyl (**4b**) substitutions on the right phenyl ring (R2) improved or maintained the β cell-protective potency (Table 3). However, replacement of the 2-methyl/ethyl group with chlorine (**4c**, **4m**) severely impaired their activity. Similarly, introduction of a carboxyl group to the para position in the right phenyl ring (**4h**, **4i**, **4j**) eliminated the β cell-protective activity, suggesting that some polar groups, such as carboxyl or amide groups, were not well tolerated. Third, given the beneficial effect of 2-methyl/2-ethyl groups in the right phenyl ring on β cell protection, various groups were introduced while retaining the 2-methyl group (Table 4). We observed that the majority of 2-methyl, 2-methyl-3-chloro, 2,6-dimethyl, and 2-ethyl-6-methyl substitutions in R2 improved or maintained the β cell-protective activity, with maximal activities ranging from 44% to 97% of β cell protection (Table 4). These activities were achieved in the presence of an array of structurally diverse substituents in R1, including 4-methyl (**5h**, **5o**, **5x**), 2-chloro (**5e**, **5m**) (Supplemental Figure 2), 4-ethyl (**5i**), and 2-methyl-3-chloro (**5q**, **5z**) (Supplemental Figure 3), indicating great latitude for variations in the R1 phenyl moiety in the presence of 2-methyl/ethyl groups in R2. We noticed that 3-chlorine (**5d–k**, Table 4) is well tolerated compared to 2-chlorine (**4c**, **4m**, Table 3) at the R2 position, probably because 2-chlorine, rather than 3-chlorine, may affect the molecular conformation and compound activity by forming a weak hydrogen bond with the adjacent

N-H. Overall, our preliminary SAR studies indicated that compounds with an *N*,1-diphenyl-5-methyl-1*H*-1,2,3-triazole-4-carboxamide backbone potently protect β cell viability against Tm-induced ER stress. We chose several such compounds (i.e., **4e**, **4k**, **5e** and **5z**) as representative 1,2,3-triazole amides for further study because of their potent β cell-protective effects against ER stress (high maximum activity with EC50 at low μ M (**4e**, **4k**, and **5e**) or near 100% maximum activity (**5z**)).

β Cell-protective Effect of 1,2,3-Triazole Derivatives Through Inhibition of ER Stress-mediated CHOP Up-regulation

We then investigated whether 1,2,3-triazole amide analogs inhibited ER stress-induced upregulation of CHOP expression in β cells. As expected, Tm treatment markedly upregulated CHOP mRNA levels in INS-1 cells, but the effect was significantly decreased by co-treatment with **4e** in a dose- and course-dependent fashion (Figures 2A and 2B). In agreement with its effects on CHOP mRNA transcription, **4e** co-treatment also decreased CHOP protein levels in Tm-treated INS-1 cells (Figure 2C). Of note, treatment with **4e** alone had no effect on CHOP mRNA levels (Figure 2D). We also observed that a number of other analogs, including **4k**, **5e** and **5z**, had a similar ability to suppress the ER stress-induced increase in CHOP mRNA expression (Supplemental Figures 4, 5 and 6). In contrast, the β cell-inactive analogs **4h** and **4i** had no effect on the inhibition of Tm-mediated CHOP induction (Figure 2E and Supplemental Figure 7). These results reveal that the β cell-protective effect of 1,2,3-triazole amide derivatives is tightly associated with the inhibition of ER stress-induced up-regulation of the CHOP gene and that 1,2,3-triazole amide derivatives suppress ER stress-mediated increase, but not basal level, of CHOP expression.

To investigate whether the inhibition of CHOP expression is critical for 1,2,3-triazole amide derivative-mediated β cell protection against ER stress, we utilized pancreatic islet β cells deficient in CHOP (CHOP^{-/-}). Induction of ER stress in these cells results in cell death through activation of IRE1 α and ATF6 pathways (but not PERK/CHOP pathway). If the 1,2,3-triazole amide derivatives function through CHOP pathway, treatment with these compounds would not prevent cell death. Indeed, Tm treatment (1 μ g/ml) induced cell death in both control (CHOP^{+/+}) and CHOP^{-/-} pancreatic islet β cells, although to a lesser degree in CHOP^{-/-} cells as expected, as analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which detects fragmentation of DNA, a marker of apoptotic cell death (Figures 3A–D). While co-treatment with compound **4e** significantly inhibited the detection of TUNEL staining in control CHOP^{+/+} insulin⁺ cells (9% of TUNEL for **4e** treatment versus 40.4% for DMSO, Figures 3A–B), it did not show an obvious effect on the TUNEL staining in CHOP^{-/-} insulin⁺ cells (14.6% of TUNEL for **4e** treatment versus 18.7% for DMSO, with no statistical significance) (Figure 3C–D). Taken together, these results indicate that **4e**-mediated inhibition of CHOP is essential for its β cell-protective effect against ER stress.

1,2,3-Triazole Derivative **4e** Inhibits ER Stress-mediated Apoptosis in β Cells

To determine whether the 1,2,3-triazole amide derivatives increased β cell viability by inhibiting apoptosis, we examined the effects of the compounds on several apoptotic markers in Tm-treated β cells. We have shown that **4e** inhibited the TUNEL staining in Tm-

treated primary CHOP^{+/+} islet β cells (Figure 3A–D). Similarly, in INS-1 β cells, the Tm-induced increase in TUNEL-positive cells was significantly reduced by treatment with **4e** from ~15% to 1.5% TUNEL-positive cells (Figure 4A, 4B). These results support **4e**-mediated inhibition of apoptosis in β cells. We used additional markers to confirm this conclusion. ER stress-induced apoptosis is manifested by marked increases in multiple key events such as cleavage of caspase-3, a critical executioner of apoptosis, and cleavage of poly (ADP-ribose) polymerase (PARP), which plays an important role in DNA repair and cell death.^{24, 25} We observed that **4e** significantly inhibited the Tm-induced cleavage of both caspase-3 and PARP in INS-1 cells (Figure 4C). Moreover, INS-1 cells treated with **4e** alone exhibited a growth rate similar to that of DMSO-treated cells (Supplemental Figure 8), arguing that **4e** did not simply increase the intracellular ATP level of Tm-treated INS-1 cells through enhanced proliferation. **4e** also protected INS-1 cells against death induced by another ER stressor, brefeldin A, which acts by disrupting the transport of proteins from the ER to the Golgi²³ (Figure 4D). We then investigated whether the hit compounds can protect β TC6 cells against a pathologically relevant stressor, the long-chain saturated free fatty acid sodium palmitate (SP). SP has previously been shown to cause β cell death at least partially through ER stress induction.²⁶ We found that **4e** treatment moderately but statistically significantly reduced SP-induced death of INS-1 cells (Figure 4E). These results are indicative of β cell protection of **4e** against overall ER stress. Furthermore, the β cell-protective effect of **4e** was not specific for INS-1 cells, as shown by its ability to increase the survival of another β cell line, β TC6 (Figure 4F), and the mouse primary islet β cells (Figures 3A–D), under Tm-induced ER stress. We notice that **4e** showed varying degrees of protection depending on both the ER stressors and the β cell lines, which might be explained in part by the different sites of action of the ER stressors or the different sensitivities of the cell lines. Taken together, these results demonstrate that the 1,2,3-triazole amide derivatives are effective in protecting β cells against ER stress-induced apoptosis.

Compound 4e Rescues ER Stress-mediated β Cell Dysfunction

ER stress has been shown to directly impair β cell functions, including their ability to respond to increases in glucose concentrations with a concomitant increase in insulin secretion (the glucose-stimulated insulin secretion (GSIS)).^{27, 28} We therefore examined whether 1,2,3-triazole derivatives improved GSIS in β cells under ER stress. As expected, insulin secretion by INS-1 cells at high glucose (20 mM) was markedly increased (~4-fold) compared with baseline secretion in 2.8 mM glucose. This increase was significantly diminished by Tm treatment, but addition of **4e** significantly reversed this effect and restored GSIS to near-normal levels (Figure 4G). In addition, **4e** appears to have no effect in insulin secretion under the condition of either glucose concentration in the absence of ER stress (Figure 4G). Thus, **4e** not only protects β cells against ER stress-induced cell death but also preserves β cell function.

Compound 4e Lowers Blood Glucose Level in STZ-induced Diabetic Mouse Model

Having established the potent effects of **4e** on β cell survival and function in cell-based assays, we next evaluated the *in vivo* efficacy of **4e** using a streptozotocin (STZ)-induced diabetic mouse model. STZ is a β cell-selective toxin that causes cell death and induces

hyperglycemia by increasing reactive oxygen species (ROS) production and causing DNA alkylating damage. It has recently been reported to induce ER stress in β cells.^{29, 30} We reasoned that **4e** should at least exert partial protective effect against STZ-induced β cell death and hyperglycemia in vivo given its inhibitory effect on CHOP expression. For this, C57BL/6J mice were injected intraperitoneally (i.p.) once daily with STZ (50 mg/kg) and either vehicle or **4e** (20 mg/kg) for 5 consecutive days. Treatment with vehicle or **4e** alone then continued for 2 more weeks. Blood glucose levels increased gradually within a week of STZ treatment; however, glucose levels were, albeit moderately but significantly, lower in **4e**-treated mice than in control (Figure 5A). Moreover, the beneficial effect of **4e** on blood glucose levels was accompanied by a concomitant increase in β cell mass. We observed that STZ dosing destroyed the majority of islets, with $21.3 \pm 4.4\%$ of islet area compared to untreated healthy controls (Figure 5B and 5C). **4e**-treated mice had approximately twice the total pancreatic islet area ($21.3 \pm 4.4\%$ for STZ + vehicle vs. $44.2 \pm 7.2\%$ for STZ + **4e**, Figures 5B and 5C) and a significantly higher number of insulin-positive cells per islet (Figures 5B and 5D) compared with STZ mice. These results indicate that **4e** ameliorates diabetes in mice by preserving β cell mass and function.

In this study, we have described a novel HTS assay for the identification of small molecules that inhibit ER stress-mediated CHOP up-regulation. A series of 1,2,3-triazole derivatives are further identified to rescue ER stress-induced β cell dysfunction and death, and we showed that one such analog **4e** significantly lowers blood glucose levels in diabetic animals. Because of the critical role of β cell dysfunction and death on the onset and progression of diabetes, HTS efforts have been made to identify small molecules that protect β cells.^{22, 31–33} These HTSs are primarily based on β cell survival-based phenotypic screens, in which β cells are subjected to stress (e.g., cytokines or ER stress)-mediated death and small molecules that suppress the stress-mediated death are identified. This approach has discovered several interesting β cell-protective chemotypes, although significant efforts are needed to identify the protein or pathway targets of these molecules.³⁴ On the other hand, to our knowledge, a systemic approach to identify small molecule modulators of known β cell survival targets or pathways has yet to be reported.

ER stress has been increasingly recognized to play an important role in pancreatic β cell dysfunction and death, in which CHOP serves as a key mediator of ER stress-induced apoptosis.^{13,14, 18, 19} Our HTS is the first to identify small molecule inhibitors of CHOP expression that are capable of protecting β cell against ER stress. The 1,2,3-triazole amide derivatives we identified in this study protect β cell against ER stress-mediated dysfunction and death in a CHOP-dependent fashion, as they are inactive in CHOP-deficient β cells. Importantly, we observed that 1,2,3-triazole amide derivatives suppress the ER stress-induced CHOP up-regulation, but do not affect the basal expression level of CHOP gene in the absence of ER stress. Although CHOP plays important role in mediating ER stress-triggered cell death and CHOP knockout mice are viable, fertile, and exhibit protective effect in response to injury or stress,^{13, 18–20} these knockout mice show some defects such as hepatic steatosis, glucose intolerance, decreased energy expenditure, and viral infection susceptibility problem.^{35–37} These detrimental effects are likely due to lack of the basal level of CHOP protein or long-term deficiency of CHOP induction in response to chronic ER

stress in these CHOP knockout animals. Our results that 1,2,3-triazole amide derivatives inhibit ER stress-mediated CHOP up-regulation only suggest that these compounds will likely provide β cell-protective effect without incurring detrimental effects associated with CHOP deficiency.

In summary, we have identified a series of 1,2,3-triazole amide derivatives capable of protecting pancreatic β cells from ER stress-induced dysfunction and death. These compounds inhibit ER stress-mediated CHOP up-regulation and hence represent first-in-class CHOP inhibitors with β cell-protective and anti-diabetic effects *in vivo*. Studies on the mechanisms by which 1,2,3-triazole amide derivatives inhibit ER stress-mediated CHOP expression are ongoing. Further optimization may lead to novel compounds that could serve as key components of a β cell protection and regeneration therapy for diabetes.

METHODS

Chemicals

The chemical libraries were obtained from ChemBridge (San Diego, CA), Maybridge (Cornwall, UK), and MicroSource (Ann Arbor, MI) and were supplied as 10 mM solutions in DMSO. All 1,2,3-triazole derivatives were obtained from ChemBridge. Chemical structures and purities were confirmed by the suppliers using NMR and HPLC. All tested compounds are at least 90% purity, with compound **4e** at 100% purity. The data of re-analyses of **4e** was performed on HPLC (Michrom Bioresources Paradigm MSRB capillary HPLC; Magic MS C18, 5 μ m, 100 Å, 0.5 $\times$ 150mm; UV 254 nm; flow rate = 20 μ L/min; Solvent A: 0.09% formic acid, 0.01% TFA, 2% CH₃CN, 97.9% water; Solvent B: 0.09% formic acid, 0.0085% TFA, 95% CH₃CN, 4.9% water; Gradient: 10%B for 2 min, 10% to 90% B in 28 min, 90% to 10% B in 5min). Purity = 100%.

Tunicamycin (Tm), brefeldin A (BFA) and streptozocin (STZ) were obtained from Sigma-Aldrich (St Louis, MO) and Tm and BFA were dissolved in DMSO for experiments whereas STZ was dissolved in Na-Citrate Buffer at the concentration of 5mg/ml.

Cell culture

HEK293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Corning Inc., Corning, NY) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and antibiotics (100 UI/mL penicillin and 100 μ g/mL streptomycin; Corning) and were maintained in a humidified 5% CO₂ atmosphere at 37°C. INS-1 cells were cultured in RPMI 1640 medium (Corning) supplemented with 10% FBS (Atlanta Biologicals), 10 mM HEPES (Gibco-Life Technologies, MD), 1 mM sodium pyruvate (Corning), 50 μ M 2-mercaptoethanol (Sigma-Aldrich), and antibiotics (100 UI/mL penicillin and 100 μ g/mL streptomycin; Corning) and maintained in a humidified 5% CO₂ atmosphere at 37°C. The mouse insulinoma cell line β TC6 (ATCC) was maintained in DMEM supplemented with 15% FBS, 1X GlutaMAX (Gibco), 1X nonessential amino acids (Gibco), and 1 mM sodium pyruvate (Gibco).

CHOP reporter cell lines

The HEK293 CHOP reporter cell line (CHOP-Luc) has been described previously.²² Cells were plated at 7×10^3 cells/well in a 384-well plate and incubated for 16 h. Test compounds at the indicated concentrations and/or Tm at 1 $\mu\text{g/mL}$ were then added. Luciferase activity was measured with a Bright-Glo kit (Promega, Madison, WI) 24 h later.

High-throughput chemical screening

HEK293 CHOP-Luc cells were seeded at 7×10^3 cells/well in 384-well plates and incubated overnight. The next day, cells were treated with 10 μM of the library compounds and incubated for a further 24 h. The medium was then aspirated and 20 μL /well of Bright-Glo luciferase assay reagent was added. Luminescence was measured with an EnVision multilabel plate reader (PerkinElmer, Waltham, MA). Hit selection was based on standard scores. 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 luminescence value of a compound - mean)/SD of the plate. Compounds that increased ATP levels >3 SD compared with control wells (standard score >3) were considered hits.

Cell survival assay

INS-1 cells or βTC6 cells were seeded at 3×10^3 cells/well in a 384-well plate and treated with compounds at the indicated concentrations. After 3 days, the medium was aspirated and 20 μL /well of CellTiter-Glo (Promega) was added. Cell viability was measured with an EnVision plate reader (PerkinElmer). For assays in which stress was induced by palmitate, a stock solution of 5 mM sodium palmitate (SP) in 5% BSA was prepared as previously reported³⁸. 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 0.2 mM.

RNA isolation and qRT-PCR

HEK293 or INS-1 cells were seeded at 4×10^5 cells/well in 6-well plates and treated with compounds for the indicated times. Total RNA was extracted using TRIzol reagent (Invitrogen-Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Samples of 2 μg of total RNA were reverse transcribed using a Super-Script® III Reverse Transcriptase kit (Invitrogen). Real-time PCR was performed in 96-well format using SYBR Select Master Mix (Applied Biosystems, Foster City, CA) with an ABI 7500 PCR system (Applied Biosystems). The PCR primer sequences (Eurofins (Edersberg, Germany) were as follows. Human CHOP: F, 5'-GCCCTTCTCTTCG-3' and R, 5'-TGTGACCTCTGCTGGTTCTG-3'. Rat CHOP: F, 5'-GAAATCGAGCGCCTGACCAG-3' and R, 5'-GGAGGTGATGCCAACAGTTCA-3'. Human cyclophilin A: F, 5'-GCCTCTCCCTAGCTTTGGTT-3', R, 5'-GGTCTGTAAAGGTGGGCAGA-3'. Rat cyclophilin A: F, 5'-GGTGACTTCACACGCCAT AA-3', R, 5'-CTTCCCAAAGACCACATGCT-3'.

Western blotting

HEK293 or INS-1 cells were seeded at 8×10^5 cells/dish in 60-mm dishes and treated for the indicated times. Cells were then washed with phosphate-buffered saline (PBS) and lysed with lysis buffer (Cell Signaling Technology, Danvers, MA) containing EDTA and phosphatase inhibitors. Aliquots of 20 μ g total protein were separated on 7% SDS-PAGE gels (Life Technologies) and transferred to PVDF membranes. The membranes were probed with primary antibodies followed by the appropriate HRP-conjugated secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG, 1:3000; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then developed. The primary antibodies and dilutions used were CHOP (1:1000; Thermo Fisher, Waltham, MA), caspase 3 (1:1000; Cell Signaling Technology), and PARP (1:1000; Cell Signaling Technology).

TUNEL assay

INS-1 cells were seeded at 4×10^4 cells/48-well and treated with the indicated compounds for 24 h. Primary islet cells isolated from pancreases of C57BL/6 and CHOP^{-/-} knockout mice were cultured in CMRL-1066 medium (Gibco-Life Technologies) containing 10% FBS. Cells were then fixed with 4% paraformaldehyde for 1 h, washed with PBS, and permeabilized for 2 min on ice with 0.1% Triton X-100 (Promega) in 0.1% sodium citrate (Thermo Fisher). TUNEL staining was accomplished using an In Situ Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany). After incubation for 1 h at 37°C, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and the images were taken using an automated high-content imaging system Image Express Micro (Molecular Devices, Sunnyvale, CA) and data analyzed by MetaXpress (Molecular Devices) or using an Olympus confocal microscope FV1000 (Olympus, Tokyo, Japan). Anti-insulin antibody (A0564 1:500 dilution; Dako, Carpinteria, CA) was used to mark insulin positive cells in mouse islets.

Glucose-stimulated insulin secretion

INS-1 cells were seeded at 1×10^5 cells/well 24-well plates and treated with the indicated compounds for 24 h. Cells were then washed with Krebs buffer (1% w/v BSA in 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, 10 mM HEPES, pH 7.4). The cells were incubated with 500 μ L Krebs buffer containing 2.5 mM glucose for 1 h and then with Krebs buffer containing 2.5 mM or 20 mM glucose for 45 min. The supernatants were collected, and secreted insulin was measured with insulin ELISA kits (ALPCO Diagnostics, Salem, NH). Cells were lysed with lysis buffer containing protease inhibitors, and total cellular protein was determined with the Bradford assay. Data are presented as the glucose-stimulated insulin secretion index: (insulin secretion stimulated by 20 mM glucose [mean of triplicate wells]/insulin secretion stimulated by 2.5 mM glucose [mean of triplicate wells]).

Animal study

All procedures involving animals were performed in accordance with the protocol approved by the Oklahoma Medical Research Foundation Institutional Animal Care and Use Committee (IACUC). C57BL/6 and CHOP^{-/-} knockout mice were obtained from The

Jackson Laboratory (Bar Harbor, Maine) and maintained on a 12 h light-dark cycle at an ambient temperature of 21°C. Mice were given free access to water and food. All experiments were performed with age-matched male mice. Procedures for islet isolation from pancreases of C57BL/6 and CHOP^{-/-} knockout mice were as previously described.²⁰ For streptozotocin (STZ)-induced diabetic model, ten-week-old C57BL/6 mice were injected intraperitoneally (i.p.) once daily for five days with STZ (dissolved in Na-Citrate Buffer; 50 mg/kg) and either **4e** (20 mg/kg body weight; 2 mg/ml in 10% DMSO in saline buffer; *n* = 7 mice) or vehicle (*n* = 6 mice). Injections of vehicle or **4e** alone were then continued for 2 more weeks. Every third day, animals were fasted for 5 h and blood was obtained by tail snip. Blood glucose levels were measured using a glucometer (Nova Statstrip Xpress; Data Science International, St. Paul, MN). At the end of the treatment period, mice were sacrificed and the pancreases were removed, fixed in formalin, and paraffin-embedded. Tissue blocks were serially sectioned at intervals of 100 µm and 6 sections were stained with an anti-insulin antibody (A0564 1:500 dilution; Dako) and DAPI (0.5 µg/ml). Insulin-positive cells were used to demarcate islets, and the total islet area was measured with an Olympus FV1000 confocal microscopy and quantified with Image-J histogram software.

Statistical analysis

Data are presented as the means ± SD of the indicated number of replicates, unless specified. Statistical comparisons were performed using a two-tailed paired Student's *t*-test. A *p* value of <0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Oklahoma Center for the Advancement of Science and Technology, and NIGMS (1P20GM103636) to W.W.

References

1. Donath MY, Halban PA. Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. *Diabetologia*. 2004; 47:581–589. [PubMed: 14767595]
2. Prentki M, Nolan CJ. Islet beta cell failure in type 2 diabetes. *J Clin Invest*. 2006; 116:1802–1812. [PubMed: 16823478]
3. Fonseca SG, Gromada J, Urano F. Endoplasmic reticulum stress and pancreatic beta-cell death. *Trends in endocrinology and metabolism: TEM*. 2011; 22:266–274. [PubMed: 21458293]
4. Scheuner D, Kaufman RJ. The unfolded protein response: a pathway that links insulin demand with beta-cell failure and diabetes. *Endocr Rev*. 2008; 29:317–333. [PubMed: 18436705]
5. Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell*. 2001; 7:1165–1176. [PubMed: 11430820]
6. Back SH, Kaufman RJ. Endoplasmic reticulum stress and type 2 diabetes. *Annual review of biochemistry*. 2012; 81:767–793.
7. Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends in biochemical sciences*. 2015; 40:141–148. [PubMed: 25656104]

8. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. *Mutat Res.* 2005; 569:29–63. [PubMed: 15603751]
9. Papa FR. Endoplasmic reticulum stress, pancreatic beta-cell degeneration, and diabetes. *Cold Spring Harbor perspectives in medicine.* 2012; 2:a007666. [PubMed: 22951443]
10. Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nature reviews. Drug discovery.* 2013; 12:703–719. [PubMed: 23989796]
11. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science.* 2011; 334:1081–1086. [PubMed: 22116877]
12. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol.* 2011; 13:184–190. [PubMed: 21364565]
13. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes & Development.* 1998; 12:982–995. [PubMed: 9531536]
14. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP, Ron D. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes & Development.* 2004; 18:3066–3077. [PubMed: 15601821]
15. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol.* 2011; 13:184–190. [PubMed: 21364565]
16. Ma Y, Brewer JW, Diehl JA, Hendershot LM. Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J Mol Biol.* 2002; 318:1351–1365. [PubMed: 12083523]
17. Lawrence MC, McGlynn K, Naziruddin B, Levy MF, Cobb MH. Differential regulation of CHOP-10/GADD153 gene expression by MAPK signaling in pancreatic β -cells. *Proceedings of the National Academy of Sciences.* 2007; 104:11518–11525.
18. Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest.* 2002; 109:525–532. [PubMed: 11854325]
19. Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ. Chop deletion reduces oxidative stress, improves β cell function, and promotes cell survival in multiple mouse models of diabetes. *The Journal of Clinical Investigation.* 2008; 118:3378–3389. [PubMed: 18776938]
20. Oyadomari S, Takeda K, Takiguchi M, Gotoh T, Matsumoto M, Wada I, Akira S, Araki E, Mori M. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc Natl Acad Sci U S A.* 2001; 98:10845–10850. [PubMed: 11526215]
21. Novoa I, Zeng H, Harding HP, Ron D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 α . *J Cell Biol.* 2001; 153:1011–1022. [PubMed: 11381086]
22. Tran K, Li Y, Duan H, Arora D, Lim HY, Wang W. Identification of small molecules that protect pancreatic beta cells against endoplasmic reticulum stress-induced cell death. *ACS Chem Biol.* 2014; 9:2796–2806. [PubMed: 25279668]
23. Osowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods Enzymol.* 2011; 490:71–92. [PubMed: 21266244]
24. Herceg Z, Wang ZQ. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat Res.* 2001; 477:97–110. [PubMed: 11376691]
25. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol.* 1999; 144:281–292. [PubMed: 9922454]
26. Osowski CM, Urano F. The binary switch that controls the life and death decisions of ER stressed beta cells. *Curr Opin Cell Biol.* 2011; 23:207–215. [PubMed: 21168319]
27. Han D, Lerner AG, Vande Walle L, Upton JP, Xu W, Hagen A, Backes BJ, Oakes SA, Papa FR. IRE1 α kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. *Cell.* 2009; 138:562–575. [PubMed: 19665977]

28. Lipson KL, Fonseca SG, Ishigaki S, Nguyen LX, Foss E, Bortell R, Rossini AA, Urano F. Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell metabolism*. 2006; 4:245–254. [PubMed: 16950141]
29. Ahn C, An BS, Jeung EB. Streptozotocin induces endoplasmic reticulum stress and apoptosis via disruption of calcium homeostasis in mouse pancreas. *Molecular and Cellular Endocrinology*. 2015; 412:302–308. [PubMed: 26003140]
30. Zhang R, Kim JS, Kang KA, Piao MJ, Kim KC, Hyun JW. Protective Mechanism of KIOM-4 in Streptozotocin-Induced Pancreatic beta-Cells Damage Is Involved in the Inhibition of Endoplasmic Reticulum Stress. *Evidence-based complementary and alternative medicine : eCAM* 2011. 2011
31. Chou DH, Bodycombe NE, Carrinski HA, Lewis TA, Clemons PA, Schreiber SL, Wagner BK. Small-Molecule Suppressors of Cytokine-Induced beta-Cell Apoptosis. *ACS Chem Biol*. 2010; 5:729–734. [PubMed: 20550176]
32. Chou DH, Duvall JR, Gerard B, Liu H, Pandya BA, Suh BC, Forbeck EM, Faloon P, Wagner BK, Marcaurelle LA. Synthesis of a novel suppressor of beta-cell apoptosis via diversity-oriented synthesis. *ACS medicinal chemistry letters*. 2011; 2:698–702. [PubMed: 21927648]
33. Scully SS, Tang AJ, Lundh M, Mosher CM, Perkins KM, Wagner BK. Small-molecule inhibitors of cytokine-mediated STAT1 signal transduction in beta-cells with improved aqueous solubility. *J Med Chem*. 2013; 56:4125–4129. [PubMed: 23617753]
34. Chou DH, Vetere A, Choudhary A, Scully SS, Schenone M, Tang A, Gomez R, Burns SM, Lundh M, Vital T, Comer E, Faloon PW, Dancik V, Ciarlo C, Paulk J, Dai M, Reddy C, Sun H, Young M, Donato N, Jaffe J, Clemons PA, Palmer M, Carr SA, Schreiber SL, Wagner BK. Kinase-Independent Small-Molecule Inhibition of JAK-STAT Signaling. *J Am Chem Soc*. 2015; 137:7929–7934. [PubMed: 26042473]
35. Maris M, Overbergh L, Gysemans C, Waget A, Cardozo AK, Verdrengh E, Cunha JP, Gotoh T, Cnop M, Eizirik DL, Burcelin R, Mathieu C. Deletion of C/EBP homologous protein (Chop) in C57Bl/6 mice dissociates obesity from insulin resistance. *Diabetologia*. 2012; 55:1167–1178. [PubMed: 22237685]
36. Grant R, Nguyen KY, Ravussin A, Albarado D, Youm YH, Dixit VD. Inactivation of C/ebp homologous protein-driven immune-metabolic interactions exacerbate obesity and adipose tissue leukocytosis. *J Biol Chem*. 2014; 289:14045–14055. [PubMed: 24662293]
37. Clase AC, Dimcheff DE, Favara C, Dorward D, McAtee FJ, Parrie LE, Ron D, Portis JL. Oligodendrocytes Are a Major Target of the Toxicity of Spongionogenic Murine Retroviruses. *The American Journal of Pathology*. 2006; 169:1026–1038. [PubMed: 16936275]
38. Mayer CM, Belsham DD. Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons: rescue of resistance and apoptosis through adenosine 5' monophosphate-activated protein kinase activation. *Endocrinology*. 2010; 151:576–585. [PubMed: 19952270]

Figure 1.

1,2,3-Triazole derivatives inhibit CHOP expression and increase cell viability. (A) CHOP mRNA levels measured by qRT-PCR in HEK293 cells treated with Tm (1 µg/ml) in the presence of 20 µM 1d or DMSO. The data are presented as the fold change after normalized to *cyclophilin A* mRNA. The results are the means $\pm$ SD of triplicates. * p <0.05 by Student's t -test. (B) Cell viability in INS-1 cells treated for 72 h in the presence of Tm (0.1 µg/ml), and of DMSO or 1d, determined by the CellTiter-Glo assay. Results are normalized and presented as the fold change with Tm alone as 1. (C) Cell viability in INS-1 cells treated for 72 h in the presence of Tm (0.1 µg/ml), and of DMSO or 4e, determined by the CellTiter-Glo assay. Results are normalized and presented as the fold change with Tm alone as 1. In (B) and (C), the results are the means $\pm$ SD of triplicates. * p <0.05 and ** p <0.01 compared to cells treated with Tm in the absence of compound by Student's t -test.

Figure 2.

1,2,3-Triazole derivatives inhibits ER stress-induced CHOP up-regulation. (A) CHOP mRNA levels by qRT-PCR in INS-1 cells treated for 8 h with **4e** at indicated concentrations in the presence or absence of Tm (0.1 μg/ml). Data are presented as the fold change after normalized to *cyclophilin A* mRNA. (B) CHOP mRNA levels by qRT-PCR in INS-1 cells treated for indicated times with or without Tm (0.1 μg/ml) in the presence of DMSO or 10 μM **4e**. Data are presented as the fold change after normalized to *cyclophilin A* mRNA. (C) CHOP protein levels by Western blotting in INS-1 cells treated with 20 μM **4e** or DMSO, and α-Tubulin was used as a loading control. (D) CHOP mRNA levels by qRT-PCR in INS-1 cells treated for indicated times in the presence of DMSO or 10 μM **4e** in the absence of Tm. Data are presented as the fold change after normalized to *cyclophilin A* mRNA. (E) CHOP mRNA levels by qRT-PCR in INS-1 cells treated for 8 h with **4h** at indicated concentrations in the presence or absence of Tm (0.1 μg/ml). Data are presented as the fold change after normalized to *cyclophilin A* mRNA.

Figure 3. CHOP-dependent protective effect of 1,2,3-Triazole derivatives on β cells under ER stress. (A) Representative images of TUNEL staining of primary mouse islet cells isolated from control C57B/6 mice and treated with 4e (10 μ M) in the presence of Tm (1 μ g/ml) for 24 h.

TUNEL (red), Insulin (green) for β cells, and nuclei stained with DAPI (blue). (B) Percentage of TUNEL⁺ Insulin⁺ cells from all islets isolated from 3 control C57B/6 mice. Results are the mean $\pm$ SD of counts from five wells of a 8-well chamber slide, approximately 50 islets. (C) Representative images of TUNEL staining of primary mouse islet cells isolated from CHOP^{-/-} knockout mice and treated with 4e (10 μ M) in the presence of Tm (1 μ g/ml) for 24 h. TUNEL (red); Insulin (green) for β cells, and nuclei stained with DAPI (blue). (D) Percentage of TUNEL⁺ Insulin⁺ cells from all islets isolated from 3 CHOP^{-/-} mice. Results are the mean $\pm$ SD of counts from five wells of a 8-well chamber slide, approximately 50 islets. *p < 0.05 and **p < 0.01. NS, no significance statistically.

Figure 4.

The 1,2,3-triazole derivative **4e** suppresses Tm-induced β cell apoptosis and dysfunction. (A) Representative images of TUNEL staining of INS-1 cells treated with **4e** (20 μ M) in the presence of Tm (0.1 μ g/ml) for 24 h. TUNEL (red); Nuclei stained with DAPI (blue). (B) Percentage of TUNEL⁺ INS-1 cells. Results are the mean $\pm$ SD of counts from five images. ## or ** $p < 0.01$ by Student's t -test. (C) Cleavage of PARP and caspase-3 determined by Western blotting in INS-1 cells treated with or without **4e** in the presence or absence of Tm. α -Tubulin was as a loading control. (D) Cell viability of INS-1 cells treated for 72 h with or

without BFA (0.2 $\mu\text{g/ml}$) in the presence of DMSO or **4e** at indicated concentrations, determined by the CellTiter-Glo assay. Results are the means $\pm$ SD of quadruplicate. $**p < 0.01$ by Student's *t*-test. (E) Cell viability of INS-1 cells treated for 72 h with or without SP (0.2 mM) in the presence of DMSO or **4e** at indicated concentrations, determined by the CellTiter-Glo assay. Results are the means $\pm$ SD of quadruplicate. $*p < 0.01$ by Student's *t*-test. (F) Cell viability in βTC6 cells treated for 72 h in the presence of Tm (0.25 $\mu\text{g/ml}$), and of DMSO or **4e**, determined by the CellTiter-Glo assay. Results are normalized and presented as the fold change with Tm alone as 1. The results are the means $\pm$ SD of triplicates. $*p < 0.05$ and $**p < 0.01$ compared to cells treated with Tm in the absence of **4e** (Student's *t*-test). (G) Insulin secretion was measured by ELISA in INS-1 cells, the values were normalized to total cellular protein, and the data are shown as the GSIS index. The results are representative of three experiments. $**p < 0.01$ by Student's *t*-test.

Figure 5.

Anti-diabetic effect of **4e** in the STZ-induced diabetic mouse model. (A) Fasting blood glucose levels were measured in STZ-injected mice treated with vehicle control ($n = 6$) or **4e** ($n = 7$). (B) C57BL/6J mice were injected once daily for 5 days with STZ (50 mg/kg body weight) and with either vehicle ($n = 6$ mice) or **4e** (20 mg/kg body weight; $n = 7$ mice). Injections of vehicle or **4e** alone were then continued for 2 more weeks. Pancreases were sectioned and slides were stained with anti-insulin antibody (green) and DAPI (blue). Slides were imaged with an Olympus FV1000 confocal microscope. Representative images shown in (B). Quantification of total islet area per section and β cell number per islet was shown in

Figure. 5C and 5D. (C) Total area of all islets per section calculated for a total of six sections using insulin-positive cells to demarcate islets for healthy control, STZ- and vehicle-treated, and STZ- and **4e**-treated mice. Data are normalized that designates healthy, control mice as 1 and presented as the means $\pm$ SEM of six sections from three mice. * $p < 0.05$ by Student's t-test. (D) Number of insulin-positive β cells per islet. Data are the means $\pm$ SEM of six sections from three mice. * $p < 0.05$ by Student's t-test.

Table 1

Effects of 1,2,3-triazole amide analogs on CHOP-Luc reporter and β cell viability against Tm.

Compd	R ₁	R ₂	IC ₅₀ (μM) ^a	Maximum activity (%) ^b and concentration (μM)	EC ₅₀ (μM) ^c
1a	H	H	1.5	12% (20)	9.5
1b	2-F	H	1.1	31% (40)	23
1c	4-iPr	2-F	0.035	29% (5)	2.1
1d	4-iPr	3-F	0.013	33% (5)	1.3

^aIC₅₀ for CHOP-Luc inhibition.

^bMaximum activity values are presented as % rescue from Tm-treated reduction in cell viability; the values under Tm treatment alone and DMSO treatment (no Tm) are designated as 0% and 100%, respectively in all tables, and the mean of three experiments were shown in all tables in this manuscript. The concentrations of the compounds at which maximum activity was achieved are shown in parenthesis.

^cEC₅₀ for INS-1 cell survival.

Table 2

The maximum protective activity of 1,2,3-triazole amide derivatives on INS-1 cell viability against Tm-induced ER stress.

Compd	R ₁	R ₂	Maximum activity (%) and concentration (μM)	EC ₅₀ (μM)
1a	H	H	12% (20)	9.5
3a	4-iPr	3-Me	35% (10)	3.2
3b	4-iPr	4-F	29% (10)	3.5
3c	4-Et	3-Me	72% (5)	2.1
3d	4-Et	4-F	82% (10)	2.7
3e	4-nBu	2-F	30% (10)	3.5

Table 3

The maximum activity of 1,2,3-triazole analogs on INS-1 viability against Tm-induced ER stress.

Compd	R	Maximum activity (%) and concentration (μM)	EC 50 (μM)
4a	2-Me	62% (10)	3.2
4b	2-Et	91% (10)	4.2
4c	2-Cl	19% (5)	0.6
4d	3-OMe	31% (10)	3.7
4e	3-F	87% (10)	2.8
4f	4-Me	18% (10)	NA ^a
4g	4-OMe	21% (5)	NA
4h	4-COOH	0% (10)	NA
4i	4-COOMe	14% (10)	NA
4j	4-NHAc	15% (10)	NA
4k	2,5-Di-Me	90% (10)	1.6
4l	2,6-Di-Me	80% (20)	4.6
4m	2,4-Di-Cl	12% (10)	NA

^aNA: not applicable or not available.

Table 4

The maximum protective activity of 1,2,3-triazole amide derivatives on INS-1 cell viability against Tm-induced ER stress.

Compd	R ₂	R ₁	Maximum activity (%) and concentration (μM)	EC 50 (μM)
5a	H	2-F	40% (40)	8.0
5b	H	3,4-Di-Me	56% (10)	3.1
5c	H	4-i-Pr	54% (10)	2.6
5d	3-Cl	2-F	92% (20)	6.5
5e	3-Cl	2-Cl	94% (20)	3.1
5f	3-Cl	2-OEt	52% (20)	8.7
5g	3-Cl	4-F	87% (20)	8.4
5h	3-Cl	4-Me	56% (10)	2.8
5i	3-Cl	4-Et	88% (10)	7.8
5j	3-Cl	4-OMe	81% (20)	5.2
5k	3-Cl	2,4-Di-Me	92% (10)	2.5
5l	6-Me	2-F	23% (40)	1.4
5m	6-Me	2-Cl	44% (40)	9.1
5n	6-Me	2-OMe	28% (10)	0.6
5o	6-Me	4-Me	73% (20)	1.7
5p	6-Me	4-Cl	57% (40)	5.2
5q	6-Me	2-Me-3-Cl	88% (40)	9.0
5r	6-Et	H	36% (40)	5.8
5s	6-Et	2-Cl	55% (40)	2.5
5t	6-Et	2-OMe	38% (20)	1.0
5u	6-Et	2-OEt	50% (40)	9.5
5v	6-Et	4-F	28% (20)	9.3
5w	6-Et	4-Cl	66% (40)	8.5
5x	6-Et	4-Me	82% (40)	9.0
5y	6-Et	4-OMe	39% (20)	5.1
5z	6-Et	2-Me-3-Cl	97% (10)	6.7