LETTER

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Small-Molecule Suppressors of Cytokine-Induced β-Cell Apoptosis

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ABSTRACT Pancreatic β-cell apoptosis is a critical event during the development of type-1 diabetes. The identification of small molecules capable of preventing cytokine-induced apoptosis could lead to avenues for therapeutic intervention. We developed a set of phenotypic cell-based assays designed to identify such small-molecule suppressors. Rat INS-1E cells were simultaneously treated with a cocktail of inflammatory cytokines and a collection of 2,240 diverse small molecules and screened using an assay for cellular ATP levels. Forty-nine top-scoring compounds included glucocorticoids, several pyrazole derivatives, and known inhibitors of glycogen synthase kinase-3β. Two compounds were able to increase cellular ATP levels, reduce caspase-3 activity and nitrite production, and increase glucose-stimulated insulin secretion in the presence of cytokines. These results indicate that small molecules identified by this screening approach may protect β cells from autoimmune attack and may be good candidates for therapeutic intervention in early stages of type-1 diabetes.

Type-1 diabetes is caused by the autoimmune destruction of insulin-producing β cells in the pancreas. β-cell apoptosis involves a set of signaling cascades initiated by interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) (*1–3*). IL-1β and TNF-α induce NFκB expression, and downstream activation of gene expression is thought to occur through nitric oxide (NO) signaling, which both increases endoplasmic reticulum stress-response pathways and decreases β cell-specific functions (*4, 5*). NO is a highly reactive molecule that inhibits the electron-transport chain, leading to decreases in glucose oxidation rates, ATP generation, and insulin production (*6*); cellular nitrite is more stable and serves as a surrogate marker for NO. NFκB activation and IFN-γ-induced STAT-1 signaling work together to effect β-cell apoptosis, mainly involving the intrinsic apoptotic pathway in both rodents and humans (*7*). The downstream effector of this cascade, caspase-3, results in apoptosis and the loss of the ability to secrete insulin in response to glucose stimulation.

Small molecules that increase β-cell survival in the presence of cytokines could be of potential clinical benefit to early stage type-1 diabetic patients. Previous studies have described small molecules with protective effects in the presence of cytokines (*8, 9*); most of these molecules were discovered because of their antioxidant or anti-inflammatory effects. For example, resveratrol in the presence of cytokines results in restoration of viability (*10*), possibly by SIRT1 activation (*11*). Further, Larsen *et al.* demonstrated that small-molecule inhibition of histone deacetylases (HDACs) with trichostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA) prevents cytokine-induced β-cell death, presumably by decreasing NFκB transactivation (*12*). Therefore, multiple mechanisms can serve to protect β cells from cytokine-induced apoptosis.

Here, we describe a phenotypic screening approach to systematically discover small molecules that increase β-cell viability and function in the presence of cytokines. Using the rat insulinoma cell line INS-1E, we screened 2,240 diverse compounds for the ability to alter cellular ATP levels in the presence of cytokines. We used secondary assays measuring caspase-3 activation, cellular nitrite production, and glucose-stimulated insulin secretion (GSIS) to detect complementary aspects of β-cell biology. We identified a number of small molecules that increase β-cell viability, including several glucocorticoids, novel pyrazole derivatives, and glycogen synthase kinase 3β (GSK-3β) inhibitors. These compounds enhanced cellular ATP levels and reduced caspase-3 activity in a dose-dependent manner. The pyrazole derivatives and one GSK-3β inhibitor, alsterpaullone, also reduced cellular nitrite production and increased GSIS in the presence of cytokines. These results suggest that small-molecule screening may provide useful compounds for therapeutic intervention in type-1 diabetes.

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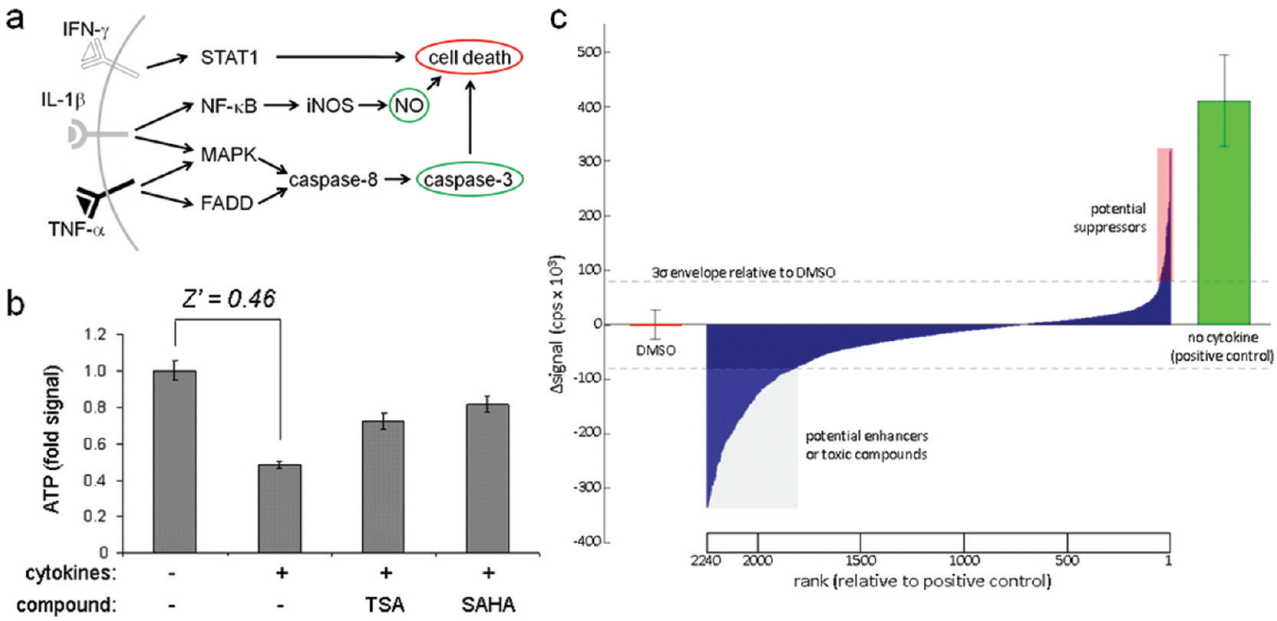

Figure 1. Assay development and screening for cellular ATP levels in the presence of inflammatory cytokines. a) Summary of the signaling pathways induced by cytokines and assay measurements used for primary (red circle) and secondary (green circles) screens. b) Rat INS-1E insulinoma cells were treated for 48 h with inflammatory cytokines (IL-1β, IFN-γ, TNF-α) in the absence or presence of HDAC inhibitors (50 nM trichostatin A (TSA) or 1 μM suberoylanilide hydroxamic acid (SAHA)). ATP levels were measured and normalized to the mean of untreated controls. Data are represented as the mean ± standard deviation of 12 independent wells. The assay *Z'* factor (*29*) was calculated on the basis of the means and standard deviations of untreated and cytokine-treated wells. c) Cellular ATP levels were assessed after treatment with each of 2,240 compounds. The signal change induced by compound treatment ("Δsignal") represents the value for each compound normalized to mock-treated wells. The means and standard deviations of mock-treated (DMSO) wells and positive-control (no cytokine) wells are shown. Potential suppressors (shaded pink) were identified as those resulting in Δsignal 3 standard deviations above the DMSO distribution.

We sought to use a physiologically relevant model of the development of type-1 diabetes (Figure 1, panel a) by using the rat INS-1E insulinoma cell line. Two-day treatment with a cytokine cocktail of IL-1β, IFN-γ, and TNF-α resulted in a 2-fold decrease in ATP levels (Figure 1, panel b). We also confirmed that the HDAC inhibitors TSA and SAHA partially suppress cytokine effects in β cells (Figure 1, panel b), at concentrations consistent with their enzymatic inhibition of HDACs in cells (*12*).

We performed a pilot screen of 2,240 compounds to identify small-molecules suppressors of this cytokine cocktail on cellular ATP levels in INS-1E cells. Compounds were considered "hits" if they increased ATP levels by 3 standard deviations relative to the mock-treatment (DMSO) distribution (Figure 1, panel c). Using this criterion, we identified 49 "hits", including 21 bioactives, 9 compounds synthesized by diversity-oriented synthesis (*13*), 5 natural products, and 14 compounds from commercial vendors. Chemical similarity analysis of the 49 "hits" revealed four clusters containing similar compounds by inspection (Figure 2, panel a). These clusters included two virtually identical compounds (alsterpaullone and kenpaullone), several pyrazole derivatives from commercial vendor libraries, nine compounds from diversity-oriented synthesis, and eight glucocorticoid derivatives.

We decided to focus initially on a set of commercially available compounds (Figure 2, panels b–d). Alsterpaullone, annotated as a GSK-3β inhibitor (*14*), completely restored β-cell ATP levels in a dose-dependent manner (Figure 3, panel a). Similarly, three pyrazole derivatives increased ATP levels to >90% of untreated controls (Figure 3, panel b). Dexamethasone, chosen as a representative glucocorticoid, was slightly less potent in enhancing ATP levels, to approximately 80% of untreated levels (Figure 3, panel c).

Caspase-3 is a downstream effector of the apoptotic pathway, and its activity is increased by a 48-h exposure of INS-1E cells to the cytokine cocktail. This increase in activity was suppressed more than 50% by 1 μM alsterpaullone (Figure 3, panel d). The pyrazole derivatives also reduced caspase-3 activity in a dose-dependent manner (Figure 3, panel e), but dexamethasone was only partially effective at reducing this activity (Figure 3, panel f). These results indi-

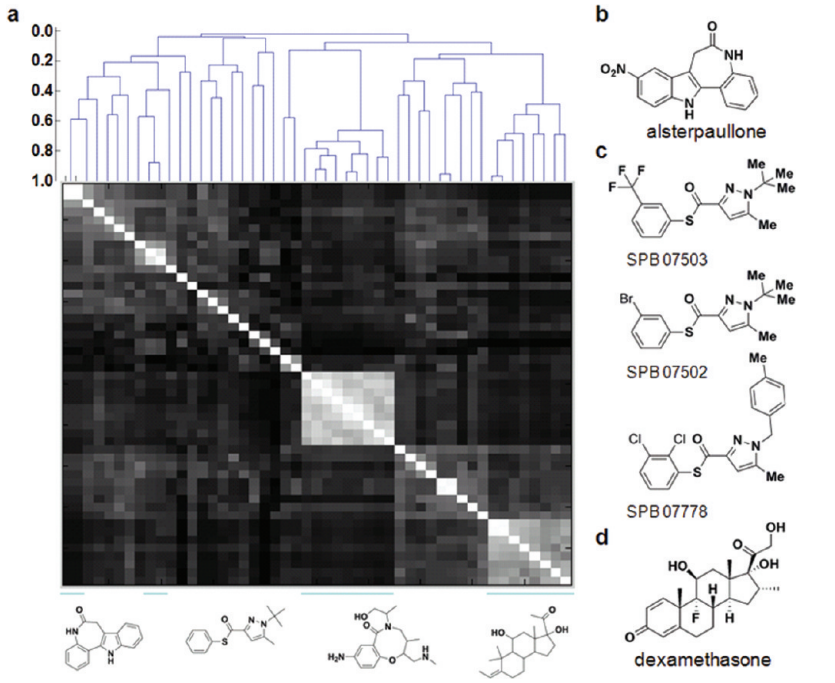

Figure 2. Chemical similarity among screening "hits". a) Forty-nine compounds exceeding the "hit" threshold (*cf.* Figure 1) were selected for cluster analysis (see Methods). Pairwise Tanimoto (*T*) similarity scores were computed among all compounds (heat map; $T = 0$, black; $T = 1$, white; linear grayscale), after which "hits" were clustered hierarchically (dendrogram). For groups with visually apparent similarities (four white/light blocks in heat map; blue bars at bottom), the maximum common substructure for each group is depicted. Structures of the compounds tested in this study: b) alsterpaullone, c) pyrazole derivatives, d) dexamethasone.

cate that screening for an increase in ATP levels can identify small molecules capable of halting the apoptotic process in the presence of inflammatory cytokines.

IL-1β induces expression of inducible nitric oxide synthase (iNOS), an effect potentiated by IFN-γ and TNF-α (*4*); the subsequent formation of NO drives β-cell death. Cellular production of nitrite, a stable oxidized product of NO used as a surrogate for NO levels, increased 3.5-fold after cytokine treatment and was completely inhibited by 2 μM alsterpaullone (Figure 4, panel a). The pyrazole derivatives were also effective in reducing nitrite production, though less so than alsterpaullone (Figure 4, panel b). Interestingly, dexamethasone had no effect on cytokine-induced nitrite production (Figure 4, panel c).

Finally, we examined the effects of these compounds on GSIS in INS-1E cells. Under normal conditions, stimulation with 15 mM glucose leads to a 3.6-fold increase in insulin secretion relative to low-glucose (2 mM) conditions (Figure 4, panel d). Cytokine treatment reduced GSIS to 1.4-fold. This loss of response was largely suppressed by the addition of 4 μM alsterpaullone to the cytokine cocktail, with stimulation elevated to 3.2-fold (Figure 4, panel d). Treatment with the pyrazole derivative SPB07503 resulted in ~50% enhancement of GSIS, while dexamethasone had no effect (Figure 4, panel d). Together, these results suggest that cellular nitrite levels are correlated with GSIS in INS-1E cells; compounds that are capable of reducing nitrite production in the face of cytokine treatment also restore GSIS.

Another GSK-3β inhibitor, Ro 31-8220, was, like alsterpaullone, among the top-scoring compounds. Although these kinase inhibitors likely interact with several targets, we reasoned that GSK-3β could be a relevant target accounting for the protective effect on β cells. Ro 31-8220 increased ATP levels, decreased caspase-3 activity, and reduced cellular nitrite production (Supplementary Figure 1). Similarly, the selective GSK-3β inhibitors lithium chloride (*15*) and CHIR99021 (*16*) increased ATP levels in the presence of cytokines (Supplementary Figure 1). However, lithium chloride completely abolished nitrite production, while CHIR99021 only decreased nitrite by 20% (Supplementary Figure 1). To evaluate the specificity of these responses, we knocked down GSK-3β in INS-1E cells with small-interfering RNA (siRNA) constructs. Knockdown of *GSK3B* resulted in ATP levels that were 75% that of the mock-transfected control, in a complete reduction of caspase-3 activity, and in a 20% reduction in nitrite production (Supplementary Figure 2). These results were similar to those achieved by CHIR99021 and suggest that selective inhibition of GSK-3β is only partially protective of cytokine-treated INS-1E β cells.

Inhibition of GSK-3β has been reported to protect β cells against glucolipotoxicity and endoplasmic reticulum stress-induced β-cell death (*17, 18*). There are key differences between these mechanisms and cytokine-induced apoptosis, so the fact that GSK-3β inhibitors could also suppress cytokine-induced β-cell apoptosis is not intuitively obvious. For example, the expression of iNOS and IκBα is upregulated by cytokines but not by high glucose concentrations (*19, 20*). Further, fatty acids such as oleate and palmitate do not activate the NFκB pathway in either INS-1E or rat islets, and fatty acid-induced β-cell death is independent of iNOS or nitrite production (*5*). Because we observed a correlation between cellular nitrite production and GSIS (Figure 4), it is likely that inhibition of

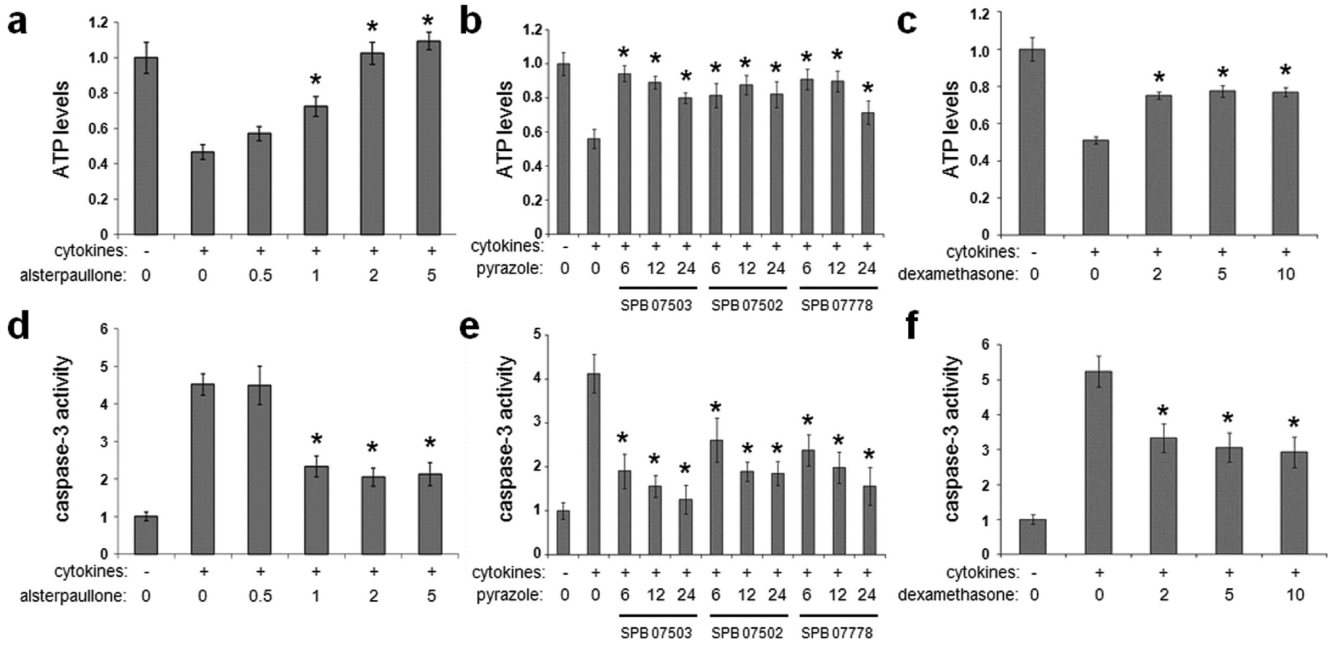

Figure 3. Suppression of cytokine-induced β-cell damage. INS-1E cells were treated with cytokine cocktail in the presence of increasing concentrations of alsterpaullone (a, d), pyrazole derivatives (b, e), or dexamethasone (c, f), all expressed as micromolar concentrations. Cellular ATP levels (a–c) and caspase-3 activities (d–f) were measured and normalized to untreated controls. Data are represented as the mean ± standard deviation of 24 independent wells. *$p < 0.01$ relative to cytokine-treated cells.

GSK-3β alone is insufficient to enhance β-cell function lost due to inflammatory cytokines. These results suggest that alsterpaullone acts through multiple mechanisms, including GSK-3β inhibition, to protect β cells from cytokine-induced apoptosis.

Glucocorticoids are a class of steroid hormones that bind to nuclear hormone receptors, which in turn translocate to the nucleus and upregulate the expression of anti-inflammatory proteins (*21*). Although glucocorticoids are generally detrimental to β-cell development and insulin secretion (*22*), a recent study showed that high doses of dexamethasone increase β-cell proliferation in rat islets (*23*). Here, we demonstrate that low-micromolar concentrations of dexamethasone increased cellular ATP levels and reduced caspase-3 activity in the presence of cytokines. However, dexamethasone neither reduced cytokine-induced nitrite production nor restored GSIS. These results indicate that glucocorticoids can only partially increase β-cell viability in this system.

Finally, the novel pyrazole derivatives were obtained from commercially available libraries. We observed that these compounds protected cells against the detrimental effects of cytokines in all assays tested. To our knowledge, there have been no previous reports of the biological activities of these or related structures. These data suggest that the pyrazole derivatives in this study could protect β cells by an as-yet unknown mechanism.

Because of the importance of inflammatory cytokines to the β cell, many efforts have been made to identify genetic or small-molecule approaches to protect β cells from cytokine-induced death (*8–10, 12, 24*). We have demonstrated the feasibility of cell-based screening to identify small molecules that prevent loss of β-cell viability in the presence of cytokines. Although screening efforts to increase β-cell number in the basal state have been described (*25*), to our knowledge, a systematic approach to discover small molecules that can prevent cytokine-induced β-cell death has yet to be reported. We also find that reduction of cytokine-induced caspase activity and nitrite production appear to be prerequisites for enhancing physiological β-cell function. The cytokines used here have numerous effects on β cells, but we believe the use of this cocktail is a relatively fair model of the development of type-1 diabetes; further studies in mice will be necessary to confirm these conclusions. This study represents a proof of concept for the discovery of novel targets and compounds for the treatment of type-1 diabetes.

METHODS

Cell Culture and Reagents. INS-1E cells (generously provided by C. Wollheim and P. Maechler, University of Geneva) were maintained in RPMI 1640 containing 11 mM glucose, 10% fetal

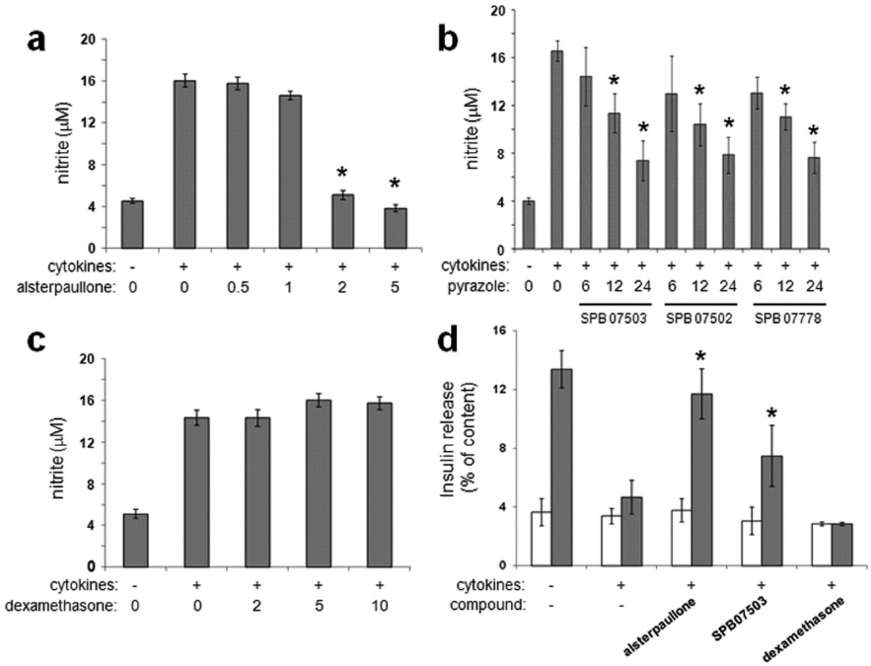

Figure 4. Reduction of nitrite production correlates with restoration of glucose-stimulated insulin secretion. Cellular nitrite production was measured after treatment with cytokine cocktail and increasing concentrations of a) alsterpaullone, b) pyrazole derivatives, or c) dexamethasone. Data represent the mean ± standard deviation of 24 independent wells. d) Glucose-stimulated insulin secretion was measured in the presence of 2 mM glucose (white bars) and 15 mM glucose (gray bars) in the absence or presence of cytokines and alsterpaullone (4 μM), the pyrazole derivative SPB07503 (12 μM), or dexamethasone (10 μM). Data are represented as the mean ± standard deviation of 8 independent wells. *$p < 0.01$ relative to cytokine-treated cells.

bovine serum, 10 mM HEPES, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, cultivated at 37 °C with 5% CO_2 in a humidified atmosphere, and split every week. Recombinant rat IL-1β and recombinant mouse TNF-α were purchased from R&D Systems. Recombinant mouse IFN-γ, Griess reagent, and dexamethasone were purchased from Sigma. CellTiter-Glo and Caspase-Glo 3/7 reagents were purchased from Promega. Alsterpaullone and Ro 31-8220 were purchased from EMD Biosciences. The pyrazole derivatives were purchased from Maybridge. CHIR99021 was synthesized as described (*26*).

High-Throughput Screening for Compounds Affecting Cellular ATP Levels. INS-1E cells were seeded at 10,000 cells per well using a Multidrop Combi (Thermo Labsystems) in white optical 384-well plates (Corning Life Sciences). After overnight incubation, medium was removed and 50 μL of RPMI containing 1% FBS and a combination of cytokines (10 ng mL^{-1} IL-1β, 50 ng mL^{-1} IFN-γ, 25 ng mL^{-1} TNF-α) was added to every well. Using libraries of compounds dissolved in DMSO and a CyBi-Well pin-transfer robot (CyBio Corp.), 0.1 μL of each compound was added. After 48 h, medium was removed and 20 μL of CellTiter-Glo reagent was added. Luminescence was measured after 10 min of incubation using an EnVision plate reader (PerkinElmer).

Screening Data Analysis. Instrument output files were processed using Pipeline Pilot (Accelrys) and input to MATLAB (The MathWorks) for data normalization. Compound performance scores relative to a distribution of mock-treated (DMSO) wells were calculated using a revised version of the scoring system underlying ChemBank (*27*). The role of replicate treatments was further developed as follows: first, mock-treatment distributions were modeled using all mock-treated wells measured on a single day, regardless of their nominal replicate; second, per-compound scores weighted each in-plate background-subtracted measurement by the uncertainty in that measurement, using the method of maximum likelihood (*28*). The uncertainty in a single background-subtracted measurement was estimated using the number of mock-treated wells on the plate and, as a measure of the assay noise, the standard deviation of the per-day mock-treatment distribution. The signal, a weighted average of differences, was scaled by the noise, the standard deviation of the mock-treatment distribution.

Chemical Similarity Analysis. Cluster analysis was performed using Pipeline Pilot extended connectivity fingerprints (unfolded ECFP_4s). Bits representing substructures present in more than 10%, and less than 90%, of the 49 compounds were selected to generate 96-bit representations for each structure. Pairwise Tanimoto similarity scores (*T*) were computed among all compounds, after which "hits" were clustered hierarchically (complete linkage), both using MATLAB. Maximum common substructures for each group with similarities apparent by inspection were determined using Pipeline Pilot.

Measurement of Cellular Nitrite Production. INS-1E cells were seeded and treated as described for high-throughput screening. After treatment with cytokine and compounds for 48 h, 10 μL of modified Griess reagent (1:1 mixture of 1% sulfanilamide in 30% acetic acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 60% acetic acid) was added to each well. After 5 min of incubation at RT, the absorbance at 540 nm was measured using an EnVision plate reader.

Caspase-3 Activity Assay. INS-1E cells were seeded at 5,000 cells per well in white optical 384-well plates and treated as described for high-throughput screening. After treatment with cytokines and compounds for 48 h, medium was removed and 20 μL Caspase-Glo 3/7 reagent was added. Luminescence was measured after 2 h of incubation using an EnVision plate reader.

Glucose-Stimulated Insulin Secretion. INS-1E cells were seeded in 96-well plates at 20,000 cells per well and incubated for 48 h in 100 μL of fresh RPMI containing 1% FBS and the cytokine cocktail, in the presence or absence of compounds. Cells were washed and incubated for 2 h in KRBH (135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4, 0.1% BSA) without glucose. Cells were subsequently incubated with KRBH containing 2 or 15 mM glucose for 1 h. The supernatant was taken for measurement of released insulin, and 100 μL of acidified ethanol was added to each well for extraction and measurement of cellular insulin content. Insulin was measured with a rat insulin ELISA kit (Alpco).

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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