Published in final edited form as:

Sci Transl Med. 2015 June 17; 7(292): 292ra98. doi:10.1126/scitranslmed.aaa9134.

Phenotypic assays identify a small molecule modulator of the unfolded protein response with anti-diabetic activity

S. Fu^{†,1,2}, A. Yalcin^{†,1}, G.Y. Lee^{†,1}, P. Li¹, Jason Fan¹, A.P. Arruda¹, B.M. Pers¹, M. Yilmaz¹, K. Eguchi¹, and G.S. Hotamisligil^{1,3,*}

¹Department of Genetics and Complex Diseases and Sabri Ülkner Center, Harvard T.H. Chan School of Public Health

²School of Life Sciences, Tsinghua University, Peking-Tsinghua Center for Life Sciences

³Broad Institute of Harvard and MIT

Abstract

Endoplasmic reticulum (ER) plays a critical role in protein, lipid and glucose metabolism, as well as cellular calcium homeostasis and signaling. Perturbation of ER function and chronic ER stress is associated with many pathologies ranging from diabetes to neurodegenerative diseases, cancer and inflammation. While targeting the ER offers therapeutic promise in preclinical models of obesity and other pathologies, the available chemical entities generally lack the specificity and other pharmacological properties required for effective clinical translation. To overcome these challenges and identify new potential therapeutic candidates, we first designed and chemically and genetically validated two high-throughput functional screening systems that independently measure the free chaperone content and the protein folding capacity of ER. With these quantitative platforms, we characterized a small molecule compound, azoramide, that improves ER protein folding ability and activates ER chaperone capacity to protect cells against ER stress in multiple systems. Remarkably, this compound also exhibits potent anti-diabetic efficacy in two independent mouse models of obesity by improving insulin sensitivity and beta cell function. Taken together, these results demonstrate the utility of this functional, phenotypic assay platform for ER-targeted drug discovery, and provide proof-of-principle that specific ER modulators can be potential drug candidates against type 2 diabetes.

* Address correspondence to: Gökhan S. Hotamisligil, M.D., Ph.D., Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA 02115, Fax: 617 432 1941, Phone: 617 432 1950, ghotamis@hsph.harvard.edu.

[†]These authors contributed equally to this work

Supplementary Materials

Fig. S1. Validation of functional reporters.

Fig. S2. Effects of azoramide on UPR reporters.

Fig S3. Effects of individual chaperones and UPR sensors on functional reporter activity.

Fig S4. Chaperone overexpression mimics the effect of azoramide pretreatment on ER function.

Fig S5. Azoramide protects against induction of ER stress by tunicamycin.

Fig S6. Long-term azoramide improves glucose metabolism in *ob/ob* mice.

Fig S7. Synthesis of azoramide.

Author contributions: SF, AY, GYL, PL, APA, BMP, MY, JF, KE designed and performed experiments, analyzed data and wrote the manuscript. GSH conceptualized the project, designed experiments, analyzed data and wrote the manuscript.

Competing interests: The authors declare that no competing interests exist.

One Sentence Summary

Here we report the development of assay platforms that enable direct measurement of ER function and protein folding capacity, which were used to identify and characterize a small molecule with the capacity to boost ER function *in vitro* and exert potent anti-diabetic effects *in vivo*.

Introduction

The endoplasmic reticulum (ER) is a central organelle for the synthesis of proteins and lipids, and is a critical regulator of cellular calcium homeostasis and signaling (1). Within the ER, protein synthesis and secretion processes are tightly and dynamically regulated at many levels to meet intrinsic needs as well as external demands (2). Generally, the rate of protein synthesis is balanced with the ER folding capacity in order to avoid accumulation of unfolded protein intermediates inside the ER lumen. When the rate of synthesis exceeds the folding capacity, unfolded proteins accumulate in the ER, triggering the unfolded protein response (UPR) (3).

Three ER transmembrane proteins act as sensors of unfolded protein accumulation in the ER: PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (4–7). Upon activation, PERK phosphorylates the translation initiation factor eIF2 α , reducing the rate of new protein synthesis (7). IRE1 is a kinase with RNA ribonuclease activity; in response to ER stress it dimerizes, undergoes autophosphorylation, and cleaves the X-box binding protein 1 (Xbp-1) mRNA (8–10). The translated product of this cleavage event (sXbp-1) is an active transcription factor that promotes expression of chaperones and other genes that support ER biogenesis (11). Similarly, in the absence of ER stress, ATF6 α is repressed through its luminal domain by binding to ER chaperones such as GRP78. The accumulation of unfolded proteins under stressful conditions displaces GRP78 from ATF6 α , which unmask the ATF6 Golgi localization sequence and subsequently releases it from the ER. In the Golgi, ATF6 α is processed by proteases that liberate its cytoplasmic domain (12, 13), allowing it to translocate to the nucleus to regulate target gene transcription (11). Together, these three canonical branches of the UPR restore the balance between protein synthesis and folding by inhibiting translation to decrease global protein synthesis, increasing degradation and disposal of unfolded protein intermediates from ER, and increasing the folding capacity of the ER by expanding its volume and increasing chaperone synthesis (14–16). However, when the ER is chronically compromised, prolonged ER stress leads to the initiation of multiple additional cellular stress and inflammatory pathways via JNK and PERK phosphorylation (17–19) as well as other signaling networks (20). Upon acute and severe ER stress conditions, the cell may even be committed to apoptosis through the upregulation of CHOP and other pathways (21, 22).

It has been well established that chronic and unresolved ER stress is not sustainable, results in defective UPR and other adaptive responses, and is strongly associated with a variety of pathologies including neurodegenerative disease, cancer and metabolic syndrome (20, 23–25). While some of these pathologies directly relate to the canonical functions of the ER, in recent years emerging evidence demonstrated that in the metabolic context, ER-associated

signaling networks also engage gluconeogenic and lipogenic pathways and influence systemic metabolic homeostasis, establishing the ER as a critical metabolic and immunometabolic hub of cells in health and disease states (20). Therefore, corrective measures targeting ER function and UPR capacity represent an attractive therapeutic strategy for many metabolic and other diseases (24, 26, 27). In support of this, small molecules with chemical chaperone activity such as 4-phenyl butyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) have been successfully applied in rodent models of metabolic disease to modulate ER responses and to improve systemic metabolism (28–31). Importantly, PBA and TUDCA have also been tested in limited, proof-of-principle human studies, and demonstrate similar activity in increasing insulin sensitivity, preventing lipotoxicity, and improving beta cell function (32, 33). However, since chemical chaperones were not generally identified based on direct screening of ER function, they may lack mechanistic specificity, and may have limited success in clinical applications due to the requirement for high doses and prolonged treatment (34). As one approach to overcome these issues, there have been recent efforts to chemically block the activity of individual UPR sensors by small molecules to modulate downstream effects, which may produce opportunities for therapeutic use in certain conditions such as cancer (35, 36). Finally, preconditioning with low dose chemical ER stress inducers has also been suggested in cell culture conditions and some *in vivo* models to boost ER function in the long-term by engaging the UPR pathways (37–39), but these noxious toxins are unlikely to have any clinical utility. Hence, there is a general scarcity of molecular or chemical tools to modulate ER capacity and the UPR for exploratory or therapeutic applications and there are no functional screening platforms to search for such chemical entities. Here we report the development of phenotypic assay platforms that enable us to search for alternative molecules that modulate ER folding capacity. Using these systems we identified and characterized a small molecule with the capacity to protect ER function *in vitro* and exert potent anti-diabetic effects *in vivo*.

Results

Complementary high-throughput, functional assay systems for real-time, continuous measurement of ER folding activity and reserve capacity

One of the key obstacles in the field of ER-based therapeutics is the lack of quantitative assay systems with a sufficient dynamic range to directly monitor ER function, which we define as the capacity of the ER to handle challenges to its functional capacity such as the influx of unfolded proteins into the ER lumen. We attempted to tackle this obstacle by utilizing components of the well-established interactions in the ER lumen that occur during the UPR or during secreted protein folding to construct high-throughput screening platforms. In two independent assays, we utilized secreted reporters and integrated internal controls for general processes that are regulated during the UPR (such as synthesis or secretion) and designed the assays such that their readouts occur in opposite directions to control for non-specific regulatory events.

As described above, the luminal domain of one of the main UPR sensors, ATF6 α , binds to the ER chaperone GRP78, which contributes to its ER retention and the silencing of ATF6 α .

transcriptional activity under non-stressful conditions (12). Therefore, we utilized a peptide derived from the ATF6 α luminal domain (ATF6LD) that is fused with *Cypridina noctiluca* luciferase (Cluc) for the measurement of ER free chaperone content and reserve capacity. We hypothesized that cells with abundant chaperone expression would retain the ATF6LD-Cluc fusion protein in the ER and thus display a lower level of luciferase secretion, while reduced chaperone expression should liberate this chimeric protein from the ER resulting in a higher level of luciferase secretion into the medium over time (Fig. 1A). Indeed, in a genetic validation experiment in HEK293 cells stably expressing the ATF6LD-Cluc reporter, siRNA-mediated suppression of GRP78 resulted in a greater than two-fold increase in luciferase secretion (Fig. 1B). Similarly, treatment of these cells with the chemical ER stress inducer thapsigargin (Tg) dose-dependently increased ATF6LD-Cluc secretion, as higher concentrations of this compound decreased chaperone availability (Fig. 1C). As predicted, in cells expressing the ATF6LD-Cluc construct, immunoprecipitation with a Cluc antibody demonstrated direct interaction between the fusion protein and GRP78, and this interaction was reduced following treatment with Tg (fig. S1A). As an internal control, the reporter construct also contains a second secreted luciferase-*Gaussia* luciferase (Gluc)- expressed under a separate CMV promoter. The secretion of the readily folded Gluc was not affected by GRP78 suppression or by Tg treatment (fig. S1B and Fig. 1C), suggesting that ATF6LD-Cluc secretion is a quantitative and inverse indicator of ER free chaperone content and reserve capacity.

Secondly, we built upon our knowledge that the membrane protein asialoglycoprotein receptor 1 (ASGR1) is a slow maturing protein whose secretion is decreased under obese, ER stressed conditions (17). Interventions that correct ER function in obese liver result in higher levels of production of this protein, indicating that it is sensitive to ER function and that it directly reflects a response to metabolic challenges that compromise the ER in obesity (17). To take advantage of these properties, we constructed an ASGR-Cluc fusion protein to monitor ER folding capacity (Fig. 1A). For this, we used the same backbone that was used for the aforementioned reporter but replaced the ATF6LD-derived cassette with the mouse ASGR1 sequence deprived of its membrane-anchoring domain and fused to *Cypridina noctiluca* luciferase. In this system, we predict that compromising ER homeostasis will lead to less efficient folding and therefore lower levels of secretion of the ASGR fusion luciferase (ASGR-Cluc). Indeed, reducing ER folding capacity by siRNA-mediated suppression of GRP78 decreased ASGR-Cluc secretion by 50% (Fig. 1D). Similarly, Tg-induced chemical ER stress also dose-dependently reduced ASGR-Cluc secretion (Fig. 1E). This construct also included a Gluc reporter as an internal control, and secretion of Gluc was not altered by GRP78 suppression or Tg treatment (Fig. S1C, Fig. 1D), indicating ASGR-Cluc is a specific and quantitative reporter of ER folding capacity. Taken together, these data validated the informative value of these reporters in examining ER functional capacity.

Identification of azoramide as a dual function ER modulator

The development of the ASGR-Cluc and ATF6LD-Cluc reporters allowed us the opportunity to directly and quantitatively measure ER folding activity and chaperone capacity in live cells. To take advantage of these systems, we first evaluated the potential ER modulating capacities of small molecular compounds identified based on their ability to engage the UPR

in the absence of cellular toxicity. One of these, N-{2-[2-(4-Chlorophenyl)-1,3-thiazol-4-yl]ethyl} butanamide (Figure 2A, referred to hereafter as azoramide) dose-dependently activated cellular UPR response element (UPRE) and ER stress response element (ERSE) luciferase reporters (fig. S2). Consistent with this activity, exposure to azoramide transiently and dose-dependently induced the expression of several chaperone genes including GRP78 and DNAJC3 (Fig. 2B, C), and induced phosphorylation of eIF2 α (Fig. 2C). However, these effects occurred to a significantly lesser degree than following thapsigargin treatment, and remarkably, even at quite high doses (25 μ M), azoramide treatment did not lead to increased expression of proapoptotic CHOP or GADD34 proteins (Fig. 2B,C). These data suggest that azoramide may have the protective effects of enhancing chaperone expression and reducing protein synthesis without inducing cytotoxicity and apoptosis.

Next, we utilized our functional assay systems to investigate the potential cytoprotective benefit of this small molecule modulator of the ER. Treatment with azoramide for 4 hours dose-dependently increased ASGR-Cluc secretion (Fig. 2D). In this setting Tg treatment dramatically suppressed ASGR reporter activity (Figure 1F), while both agents increased the expression of chaperones. These data illustrate that unlike a chemical ER stress inducer, azoramide acutely enhances the folding capacity of the ER. In the ATF6LD-Cluc assay, we found that 24-hour treatment with azoramide decreased ATF6LD-Cluc secretion (Fig. 2E). Furthermore, overexpression of chaperones resulted in a dramatic decrease in ATF6LD-Cluc secretion that was not further affected by azoramide treatment (Fig. 2F), suggesting that increased chaperone expression may mediate the azoramide-induced block of ATF6LD-Cluc secretion. Interestingly, siRNA-mediated inhibition of individual chaperones was insufficient to block the effect of azoramide on ATF6LD-Cluc secretion (fig. S3A), indicating that expression of multiple chaperones or the changes in total chaperone capacity may underlie this effect, as would be predicted to be monitored by this readout.

We next asked whether activity of a specific UPR branch was required for the azoramide effect on ATF6LD-Cluc secretion. In the absence of azoramide, suppression of XBP1 resulted in a potent increase in ATF6-Cluc secretion, while reducing the level of ATF6 α resulted in a mild decrease in reporter activity (fig. S3B). However, reducing expression of XBP1 and PERK abrogated the effect of azoramide, while suppression of ATF6 α appeared to have no effect (Fig. 2G). In agreement with this, treatment of cells with the PERK inhibitor GSK2606414 or the IRE1 inhibitor 4 μ 8C completely abrogated the effect of azoramide on ATF6LD-Cluc secretion (Fig. 2H). These data suggest that azoramide may require the presence of intact IRE1 and PERK branches of the UPR to fully increase chaperone capacity. Taken together, these experiments identify azoramide as a first of its kind compound with the dual property of not only boosting ER folding acutely but also activating ER chaperone capacity chronically to promote ER homeostasis.

Azoramide protects cells from induced ER stress

Since azoramide induces chaperone capacity and improves ER function, we next asked whether these effects could protect cells against ER stress conditions. Tunicamycin (Tm) is a chemical compound that blocks protein glycosylation and causes the accumulation of misfolded proteins in the ER. Treatment of reporter cells with Tm dose-dependently

increased ATF6LD-Cluc secretion and decreased ASGR-Cluc secretion (Fig. 3A, black lines), consistent with the known deleterious effect of this chemical on ER function. Co-treatment of azoramide and Tm diminished the ER-stress induced reduction in ER folding capacity and chaperone abundance (Fig. 3A, orange lines), and 5 hours of pretreatment with azoramide was sufficient to nearly abrogate the effect of Tm, as reflected in the patterns of ATF6LD-Cluc and ASGR-Cluc secretion (Fig. 3A, red lines). Suppression of chemically-induced stress was also observed in cells overexpressing GRP78 (fig. S4), further supporting our hypothesis that the protective effect of azoramide treatment is related to induced chaperone capacity.

The cytoprotective effects of azoramide pretreatment were also evident biochemically. As shown in Figure 3B, treatment of Huh7 cells with Tm in the absence of azoramide markedly induced GRP78 and CHOP protein expression, but pre- and co-treatment with azoramide protected cells from CHOP induction. The changes in CHOP and GRP78 protein level were reflected in the transcript level of these genes upon azoramide and Tm treatment (fig. S5), indicating prevention of the response typically elicited by Tm treatment. In azoramide treated cells, exposure to Tm resulted in a markedly reduced induction of GRP78 protein compared to untreated controls, indicating a lower overall level of ER stress in pretreated cells (Fig. 3B). These data provide molecular and functional evidence that azoramide treatment potently protects cells against chemically-induced ER stress conditions.

We also examined the potential of azoramide to protect cells against two additional physiological models of ER stress. First, we expressed the ATF6LD-Cluc reporter in HEK293A cells, and transitioned the cells from 21% O₂ to 1% O₂ to trigger hypoxia-induced ER stress. Hypoxia induced a significant increase in ATF6LD-Cluc secretion, which was completely prevented by pretreatment with azoramide (Fig. 3C). Next, we analyzed ER stress and cell survival in HEK293A cells transfected with a mutant form of Rhodopsin. The P23H Rhodopsin mutant is a misfolding form of the protein that has been identified in patients with autosomal dominant retinitis pigmentosa (40); accumulation of this misfolded protein activates the UPR in retinal cells (41), and in an animal model of the disease, vision can be restored by overexpression of GRP78 (42). We found that expression of mutant Rhodopsin significantly induced CHOP expression and, following treatment with MG132 to enhance accumulation of the misfolded protein, P23H Rhodopsin-expressing cells exhibited significantly reduced viability. Remarkably, these effects were dose-dependently ameliorated by treatment with azoramide (Fig. 3D, E), indicating the broad protective effects of this compound against ER stress due to various etiologies.

A critical component of ER function in all conditions is the ability to preserve calcium stores, as calcium plays a critical role in promoting protein folding and ER homeostasis (43). Hence, we examined whether the broad impact of azoramide on ER function involved mechanisms related to intraluminal calcium levels. To evaluate this, we expressed an ER-targeted Ca²⁺ FRET reporter in Hepa 1–6 cells followed by treatment with azoramide. As depicted in Figure 4A and B, azoramide treatment modestly but significantly increased baseline ER Ca²⁺ level. In control cells, Tg treatment induced rapid Ca²⁺ release from the ER, as measured by a decline in the FRET ratio (slope), while azoramide pretreated cells retained a greater fraction of Ca²⁺ in the ER (lag time) (Fig. 4C).

In a complementary approach, we measured the Tg-induced Ca^{+2} rise in the cytosol using the fluorescent dye Fura-2. In accordance with the observed improvement of ER calcium retention, azoramide treatment induced a higher cytosolic Ca^{+2} peak following Tg stimulation compared to control (Fig. 4D and E). These data indicate that azoramide-mediated protection against chemically induced ER stress is reflected by enhanced calcium retention in the ER, thus increasing ER calcium concentrations. To understand this effect further, we asked whether azoramide treatment altered expression of enzymes required to maintain the high ER/cytoplasm Ca^{+2} concentration gradient. Indeed, azoramide treatment of a hepatocyte cell line induced a significant increase in protein level of the sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase (SERCA) (Fig. 4F) indicating that its mechanism of action in ER calcium retention, at least in part, involves enhancement of SERCA activity, which is known to be disturbed in obesity and diabetes (17).

Azoramide improves glucose homeostasis in mice with genetic obesity

The identification of azoramide as a compound with the dual properties of acutely promoting ER folding and boosting long-term ER reserve capacity suggested that it may harbor activity as an ER-based therapeutic agent. We first chose to study the effect of azoramide in a genetic model of severe obesity and diabetes (*ob/ob* mice) due to the well-established involvement of ER dysfunction in disease pathogenesis in both experimental models and humans, and the mechanistic connection between ER stress and glucose metabolism (19, 44–51). Similar to our observations in cell culture, daily oral administration of 150mg/kg azoramide for 1 week resulted in improved ER function in the liver, as evidenced by induced expression of GRP78 (Fig. 5A). This brief treatment did not alter body weight (Fig. 5B) but was sufficient to significantly improve fasting glucose (Fig. 5C), and glucose tolerance (Fig. 5D). In addition, azoramide was well tolerated, and 5 weeks of dosing did not induce weight loss (fig. S6A), but did result in significantly reduced fasting glucose (fig. S6B) and dramatically improved glucose tolerance (fig. S6C). Furthermore, the early induction of GRP78 in liver lysates was no longer apparent after 5 weeks of treatment (fig. S6D), supporting the conclusion that ER folding capacity was improved in this setting.

Azoramide preserves beta cell function and survival during metabolic ER stress

We next asked whether the azoramide-induced improvement in glucose homeostasis in obese mice also involved improved beta cell function or survival, as these cells are known to be susceptible to ER stress in the setting of increased insulin demand (52–54). In islets isolated from *ob/ob* mice treated with azoramide for 10 days, we observed higher levels of expression of insulin pre mRNA and Pdx1 (Fig. 6A), potentially indicating improved beta cell function. In vehicle-treated *ob/ob* mice, glucose stimulated insulin secretion (GSIS) was absent, and in fact there was a trend toward decreased circulating insulin 15 minutes following the glucose bolus (Fig. 6B). However, one week of azoramide treatment significantly improved *in vivo* GSIS in this model (Fig. 6B). To further investigate the beta cell-intrinsic effects of azoramide, we used beta cell lines. In Min6 cells, azoramide treatment significantly rescued the blunted GSIS observed in the setting of palmitate-induced ER stress (Fig. 6C), and in Ins-1 cells, azoramide treatment improved viability in the setting of gluco-lipotoxicity (Fig. 6D). These results demonstrate that the metabolic

improvements observed upon azoramide treatment may, at least part, be mediated by improvements in beta cell function and survival.

Azoramide improves glucose homeostasis in mice with diet-induced obesity

Observing these dramatic effects on glucose metabolism prompted us to perform more detailed metabolic analysis in an additional model with diet-induced obesity, a mouse model that is more relevant to human disease and also characterized by ER stress (46–49, 55, 56). Male mice fed 60% high fat diet (HFD) for twenty weeks were treated with 150mg/kg azoramide by gavage once per day for one week at the University of Massachusetts national mouse metabolic phenotyping center. This brief treatment was sufficient to markedly improve fasting blood glucose (Fig. 7A) and reduce body weight (Fig. 7B).

Hyperinsulinemic euglycemic clamps were then performed in these mice in order to determine whole body glucose fluxes and examine target tissues influenced by this treatment. There was a striking increase in the glucose infusion rate (GIR) in mice receiving azoramide (Fig. 7C), resulting in significantly elevated glucose disposal rates (Rd) (Fig. 7D). Hepatic glucose production (HGP) during the clamp was also markedly reduced (Fig. 7E). In fact, under the clamp conditions, insulin essentially completely suppressed hepatic glucose production in the azoramide-treated group. Consistent with the increased rates of disposal, glucose uptake to peripheral tissues, such as muscle (Fig. 7F) and white adipose tissue (Fig. 7G) was significantly increased by azoramide, as was the rate of glycogen synthesis (Fig. 7H), demonstrating increased insulin action in liver and in peripheral tissues. Taken together, these data suggest that systemic administration of azoramide has potent insulin sensitizing and anti-diabetic properties in multiple preclinical models of obesity and that these effects are readily reproducible by independent facilities and scientists.

Discussion

Chronic ER stress and defective UPR are important mechanisms in the pathogenesis of metabolic diseases (20). Importantly, defective ER function and UPR are features of obesity and both type 1 and type 2 diabetes in humans (30, 49–51, 53, 57–59). Therefore interventions targeting ER function and/or designed to improve the UPR capacity may be useful therapeutic strategies for metabolic and other pathologies. In fact, limited studies in humans using chemical chaperones provide proof-of-principle that such interventions may have translational implications (32, 33). However, there is a significant limitation in these existing chemical entities and a shortage of strategies, including cellular assay systems, to identify and develop a diverse set of new and more effective molecular entities. Here, we utilized a screening platform with ER functional reporter assays to characterize azoramide, a small molecule UPR regulator. We found that in cells, azoramide has the unique dual property of acutely increasing ER folding output while chronically inducing chaperone expression and promoting ER homeostasis. Treatment of cells with azoramide was strongly protective against chemical (thapsigargin and tunicamycin-induced), hypoxia, lipotoxicity, and protein misfolding-induced ER stress. Remarkably, azoramide treatment significantly improved insulin sensitivity and glucose tolerance and beta cell function in obese mice in multiple preclinical models. These data provide proof-of-principle that small molecule modulators of adaptive UPR pathways can be identified with functional phenotypic screens

targeting the ER, and that these may be beneficial in the treatment of ER stress-mediated pathologies, particularly metabolic disease. In addition, the compound described in this study was effective in preventing the death of cells expressing a mutant form of Rhodopsin identified in autosomal dominant human retinitis pigmentosa associated with protein misfolding and ER stress (41). Since azoramide protects cells expressing this mutant protein from death, it may represent a therapeutic opportunity against forms of progressive neurodegenerative disorders including retinitis pigmentosa that feature cell death induced by mutant, misfolding proteins. Furthermore, the unique approach described here to identify therapeutic candidates opens up the possibility of discovery of similar compounds that help alleviate ER stress by capitalizing on the adaptive features of the UPR.

Currently, there are two strategies guiding the development of ER-based therapeutics. The first is preconditioning. For example, a sub-toxic dose of classical ER stress inducers has previously been shown to be protective against oxidative stress in cell lines (37), to prevent cell death in response to nephrotoxins (38) and methylmercury (39), and to prevent the development of immune-mediated nephritis (60) and vascular leakage (61) in mouse models. The second is chemical chaperones (62). Small molecule chemical chaperones such as PBA and trimethylamine N-oxide dehydrate (TMAO) relieve ER stress by aiding protein folding in the ER lumen (63). These molecules and other chemical chaperones like TUDCA can improve glucose homeostasis in a mouse model of diabetes (28), and may potentially be therapeutic in humans (32, 33). Recently, a new class of ER stress-relieving small molecule chemical chaperones was identified by a high throughput chemical screen (64). However, the common feature of all these chemical chaperones is that they blunt the UPR pathways, as evidenced by decreased GRP78 protein expression. As demonstrated here, the newly identified chemical compound azoramide has distinct advantages over both preconditioning and chemical chaperone approaches. First, unlike chemical toxins, azoramide pretreatment does not impair ER function as part of its initial action. Second, azoramide can be used at much lower concentrations than those of chemical chaperones with the added benefit of engaging endogenous ER homeostasis resolution programs. The remarkable potency of azoramide as an anti-diabetic agent also raises the potential utility of the functional screening systems described here to generate additional such molecules to target metabolic diseases.

Maintaining a high calcium concentration in the ER is critical for various ER functions, such as protein folding and secretion. Calcium can bind to some amino acid side chains and may directly alter protein folding, and is required for the function of multiple chaperones, including GRP78 (65). The high luminal calcium concentration is established by a balance between calcium uptake by the action of sarcoplasmic/endoplasmic reticulum Ca^{+2} ATPase (SERCA) enzymes and calcium release by IP3 receptors (66), and it has previously been shown that reducing ER calcium release by the IP3 receptor improves glucose homeostasis in obese diabetic mice (67, 68). We recently demonstrated that SERCA activity is compromised in the livers of genetically obese mice, resulting in reduced Ca^{+2} storage in the ER lumen (17). Here we show that azoramide treatment leads to increased SERCA expression, resulting in enhanced retention of Ca^{+2} within the ER. Thus it is possible that the induction of chaperone gene expression *in vitro* and the anti-diabetic effect of azoramide in obese mice are related (directly or indirectly) to modulation of calcium homeostasis. Our

efforts to dissect this molecular mechanism indicate that azoramide interacts with UPR pathways to promote resolution of ER stress and improve ER function. Specifically, genetic suppression and chemical inhibitor experiments suggest that the PERK and IRE1/XBP-1 arms of the UPR may be involved in azoramide action, although at this time it is unclear whether the effects of azoramide are due to direct interactions with these ER sensors, and to what extent its actions are dependent on an intact UPR system. Further studies will be required to elucidate the exact mechanisms of azoramide function and its translational possibilities.

In summary, the high-throughput functional assay platform described here demonstrates the feasibility to monitor ER function and identify potent molecular entities that act on the ER and are effective in the treatment of diabetes in preclinical models. These systems should facilitate the efforts to chemically target ER stress and identify small molecules that may have utility in many other disease states.

Materials and Methods

Study Design

The objective of the study was to develop assays to dynamically monitor ER function and to characterize the cellular and physiological effects of a small molecule modulator of ER function. For the *in vitro* experiments: the dose response curves for reporter assays were performed in duplicate; quantitative, real-time PCR (QPCR) samples were analyzed in technical duplicates and experimental duplicates. All the experiments were done at least twice. *In vivo* results shown are representative of two independent cohorts. All *in vivo* experiments (GTT, ITT, blood glucose measurements) were performed blinded. The elimination criteria for the outliers were based on the visible health of the individual mice. Mice appearing sick or that underwent significant weight loss were eliminated from analysis.

Biochemical Reagents

Azoramide was synthesized at Syncore laboratories (Shanghai, China), the details of which are described in Fig. S7. Tunicamycin and thapsigargin were obtained from Sigma. Lipofectamine, siRNAs, RNAiMax, Taqman primers and probes were from Life Technologies. shRNA constructs were from Sigma. Cypridina luciferin and native coelenterazine (CTZ) and were procured from Prolume, Ltd.

Reporter construction

Functional reporter plasmids pGluc/ATF6LD-Cluc and pGluc/ASGR-Cluc were generated as follows: full length coding sequence of *Cypridina* luciferase (Cluc) (Genbank Accession: AB159608) protein was cloned in pCDNA3.1(-) cloning plasmid (Invitrogen Cat #V795-20) between NheI and XbaI restriction sites downstream of cytomegalovirus (CMV) promoter. Then, the *Gaussia* luciferase (Gluc) (Genbank Accession: AY015993) coding sequence controlled by CMV promoter (CMV-Gluc) was cloned using a MfeI restriction site using PCR primers (Fw: TGCTTAGGGTTAGGCGTTTT, Rv: TGGCAAGTGTAGCGGTCA). ATF6LD, luminal domain (amino acids 400 to 670) of human ATF6 α protein (Genbank

Accession AAB64434.1) was cloned inside *Cypridina* luciferase gene (Genbank Accession: AB159608) after signal peptide sequence using primers (Forward: CAGGATTCCAGGAGAATGAA and Reverse: AGGACAGTCCTG-TGTGCCTC) to generate ATF6LD-Cluc expressing plasmid pGluc/ATF6LD-Cluc. ASGR coding sequence (amino acids 62 to 284) of mouse Asialoglycoprotein receptor 1 protein (ASGR) (Genbank Accession: NP_033844.1) was cloned inside *Cypridina* luciferase gene after signal peptide sequence using primers (Forward: AATTCCCAACTCCGGAAGA and Reverse: ATTAGCCTTATCCAACCTTTGTCTCA) to generate ASGR-Cluc expressing plasmid pGluc/ASGR-Cluc.

Cell line experiments

Compound treatment of reporter-expressing HEK293 cells was performed in 96-well plates. The cells were seeded before the treatment at a density of 1.5×10^4 cells/well. The next day, the cells were changed to fresh media containing various concentrations of compounds. At the end of the treatment, 10 μ l medium was transferred into two 96-well white plates for luciferase assays following the manufacturer's protocol. Briefly, 50 μ l of luciferase substrate (1 μ M Cypridina or 10 mM CTZ in 100 mM Tris buffer, pH7.5) was added to the 10 μ l medium and incubated in the dark for 5–10 minutes. The luminescence was read on EnVision plate reader (Perkin Elmer).

Inducible ATF6LD-Cluc reporter cells were generated using Tet-Express inducible expression system (ClonTech). The cells were induced overnight according to the manufacturer's protocol and incubated either in normoxia (21% O₂) or hypoxia (1% O₂) (H35 Hypoxystation, Don Whitley Scientific) for 16 hours. Luminescence was read as described above.

HEK293A cells were transfected with Bovine Rhodopsin^{WT}, Rhodopsin^{P23H} (a kind gift from Dr. Michael Cheetham, UCL institute of Ophthalmology) or GFP control plasmids using Fugene HD. Eight hours after transfection, cells were washed with fresh medium and treated with azoramide or vehicle. For gene expression analysis, cells were harvested after 24 hours. For viability analysis, 1 μ M MG132 was added to the medium 16 hours after transfection. 24 hours after treatment, cells were analyzed using the CellTiterGlo Luminescent cell viability assay system (Promega) following the manufacturer's protocol.

INS1 cells were treated with 25mM Glucose and 500 μ M Palmitate (G/P) in the absence or presence of 20 μ M azoramide for 60 hours. At the end of the incubation, viability was measured using the CellTiter-Glo cell viability assay system.

Analysis of glucose-stimulated insulin secretion (GSIS)

Insulin release from MIN6 cells was measured as previously described (69). Briefly, MIN6 cells treated with BSA or 200 μ M palmitate with or without azoramide for 48 hours were pre-incubated for 30 min in Krebs-Ringer bicarbonate (KRB) buffer containing 2.8 mM glucose and 0.2% BSA, after which they were incubated for 1 h with KRB buffer containing either 22.4 mM glucose or 2.8 mM glucose and 0.2% BSA. The secreted insulin was analyzed using an ELISA system (Crystal Chem).

ER calcium measurements—ER calcium levels were determined using the D1ER (Cameleon) calcium sensor kindly provided by Dr. Roger Tsien (University of California, San Diego) as previously described (68). Briefly, Hepa 1–6 cells were seeded on 3.5-cm imaging dishes and transiently transfected with D1ER plasmid using lipofectamine. Twelve hours after transfection, cells were treated with 20 μ M of azoramide compound or DMSO (vehicle) for 48 hours in growth medium (DMEM, 10% CCS). For imaging, cells were washed and maintained in a medium as follow: 10 mM Hepes, 150mM NaCl, 4mM KCl, 2mM CaCl_2 , 1mM MgCl_2 , 10mM D-glucose, pH 7.4. Ca^{2+} free medium was based on the formula above, in the absence of CaCl_2 and adding 2mM EGTA, 3 mM MgCl_2 . Fluorescent images of single cells were obtained using an Olympus IX81 microscope and an ORCA-AG camera from Hamamatsu with a filter cube Chroma 71007A. Emission ratio of the Cameleon was obtained using two emission filters (485/40 for CFP and 535/25 for citrine) controlled by a Lambda 10-2 filter changer (Sutter Instruments, Novato, CA). Exposure times were typically 250ms and images were collected every 5 seconds, background corrected and analyzed with Slidebook (Intelligent Imaging Innovations).

Cytosolic calcium measurements—Cytosolic Ca^{2+} levels were determined using Fura-2 AM (Invitrogen) as previously described (68). Briefly, cells were loaded with 4 μ M Fura-2AM and 1 μ M Pluronic F-127 in HBSS for 60 min at room temperature and then washed and maintained in the same buffer as described for ER Ca^{2+} measurements. Fluorescence images were obtained using an Olympus IX70 with 40x objective and alternately illuminated with 340 and 380 nm light for 250ms (Lambda DG-4; Sutter Instrument Co.). Emission light > 510 nm was captured using a CCD camera (Orca-ER; Hamamatsu Photonics). Images were collected every 5 seconds, background corrected and analyzed with Slidebook (Intelligent Imaging Innovations).

Mice—All animal care and experimental procedures were performed under approval of animal care committees of Harvard University. Male mice with genetic obesity were obtained from Jackson Laboratories (Strain: B6.Cg-*Lep^{ob}*/J, stock number: 000632) at 9–12 weeks of age and kept on regular chow diet. Male mice for the diet induced obesity model (HFD) were also obtained from Jackson Laboratories (Strain: C57BL/6J DIO, stock number: 380050), which were fed *ad libitum* for 20 weeks with 60% kcal fat diet (D12492i, Research Diets) after weaning. The mice were kept on a 12 hour day/night cycle and azoramide compound was fed to mice once a day at 3:00 pm by oral gavage (150mg/kg in 200 μ l vehicle solution). The recipe for the vehicle solution was 10% (vol/vol) ethanol, 0.1% (vol/vol) Acetic Acid, 40% (vol/vol) PEG400 and 0.05% (weight/vol) CMC (carboxymethylcellulose). Glucose and weight measurements were taken at 3:00pm, after 6 hours food withdrawal. Glucose tolerance tests (GTT) were performed by intraperitoneal glucose injection (1 g/kg) following an overnight food withdrawal as described previously (18). Metabolic cage and euglycemic hyperinsulinemic clamp studies were performed as described previously (70, 71).

Islet isolation

The methods for isolating islets from mice were described previously (69). Briefly, the pancreatic duct was perfused with 2.5 ml of 3 mg/ml Liberase RI (Roche), after which it was

excised and then disaggregated by shaking for 24 min at 37°C. The islets were partially isolated by sedimentation and then hand picked from the acinar tissue debris under a dissecting microscope.

Statistical analysis

Graphical data are presented as mean $\pm$ SEM or SD as indicated in the figure legends. Differences between groups were determined by student's t-test or repeated measures ANOVA as indicated, and considered significant when $p < 0.05$. Data were normally distributed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the former employees of Syndexa Pharmaceuticals Corp. for their initial discovery of azoramide and the generous donation of this compound, and to Dr. Jason Kim and the National Mouse Metabolic Phenotyping Center at the University of Massachusetts for *in vivo* analysis of the HFD-fed mice. We thank Dr. Kathryn Claiborn for critical reading and editing of this manuscript and Dr. Martin P. McGrath for expert advice on chemical structures.

Funding: This work was supported in part by a grant from the JDRF (17-2012-346) to GSH.

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Figure 1. Reporter systems monitor ER chaperone availability and activity

A. Two dual luciferase reporter constructs were generated, expressing the ATF6LD (yellow) or ASGR (black) fused to Cluc (red). Gluc (blue) was expressed from a separate promoter as an internal control. ER chaperones (green) mediate retention of ATF6LD-Cluc in the ER lumen by protein-protein interaction through a sensor domain in the ATF6LD reporter. ASGR-Cluc secretion is mediated by the folding capacity of the ER. **B.** siRNA-mediated suppression of GRP78 increases ATF6LD-Cluc secretion compared to non treated and nonspecific controls (siCont). Efficiency of GRP78 suppression was validated by western

blot analysis (lower panel). **C.** Thapsigargin (Tg) induced a dose-dependent increase in secretion of ATF6LD-Cluc compared to control (Gluc). **D.** siRNA-mediated suppression of GRP78 reduces ASGR-Cluc secretion compared to non treated and nonspecific controls. **E.** Tg induced a dose-dependent decrease in secretion of ASGR-Cluc compared to control (Gluc). For B and D, n=6 per group, graphs represent mean $\pm$ SEM. For C and E, the mean of two technical replicates is shown. Experiments are representative of at least three independent experiments.

Figure 2. Azoramidate regulates ER folding and secretion capacity without inducing ER stress
A. Chemical structure of azoramidate (Azo). **B.** Expression profile of UPR genes in Huh7 cells following Tg and Azo treatment, average of two experimental duplicates is shown. CHOP induction in the 1 μ M Tg condition was 23.6 fold higher than control. **C.** Time course demonstrates differential regulation of GRP78 and CHOP expression and phosphorylation of eIF2 α in Tg (upper panel) or Azo-treated Huh7 cells (lower panel). **D.** Dose response of Azo-induced increase of ASGR-Cluc secretion compared to control (Gluc). **E.** Dose response of Azo-induced decrease of ATF6LD-Cluc secretion compared to control (Gluc).

F. ATF6LD-Cluc secretion in Azo-treated cells overexpressing GRP78 and ERDJ3. **G.** ATF6LD-Cluc secretion in Azo-treated cells following siRNA-mediated reduction of PERK, XBP-1 or ATF6. **H.** ATF6LD-Cluc secretion in Azo-treated cells following treatment with the PERK inhibitor GSK2606414 or the IRE1 inhibitor 4μ8C. For D–H, the mean of two technical replicates are shown. Experiments are representative of at least three independent experiments.

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Figure 3. Azoramide protects against chemically-induced ER stress in vitro

A. Azoramide co- and pre-treatment counteracts tunicamycin (Tm)-induced ATF6LD-Cluc secretion and Tm-induced decrease of ASGR-Cluc secretion. The mean of two technical replicates for each condition is shown. Experiments are representative of at least two independent experiments. **B.** Azo pretreatment suppresses Tm-induced GRP78 and CHOP protein expression. **C.** Hypoxia induces ER stress, as measured by increased ATF6LD-Cluc secretion. Pretreatment with Azo abrogates this effect. Graph indicates mean $\pm$ SD, $n=8$ per treatment. **D.** Expression of the P23H Rhodopsin mutant in HEK293A cells induces ER

stress, as measured by CHOP expression. Treatment with Azo dose-dependently abrogates this effect. Graph indicates mean $\pm$ SEM, n=4 per treatment. **E.** Azo treatment dose-dependently restores viability in Rhodopsin^{P23H}-expressing cells. Graph indicates mean $\pm$ SEM, n=8 per treatment, **p<0.01 by t-test.

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Figure 4. Azoramide treatment alters ER calcium homeostasis

A. Azoramide treatment increased the basal ER calcium concentration compared to vehicle in Hepa1-6 cells. Graph represents mean $\pm$ SEM, $n=91-184$ per group. **B,C.** Azo pre-treatment increased ER calcium retention following Tg treatment. Graph represents mean $\pm$ SEM, $n=40-55$ per group. **D,E.** Azo treatment delays the influx of calcium into the cytoplasm induced by Tg treatment. Graph represents mean $\pm$ SEM, $n=3-4$ independent experiments (panel D), and $n=63$ (panel E). **F.** Azo treatment induces SERCA2 expression in Hepa 1-6 cells. * $P<0.05$, ** $P<0.01$ by t-test.

Figure 5. Azoramide reduces ER stress and improves metabolism in *ob/ob* mice

A. Evaluation of ER stress markers in liver lysate from the *ob/ob* mice treated with oral Azo for one week. **B.** Body weights of the *ob/ob* mice during treatment with Azo or vehicle. **C.** Overnight fasting blood glucose levels, and, **D.** Glucose tolerance tests performed in the same groups of mice illustrate the impact of Azo treatment on glucose metabolism. In panels B–D, graphs represent mean $\pm$ SEM, $n=10$ mice per group. * $P<0.05$, ** $P<0.01$ by t-test (panel C), or repeated measures ANOVA (panel D).

A.

B.

C.

D.

Figure 6. Azoramide improves ER function, insulin secretion and survival in beta cells

A. Measurement of insulin and Pdx1 mRNA levels in islets isolated from *ob/ob* mice treated with vehicle or Azo for one week. Graph indicates mean $\pm$ SEM, $n=3$. **B.** Glucose stimulated insulin secretion *in vivo* during glucose tolerance test of Azo- or vehicle-treated *ob/ob* mice, presented as change from fasting insulin level. Graph represents mean $\pm$ SEM, $n=9-10$ mice/group. **C.** Glucose stimulated insulin secretion in Azo- or vehicle-treated Min6 cells. Graph indicates mean $\pm$ SEM, $n=4$. **D.** Survival of vehicle and Azo-treated Ins-1 cells in the context of gluco-lipotoxicity (25mM Glucose and 500 μ M Palmitate). Graph indicates mean $\pm$ SEM, $n=8$. * $p<0.05$ by t-test.

Figure 7. Azoramide induces weight loss, changes in energy expenditure and improved metabolic profile in mice with diet-induced obesity

A. Daily oral dosing of Azo for 1 week in HFD-induced obese mice decreased 6-hour fasting blood glucose. **B.** Body weight of HFD-fed mice before and after one week of Azo or vehicle treatment. **C.** Glucose infusion rate during hyperinsulinemic euglycemic clamp. **D.** Glucose disposal during clamp. **E.** Hepatic glucose production during clamp **F.** Glucose uptake to muscle (gastrocnemius) during clamp. **G.** Glucose uptake to white adipose (WAT)

during clamp. **H.** Glycogen synthesis during clamp. Graphs represent mean $\pm$ SEM, n=8–10/group, *P<0.05, **P<0.01 by t-test.

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