JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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# Small-Molecule Inducer of $\beta$ Cell Proliferation Identified by High-Throughput Screening

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*Supporting Information*

**ABSTRACT:** The identification of factors that promote $\beta$ cell proliferation could ultimately move type 1 diabetes treatment away from insulin injection therapy and toward a cure. We have performed high-throughput, cell-based screens using rodent $\beta$ cell lines to identify molecules that induce proliferation of $\beta$ cells. Herein we report the discovery and characterization of WS6, a novel small molecule that promotes $\beta$ cell proliferation in rodent and human primary islets. In the RIP-DTA mouse model of $\beta$ cell ablation, WS6 normalized blood glucose and induced concomitant increases in $\beta$ cell proliferation and $\beta$ cell number. Affinity pulldown and kinase profiling studies implicate Erb3 binding protein-1 and the IκB kinase pathway in the mechanism of action of WS6.

$\beta$ cells are a subset of cells in the pancreatic islets, which themselves comprise only a few percent by mass of the pancreas. Despite their small numbers, $\beta$ cells play a dominant role in metabolism by regulating glucose homeostasis through the production of insulin. In type 1 diabetes (T1D), a combination of genetic and environmental factors results in the selective targeting of $\beta$ cells by the immune system, ultimately leading to loss of functional $\beta$ cell mass.[1,2] The current standard of care for T1D involves stringent patient self-monitoring to maintain glucose homeostasis via insulin injection.[3] A more robust therapy can be envisioned that addresses $\beta$ cell dysfunction directly by promoting regeneration of $\beta$ cell mass. Small molecules,[4−6] growth factors,[7] and ectopic expression of transcription factors[8] and cell cycle genes[9,10] have been described that are capable of inducing $\beta$ cell proliferation, but robust, pharmacological proliferation of human islets remains elusive. Herein we report the identification of WS6, a small molecule capable of inducing proliferation of primary rodent and human islets *in vitro* and mouse $\beta$ cells *in vivo*, and correcting hyperglycemia in the RIP-DTA mouse model of $\beta$ cell ablation.

In previous studies, we described a high-throughput screen for the identification of proliferative small molecules using a growth-arrested $\beta$ cell line. Further screening efforts have identified a number of additional chemotypes capable of inducing proliferation of R7T1 cells, including the diarylurea WS1[11,12] (Figure 1A). Preliminary analogue synthesis (see detailed synthetic scheme and procedures in the Supporting Information) yielded diarylurea WS3 and diarylamide WS6, which induced R7T1 proliferation in dose response, with EC50 values of 0.074 $\mu$M and 0.28 $\mu$M, respectively (Figure 1B). Proliferation of R7T1 cells, which are cultured in suspension and grow as clusters, was apparent by visible inspection (Figure 1C).

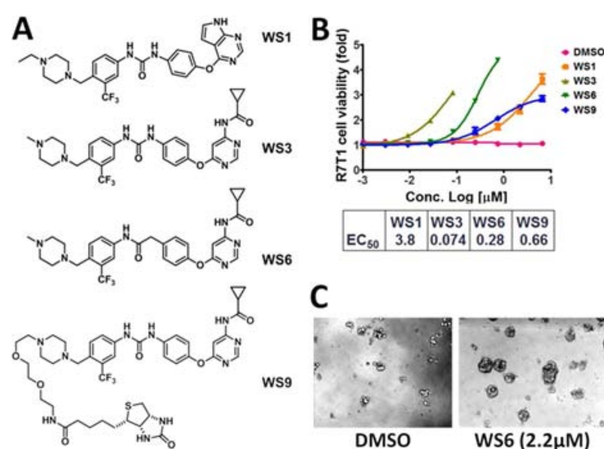


**Figure 1.** Diarylurea compounds induce proliferation of the mouse $\beta$ cell line R7T1. (A) Chemical structures of diarylurea compounds WS1, WS3, WS6, and WS9. (B) Dose-dependent effects of WS6 and analogues on R7T1 $\beta$ cell proliferation. (C) Expanded R7T1 cell clusters after 5 days of WS6 treatment (bright field image).

Unlike the previously reported molecule, BAY K8644, which displayed moderate activity in primary islets, initial experiments suggested that WS6 was robustly active in primary rodent and

**Received:** September 19, 2012
**Published:** January 19, 2013

 © 2013 American Chemical Society  dx.doi.org/10.1021/ja309304m | *J. Am. Chem. Soc.* 2013, 135, 1669−1672

human islets. In order to quantitatively characterize WS6's activity in primary cells, we sought to develop a high-throughput islet proliferation assay. However, intact islets clusters are not amenable to traditional automated manipulation because of their large and heterogeneous size distribution (50−300 μm). To overcome this, we developed a dispersed islet proliferation assay, wherein intact islets are dissociated with trypsin to yield a single-cell suspension, plated in monolayer on poly-D-lysine-coated 384-well plates in standard islet medium (10% FBS, 5.5 mM glucose RPMI), and then assayed by automated high-content imaging for EdU incorporation into insulin-positive cells. The addition of the antibiotic Geneticin helps to suppress fibroblast outgrowth of the dispersed human islet cultures, whereas this is not required for the analogous rat islet culture.[13] In this assay format, proliferation of both rat and human islets is induced by overexpression of cell cycle activators CDK7, cyclin D1, and cyclin H[9] (Figure S1). Encouragingly, WS6 induced up to 4% of rat β cells to proliferate, with an EC50 of 0.4 μM (Figure 2A). In the same format, WS6 also induced 3% of human β

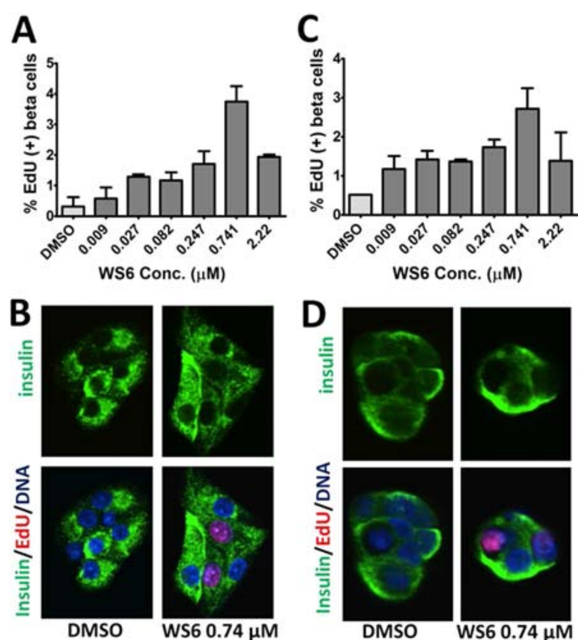


**Figure 2.** WS6 induces primary β cell proliferation in dissociated format. (A) WS6 causes proliferation of rat dissociated islets in dose response. (B) Representative images of proliferating rat β cells. (C) WS6 induces human β cell proliferation in dose response. (D) Representative images of proliferating human β cells in dissociated format.

cells to proliferate, with a similar potency to the rat β cells (Figure 2C). For illustration purposes, high magnification confocal microscope images show the proliferating rat (Figure 2B) and human (Figure 2D) β cells in dissociated format.

We also tested the activity of WS6 in intact islet cultures.[10] Islets were cultured with WS6 for 4 days in standard islet media, fixed and embedded in paraffin, and serial sections were cut and stained for insulin and Ki67, a marker of proliferation present during the G1, S, G2, and M phases of cell cycle. β cell proliferation was quantified by counting the percentage of insulin-positive cells that colocalize with Ki67. Encouragingly, WS6 induced significant proliferation compared to controls in both rat (Figure 3A) and human (Figure 3C) intact islets. Images representative of those used for quantification are shown in Figure 3B (rat) and Figure 3D (human).

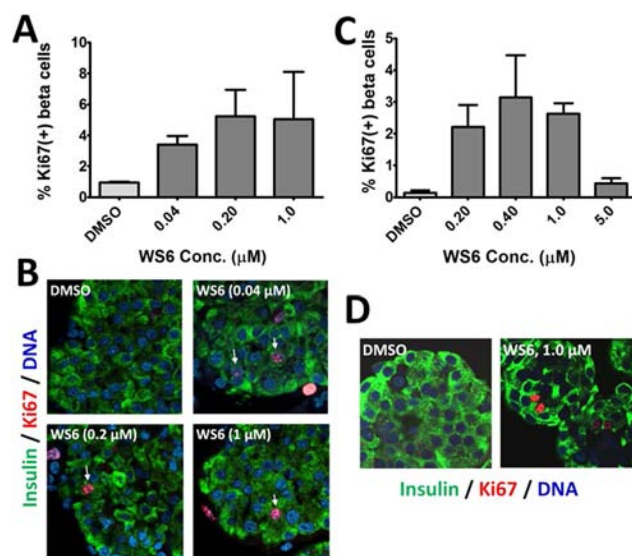


**Figure 3.** WS6 causes proliferation of primary rat and human β cells in intact islet format. (A) WS6 induces primary rat β cell proliferation in dose response after 4 day treatment in standard islet media. (B) Representative images of proliferating rat β cells with WS6 treatment. (C) WS6 proliferates human primary β cells in dose response after 4 day treatment. (D) Representative images of proliferating human β cells treated with WS6.

Next, we set out to evaluate the effect of the compound on β cell proliferation and hyperglycemia in a rodent model of T1D. RIP-DTA mice are double transgenic mice that express both the reverse tetracycline transactivator (rtTA) under control of the rat insulin promoter (RIP) and diphtheria toxin A (DTA) under control of a tetracycline-sensitive operon.[14] Treatment of these mice with doxycycline (Dox) causes selective expression of DTA in their pancreatic β cells, which leads to β cell ablation and onset of hyperglycemia. RIP-DTA mice were fed Dox in the drinking water until the onset of overt diabetes (blood glucose reading >300 mg/dL, typically 4−10 days), at which point Dox treatment was discontinued and treatment with WS6 was initiated (5 mg/kg every other day via intraperitoneal injection). Pharmacokinetic studies with WS6 at 50 mg/kg revealed a $C_{MAX}$ of ∼5 μM and $T_{1/2}$ of ∼2 h (Figure S4); the dose used in the RIP-DTA study was lowered to 5 mg/kg for optimal tolerability during the 45 day chronic treatment. Treatment with WS6 caused a progressive reduction of blood glucose over time, starting around 2 weeks (Figure 4A). The reduction in glucose occurred with a concomitant increase in β cell proliferation from 3.8% to 6.4%, as assessed by $K_i$-67/insulin costaining of pancreatic sections in mice sacrificed at the end of week 1, when we hypothesize the proliferation rate increase relevant to blood glucose lowering could be optimally measured (Figure 4B, left panel). At the termination of the study, we observed ∼50% increase in β cell mass in WS6-treated mice (Figure 4B, right panel).

In order to understand the mechanism of action of WS6, a biotin-conjugated analogue (WS9) was synthesized for affinity-based target identification experiments. WS9 proliferates R7T1 β cells 3-fold with an EC50 of 0.66 μM, comparable to WS6 but with somewhat reduced maximal efficacy (Figure 1B). To identify putative binding partners of WS6, affinity chromatog-

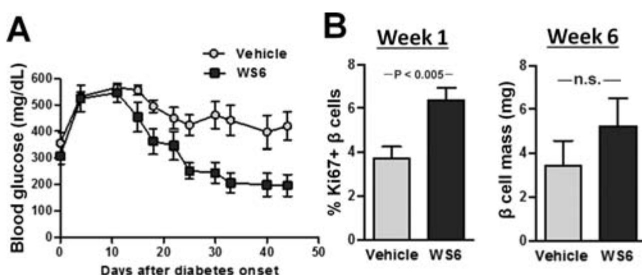


**Figure 4.** WS6 ameliorates diabetes in RIP-DTA mice. (A) Fed blood glucose levels (mean ± SEM) over time in mice treated with either 5 mg/kg i.p. WS6 (black squares) or water (gray circles). (B) Percentage of $K_i$-67-expressing $\beta$ cells (left) and $\beta$ mass (right) (mean ± SEM) in the pancreas of RIP-DTA mice after 1 and 6 weeks of treatment, respectively, with either WS6 (black bars) or water (gray bars).

raphy was performed by incubating WS9 with R7T1 cell lysate followed by streptavidin pulldown. Two-dimensional polyacrylamide gel electrophoresis and silver staining (Figure S5) revealed several spots that were pulled down by WS9 (5 $\mu$M) but were competed off by incubation with WS6 (500 $\mu$M). Tandem mass spectrometry identified IKK$\varepsilon$ and EBP1 among the proteins that were differentially bound to WS9. The identity of these two proteins was further supported by Western blotting of WS9-enriched lysate with anti-EBP1 and anti-IKK$\varepsilon$ antibodies (Figure 5A).

Erb3 binding protein-1 (EBP1, also called PA2G4) is classified as a tumor suppressor on the basis of its ability to repress cell cycle regulated genes through binding to histone deacetylase and retinoblastoma protein.[15,16] The I$\kappa$B kinase IKK$\varepsilon$ plays a role in regulating the NF$\kappa$B pathway, which is involved in the proinflammatory stress response. To confirm a role for these proteins in the $\beta$ cell proliferation activity of WS6, we analyzed the effects of blocking their activity either pharmacologically or with shRNAs. Multiple candidate shRNAs (Sigma) for knockdown of EBP1 in R7T1 cells were generated (Figure 5B); the best construct, shRNA-92, induced significant proliferation of R7T1 cells (Figure 5C). In contrast, the IKK inhibitor IKK-16 (Tocris) alone had little effect on R7T1 proliferation. To evaluate a possible synergistic effect of EBP1 and IKK, we assayed IKK-16 in combination with shRNA-92 and saw that this combination fully recapitulated the effect of WS6 on R7T1 proliferation (Figure 5C), suggesting both pathways contribute to the activity of WS6. In the presence of EBP1 shRNA, WS6 and IKK-16 cotreatment had similar effects on proliferation. Overexpression of EBP1 in R7T1 cells reduced the ability of WS6 to induce proliferation (Figure 5D), implicating a direct involvement of EBP1 in the mechanism of action of WS6. Given EBP1's antiproliferative role in other cell types via suppression of E2F-mediated cell cycle gene expression,[16] we examined expression of E2F, cyclin-D1 and -D2, and EBP1 in R7T1 cells treated with WS6 (1 $\mu$M) for various times (Figure 5E). E2F mRNA was induced 4.5-fold at 12 h, while cyclin-D1 was induced 3.5-fold at 8 h. Cyclin-D2 was modestly induced (1.8-fold) at 12 h, while changes in EBP1 expression were not statistically significant. We also observed a 2-fold increase in an E2F promoter-driven luciferase reporter gene assay carried out in R7T1 cells (Figure S3). We tested the possibility that WS6 invoked the GLP1R pathway, a known regulator of cyclin-D expression and $\beta$ cell proliferation,[20] but no cAMP response element activation was observed (Figure S5).

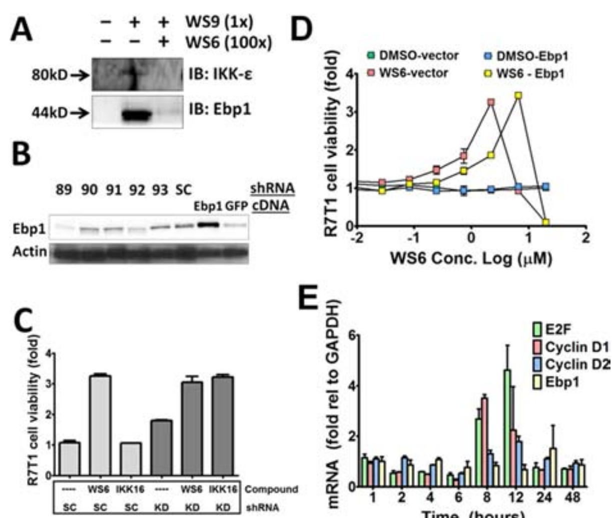


**Figure 5.** WS6 target identification and validation. (A) Affinity pulldown and WB confirmation of IKK$\varepsilon$ and EBP1 after enrichment of R7T1 lysate with WS9 (5 $\mu$M); both were competed off in the presence of excess WS6 (100×). (B) Western blot of EBP1 knockdown with shRNA 89−93 (EBP1 knockdown constructs) and scrambled control lentivirus, and lentiviral EBP1 overexpression in R7T1 cells. (C) EBP1 knockdown with shRNA 92 in R7T1 causes proliferation, which is enhanced with either WS6 (1 $\mu$M) or IKK inhibitor IKK-16 (1 $\mu$M) cotreatment. (D) EBP1 overexpression shifts the potency of WS6 in R7T1 proliferation. (E) Transcriptional changes induced by WS6 treatment (1 $\mu$M); for each target gene, change relative to GAPDH was calculated and then normalized to change induced by DMSO at each time point.

In summary, we have identified a novel small molecule capable of inducing proliferation of pancreatic $\beta$ cells. WS6 is among a few agents reported to cause proliferation of $\beta$ cells *in vitro* or *in vivo*.[6,17,18] While the extensive medicinal chemistry that would be required to improve the selectivity, efficacy, and tolerability of WS6 is beyond the scope of this work, further optimization of WS6 may lead to an agent capable of promoting $\beta$ cell regeneration that could ultimately be a key component of combinatorial therapy for this complex disease.[19]

## ■ ASSOCIATED CONTENT

### Supporting Information

Detailed protocols for cell culture, *in vitro* $\beta$ cell proliferation assays in cell line and primary rat and human islets, affinity pulldown, shRNA knockdown, and organic synthesis of WS3 and WS6. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The authors acknowledge the efforts of John Walker and Eric Peters for experimental support. This work was supported by the JDRF and the Novartis Research Foundation. M.S.T. and J.G.S. were supported by postdoctoral fellowships from the JDRF. The authors also thank Patricia Kilian and Richard Insel of JDRF for their support and insight.

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