EXPERIMENTAL STUDY

Efficacy of 1400 W, a novel inhibitor of inducible nitric oxide synthase, in preventing interleukin-1β-induced suppression of pancreatic islet function in vitro and multiple low-dose streptozotocin-induced diabetes in vivo

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Abstract
Objective: Nitric oxide (NO), generated by inducible nitric oxide synthase (iNOS), has been implicated in β-cell destruction in type 1 diabetes. In the present study, we tested a highly selective iNOS inhibitor, 1400 W, against interleukin-1β (IL-1β) induced suppression of rat pancreatic islets, and investigated whether 1400 W could prevent multiple low-dose streptozotocin (MLDS) induced diabetes in mice. Furthermore, we studied if 1400 W affected lipopolysaccharide (LPS) induced increase in plasma nitrite + nitrate (NO\textsubscript{x}) in mice.

Design and methods: Precultured rat pancreatic islets were exposed for 48 h to 0, 1, 10 or 50 μmol/l 1400 W in the presence or absence of 25 U/ml IL-1β, whereupon islet functions were analyzed. MLDS-treated mice were given 5.9 mg/kg body weight of 1400 W intraperitoneally daily or 14 mg/kg body weight twice a day. Blood glucose was monitored and degree of pancreatic mononuclear infiltration was determined. Mice previously injected intraperitoneally with LPS (500 μg) were given 1400 W (14 mg/kg body weight) intraperitoneally and plasma NO\textsubscript{x} was determined after 3, 6 and 10 h.

Results: The inhibitor alone did not affect islet functions. 1400 W (50 μmol/l) fully counteracted both the suppression of glucose oxidation rate, (pro)insulin biosynthesis and nitrite accumulation caused by IL-1β. Cytokine-induced decrease in medium insulin accumulation and glucose-stimulated insulin release was partly counteracted by 1400 W, suggesting that inhibition of insulin release was partially NO independent. LPS-induced increase in plasma NO\textsubscript{x} was markedly inhibited for up to 10 h after 1400 W administration. Irrespective of 1400 W treatment, animals treated with MLDS developed hyperglycemia and pancreatic insulitis.

Conclusions: 1400 W counteracted IL-1β-induced suppression of rat islets in vitro and LPS induction of NO\textsubscript{x} in vivo, however, it failed to protect against MLDS diabetes in vivo. The latter might be due to a failure by 1400 W in vivo to inhibit NO formation at the level of the pancreatic islet.

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Introduction

Type 1 diabetes mellitus is believed to be an autoimmune disease in which the T-cells and macrophages invade the islets of Langerhans in the pancreas. It is suggested that these mononuclear cells, either directly or through the release of inflammatory mediators such as cytokines, e.g. interleukin-1β (IL-1β) and interferon-γ (IFN-γ), and free radicals cause the specific β-cell destruction that occurs in type 1 diabetes (1).

The radical nitric oxide (NO) is produced, directly or indirectly, from L-arginine by the enzyme NO synthase (NOS) (2) in many mammalian cells. The neuronal NOS (nNOS) and the endothelial NOS (eNOS) isoforms are Ca\textsuperscript{2+} dependent and mostly expressed constitutively with a relatively low NO output (2, 3). The inducible NOS (iNOS) isoform, which has been suggested to be involved in β-cell destruction (4, 5), is largely or completely independent of Ca\textsuperscript{2+} (2, 3). This isoform is mostly not expressed under normal conditions but can be induced by, for instance, cytokines and endotoxins to produce large amounts of NO that in this case often function as a cytostatic and a cytotoxic molecule (2, 3, 6).

It has been found that both human and rodent pancreatic β-cells express iNOS and produce NO in
response to cytokines in vitro (7, 8). Furthermore, it has been shown that iNOS is expressed by both β-cells and activated macrophages in the pancreatic islets during the pre-diabetic insulitis in certain strains of mice and rats that spontaneously develop type 1 diabetes (9, 10). Flodström et al. also showed that iNOS knockout mice are resistant to diabetes induced by multiple low doses of streptozotocin (MLDS) (11).

These findings have raised interest in the question of whether inhibitors of iNOS could be used to prevent the development of type 1 diabetes. However, if this were the case, a high selectivity for iNOS vis-à-vis nNOS and eNOS seems important to prevent side effects that might arise from inhibiting the other isoforms (2). Recently, Suarez-Pinzon et al. reported that an iNOS inhibitor and scavenger of peroxynitrite, guanidinoethyldisulfide (GED), delayed the onset of diabetes in non-obese diabetic (NOD) mice from 12 to 22 weeks (12). However, according to the criteria put forward by Alderton et al., this inhibitor is at best a ‘partially’ selective iNOS inhibitor (2).

Against this background we decided to test if a new and ‘highly’ selective inhibitor of iNOS called 1400 W (N-(3-(aminomethyl)benzyl)acetamidine) (2, 13) could prevent the IL-1b (N-(3-(aminomethyl)benzyl)acetamidine) (2, 13) could prevent the IL-1b-induced suppression of pancreatic islet function in vitro. It has been shown that inhibition of human iNOS by 1400 W competes with l-arginine and the reaction is NADPH dependent (2). The mechanism for inhibition is considered to be either irreversible or very slowly reversible (2). Depending on our results with 1400 W, we also aimed to examine if the compound could prevent MLDS-induced diabetes in vivo.

Materials and methods

Isolation and culture of pancreatic islets

Adult Sprague–Dawley rats were anesthetized and killed by cervical dislocation, pancreatic tissue removed and digested by collagenase. Pancreatic islets were then handpicked under a stereomicroscope. Islets were cultured free-floating in medium RPMI-1640 supplemented with 10% fetal calf serum (FCS, vol/vol). Islet cultures were kept at 37°C in air + 5% CO2 for 6–7 days with medium changes every second day.

Islet treatment

Islets were subsequently divided into groups of 50 and cultured in a volume of 2.5 ml for 48 h as described above, with different additives. Three groups were cultured with 1400 W (1, 10 and 50 μmol/l; Alexis Biochemicals, Lausen, Switzerland), one group with IL-1β (25 U/ml), and another three groups with both IL-1β and 1400 W. Islets cultured without any additives served as a control group. The islets were then subjected to further experiments and analysis as described below. Medium without islets was also kept under the same conditions, to use as a blank for the analysis of medium nitrite content and medium insulin accumulation.

Analysis of medium nitrite content

The determination of medium nitrite content was performed using the Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 5% sulfanilamide, and 25% H2PO4) (14). A 10 μl volume of recently prepared Griess reagent was added to 100 μl samples of culture medium and incubated at 60°C for 2 min. The reaction products were then measured spectrophotometrically (λ = 546 nm) and the amount of nitrite in the samples calculated by the use of a standard curve. All determinations (including the standard curve) were performed in triplicates.

Determination of islet glucose oxidation rate

Groups of 10 islets were incubated in glass vials containing 100 μl of Krebs–Ringer buffer supplemented with 10 mmol/l HEPES (Sigma), D-[U-14C]glucose (Amersham-Pharmacia Biotech, Amersham, UK) and non-radioactive glucose to give a final concentration of 16.7 mmol/l. The vials were placed in glass scintillation vials, with its shafts squeezed through rubber membranes, gassed with 95% O2 and 5% CO2 and sealed tightly. The vials were then incubated in a slow-shaking water bath (30 strokes/min) at 37°C for 90 min before the glucose oxidation was terminated with 100 μl of 0.05 mmol/l antimycin A (Sigma) (dissolved in 99.9% ethanol), injected through the membrane into the inner vial. In the same way, 100 μl of 0.4 mol/l Na2HPO4 (pH 6.0) was injected to release the CO2 formed. The CO2 was then trapped by injection of 250 μl Hyamine 10-X (New England Nuclear, Boston, MA, USA) into the outer vial before incubating for another 2 h at 37°C. Finally 5 ml of Ultima Gold scintillation liquid (Packard Instrument, Meriden, CT, USA) was added and the radioactivity was measured by liquid scintillation counting. Triplicate samples were used for all determinations, and vials with medium but without islets were used as blank.

Determination of medium insulin accumulation, glucose-induced insulin release, insulin, and DNA contents

For all of the insulin determinations we used a rat insulin ELISA (Mercodia, Uppsala, Sweden). To determine the glucose-induced insulin release, duplicate groups of 10 islets were first incubated in 100 μl Krebs–Ringer buffer, supplemented with 10 mmol/l HEPES, 16.7 mmol/l glucose and 2 mg/ml BSA (ICN Biomedicals, Aurora, OH, USA), for 90 min at 37°C.
To determine the insulin and DNA contents from these glucose-stimulated islets, the islets were ultrasonically disrupted in 200 µl redistilled water. A 50 µl volume of the homogenate was then mixed with 125 µl of acid ethanol (0.18 mol/l HCl in 96% (vol/vol) ethanol) and insulin was extracted at 4°C overnight before determination. Another two 50 µl aliquots from the homogenate were used to determine the DNA contents by a fluorophotometric method (15).

**Determination of islet (pro)insulin biosynthesis and total protein biosynthesis**

Groups of 10 islets were incubated in duplicate for 2 h in 100 µl of Krebs–Ringer buffer containing 2 mg/ml BSA, 16.7 mmol/l glucose and 50 µCi/ml L-[4.5-3H]leucine (Amersham-Pharmacia Biotech, Uppsala, Sweden) at 37°C in air + 5% CO2. After incubation the islets were washed, in Hanks’ balanced salt solution containing 10 mmol/l non-radioactive leucine, and then sonicated in 200 µl redistilled water.

From each sample, 4 × 10 aliquots were transferred to Eppendorf tubes for (pro)insulin analysis according to Halban et al. (16). To the tubes, 100 µl of glycine buffer with Triton X-100 were added before adding 10 µl of guinea pig anti-bovine insulin serum (ICN Biochemicals, Cosa Mesa, CA, USA) to two of the tubes and 10 µl of guinea pig serum (for non-specific antibody binding) to the other two. The tubes were mixed vigorously and incubated at room temperature for 1 h. The samples were then mixed again and 100 µl of protein A-Sepharose (Amersham-Pharmacia) solution were added before incubating for another 15 min during slow shaking. The samples were then centrifuged (30 s at 500 g) and washed twice with 0.5 ml of glycine buffer containing Triton X-100. The pellets were resuspended in 2 × 250 µl acetic acid solution and transferred to scintillation vials before 4 ml Ultima Gold scintillation liquid was added and the samples counted in a liquid scintillation counter.

In vivo protocol 1

Inbred, 10–13-week-old male C57BL/Ks mice (Biomedical Center, Uppsala, Sweden) were used. The mice had free access to tap water and pelleted food (R36; AnaLyzen, L工业园ping, Sweden). The mice were divided into four groups (five mice per group) with different kinds of treatment: citrate buffer + saline; citrate buffer + 1400 W; streptozotocin (STZ) + saline; and STZ + 1400 W. Once a day, the mice first received an intraperitoneal injection of either citrate buffer (pH 4.5; 0.2 ml) or STZ (40 mg/kg body weight; 0.2 ml) dissolved in citrate buffer, and then after 30 min they received another intraperitoneal injection of either saline (9 g/l; 0.2 ml) or 1400 W (5.9 mg/kg body weight; 0.2 ml) dissolved in saline. This treatment was maintained for five consecutive days after which only the saline/1400 W injections were given for another 5 days. Blood glucose determinations were performed on blood samples taken from the tail tip of non-fasted mice on day 0, before any injections, and on days 3, 7, 10 and 14. Blood glucose was measured by a blood glucose meter (Medisense, London, UK). The mice were weighed on days 0, 7 and 14 and killed for morphologic examination of the pancreatic glands on day 14.

In vivo protocol 2

Based on the results from protocol 1 and a study by Parmentier et al. (17), we tested another protocol. This time we increased the injected dose of the iNOS inhibitor to 14 mg/kg body weight and gave the saline/1400 W injections twice a day (~10 h between the first and second injection every day), thus increasing the daily dose of the inhibitor by almost five times. The mice in this study were 14–18 weeks old.

Morphologic examination

The mice were killed by cervical dislocation, the pancreatic glands removed and fixed in 10% formalin for 24 h, after which they were embedded in paraffin and cut into 5 µm thin sections. These sections were stained with hematoxylin and eosin and examined under a microscope. The pancreatic islet histology was ranked into four arbitrary classes where class A denotes normal islet structure; class B mononuclear cell infiltration in the islet peri-insular area; class C heavy mononuclear cell infiltration into a majority of islets, i.e. insulitis; and class D only a few residual islets present, often showing an altered architecture and pyknotic cell nuclei (18). The examiner was unaware of the origin of the sections.
Determination of total plasma nitrite and nitrate \((\text{NO}_x)\) in vivo

The same strain of inbred C57BL/Ks mice were used, as in protocol 1 and 2, with free access to food and water, although, in this study we used both males and females and their ages ranged from 10 to 17 weeks. The mice were divided into three groups with different kinds of treatment: saline + saline, lipopolysaccharide (LPS from \(E.\) coli (Sigma-Aldrich, Stockholm, Sweden)) + saline and LPS + 1400 W. First the mice received an intraperitoneal injection of either saline \((9\,\text{g/l}; 0.2\,\text{ml})\) or LPS \((500\,\mu\text{g}; 0.2\,\text{ml})\) dissolved in saline, and then after 3 h they received another intraperitoneal injection of either saline or 1400 W \((14\,\text{mg/kg body weight}; 0.2\,\text{ml})\) dissolved in saline. After another 3, 6 or 10 h, retro-orbital venous blood samples were collected before mice were killed by cervical dislocation. Samples were centrifuged at 4000 \(g\) for 5 min and plasma collected. NO\(_x\) levels were determined by the use of the spectrophotometric assay kit BIOXYTECH NO-540 (Oxis Research, Portland, OR, USA) according to the manufacturer’s instructions. This assay kit uses cadmium to reduce nitrate to nitrite before using the Griess reaction and measuring the absorbance at 540 nm.

Statistical analysis

Data are presented as means±S.E.M. and groups of data were compared by one-way repeated ANOVA, with subsequent all pairwise comparison procedures by Bonferroni \(t\)-test or Student–Newman–Keuls method using SigmaStat (SPSS Inc., Chicago, IL, USA).

Results

Pancreatic islets, cultured with IL-1β for 48 h, showed a marked inhibition of glucose oxidation rate \((58\%\) of the control group), which was fully counteracted by 1400 W at the highest concentration \((50\,\mu\text{mol/l})\) (Fig. 1). However, no such significant counteraction was seen at the lower concentrations \((1\,\text{and}\,10\,\mu\text{mol/l})\) (data not shown). Incubations with 1400 W alone showed no significant changes in glucose oxidation rate. In fact, the inhibitor alone did not cause any significant change, compared with the control, in any of our in vitro experiments. Neither did we, in any of the experiments, observe any significant counteraction of the IL-1β-induced effects at the lowest concentration of 1400 W \((1\,\mu\text{mol/l})\).

The medium nitrite accumulation of the islets cultured with IL-1β was increased about threefold compared with the control value. This increase was also fully prevented by the addition of 1400 W \((50\,\mu\text{mol/l})\) with no significant difference between control and IL-1β + 1400 W (Fig. 2). With the addition of the intermediate concentration \((10\,\mu\text{mol/l})\), only a minor decrease (about 25% less nitrite accumulation) was seen (not shown).

The amount of insulin accumulation in the medium during a 48-h culture of islets with IL-1β was decreased to 23% of the control value (Fig. 3). This was partly prevented by 50 \(\mu\text{mol/l}\) of 1400 W to 63%.

Similarly, the amount of insulin released by islets (previously exposed to IL-1β) during incubation in 16.7 \(\text{mmol/l}\) glucose was decreased to 12% of the control value. Using the highest concentration of 1400 W, we observed an increase of this value to about 60% of the control value (Fig. 4).

Figure 1 Glucose oxidation rates in islets cultured for 48 h with or without 1400 W \((50\,\mu\text{mol/l})\) and in the presence or absence of IL-1β \((25\,\text{U/ml})\). Values are means±S.E.M. for four experiments.

* denotes \(P < 0.01\) vs control and # denotes \(P < 0.01\) vs IL-1β value, using one-way repeated ANOVA with subsequent all pairwise comparison procedures by Bonferroni \(t\)-test.

Figure 2 Medium nitrite accumulation after a 48-h culture of islets with or without 1400 W \((50\,\mu\text{mol/l})\) and in the presence or absence of IL-1β \((25\,\text{U/ml})\). Values are means±S.E.M. for 10 experiments.

* denotes \(P < 0.001\) vs control value and # denotes \(P < 0.001\) vs IL-1β value, using one-way repeated ANOVA with subsequent all pairwise comparison procedures by Bonferroni \(t\)-test.
Islets exposed to IL-1β had an insulin content which was 72% compared with control. With the addition of 1400 W (50 μmol/l), the insulin content increased to 126% compared with control; this value was higher both compared with the IL-1β value and the control value (Fig. 5).

Analysis of the islets (pro)insulin biosynthesis during a 2-h incubation in 16.7 mmol/l glucose showed that islets previously cultured with IL-1β had a decreased synthesis to 58% compared with control. With the addition of 1400 W (50 μmol/l) this decline was completely counteracted (Table 1). Although similar trends were observed for determinations of total protein biosynthesis and % (pro)insulin of total protein, the only statistically significant difference seen here was an increase in total protein biosynthesis in islets exposed to IL-1β + 1400 W compared with islets exposed to only IL-1β (Table 1).

Based on our in vitro results, we decided to test 1400 W in vivo to explore if the inhibitor could prevent the onset of diabetes in mice, induced by MLDS. Two different protocols were studied, and in both, the mice treated with STZ gradually developed hyperglycemia. None of the concentrations of 1400 W used in these experiments (5.9 mg/kg body weight once a day, in the first, and 14 mg/kg body weight, twice a day, in the second) were able to prevent this (Figs 6 and 7). The administration of the inhibitor alone did not cause any change in blood glucose levels compared with the control group (treated only with saline). The inhibitor did not affect the body weight in any of the experiments.

Moreover, the administration of 1400 W did not appear to influence the progression of mononuclear cell infiltration or insulitis (Table 2).

To examine if 1400 W (14 mg/kg body weight) did function in vivo to reduce NO levels, we decided to determine NO₃ plasma levels in mice exposed to different treatments (saline + saline, LPS + saline or LPS + 1400 W). LPS was adopted as a strong inducer of iNOS. NO₃ plasma levels were determined...
Table 1  Effect of 48-h exposure of rat pancreatic islets to 1400 W, IL-1β, or both, on (pro)insulin (PI) biosynthesis, total protein biosynthesis (TOT) and fraction PI of TOT compared with control. The concentrations used were 50 μM 1400 W and 25 U/ml IL-1β. The results are presented as means±S.E.M. for five experiments.

<table>
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<tr>
<th>Exposure</th>
<th>PI biosynthesis</th>
<th>TOT biosynthesis</th>
<th>Fraction PI of TOT</th>
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<tr>
<td></td>
<td>(dpm/10 islets h)</td>
<td></td>
<td>(%)</td>
</tr>
<tr>
<td>Control</td>
<td>934±271</td>
<td>6065±1325</td>
<td>14.47±1.21</td>
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<tr>
<td>1400 W</td>
<td>1084±383</td>
<td>6745±1859</td>
<td>15.01±1.25</td>
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<td>IL-1β</td>
<td>542±205*</td>
<td>4638±1279</td>
<td>10.93±2.33</td>
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<tr>
<td>IL-1β + 1400 W</td>
<td>1005±258#</td>
<td>7527±1663##</td>
<td>13.19±1.07</td>
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* denotes P < 0.05 vs control, while # and ## denote P < 0.05 and P < 0.01 respectively vs IL-1β, using one-way repeated ANOVA with subsequent all pairwise comparison procedures by Student–Newman–Keuls method.

Figure 6  Blood glucose concentrations in mice treated with injections of either buffer + saline (black bars), STZ + saline (gray), buffer + 1400 W (5.9 mg/kg body weight) (dark gray) or STZ + 1400 W (light gray). Values are means±S.E.M. for five animals and * and # denote P < 0.001 and P < 0.05 respectively vs the corresponding value on day 0, using one-way repeated ANOVA with subsequent all pairwise comparison procedures by Bonferroni Hest.

Figure 7  Blood glucose concentrations in mice treated with injections of either buffer + saline (black bars), STZ + saline (gray), buffer + 1400 W (14 mg/kg body weight) (dark gray) or STZ + 1400 W (light gray). Values are means±S.E.M. for five animals and * and # denote P < 0.001 and P < 0.01 respectively vs the corresponding value on day 0, using one-way repeated ANOVA with subsequent all pairwise comparison procedures by Bonferroni Hest.
3, 6 and 10 h after the last injection. LPS caused a time-dependent and marked increase in plasma NO\textsubscript{x} levels. Moreover, we found that the production of NO\textsubscript{x} was effectively inhibited by 1400 W after 3, 6 and 10 h (Fig. 8). There was no statistically significant difference between the groups that received LPS + 1400 W and the control groups.

### Discussion

The results from this study show that the new and selective iNOS inhibitor 1400 W can counteract the suppressive effect, induced by IL-1\textbeta, on the function of rat islet \textbeta-cells in vitro. It was able to prevent completely the induced increase in medium nitrite accumulation and also to fully restore the reduction in glucose oxidation rate as well as the (pro)insulin biosynthesis. Since the elevation in nitrite formation was fully prevented at 50 μmol/l 1400 W, we did not think it was worthwhile to use any higher concentrations of the drug. In the analysis of glucose-stimulated insulin release and medium insulin accumulation, however, only a partial protection was seen. This could suggest that the damage to the islet insulin release mechanism that could not be counteracted by 1400 W was caused by an NO-independent mechanism. This is in line with earlier findings that cytokines can cause NO-independent suppressive actions in \textbeta-cells (8, 19–21).

Although our in vitro results with 1400 W were promising, in the sense that IL-1\textbeta actions could be reduced at μmol/l concentrations, rather than the mmol/l concentrations often required with other NOS inhibitors, 1400 W failed to protect against MLDS-induced diabetes and the drug did not influence the progression of mononuclear cell infiltration in C57BL/Ks mice. However, we found that 1400 W effectively inhibited the LPS-induced increase in NO\textsubscript{x} production \textit{in vivo}, even 10 h after administration of 1400 W. Systemic LPS administration leads to a potent iNOS activation by immune cells throughout the body. A similar systemic injection of 1400 W was in this context efficient in reducing plasma NO\textsubscript{x} levels.
The question remains of how effectively 1400 W can inhibit iNOS activity at the level of the pancreatic islet, especially during insulitis. Furthermore, if iNOS is also expressed within β-cells leading to intracellular NO formation (22), it would be required that a sufficient amount of 1400 W is taken up by these cells. To our knowledge, no in vivo studies have been performed concerning the bioavailability of 1400 W in β-cells. We can therefore not exclude the possibility that our in vivo experiments failed due to an insufficient uptake of 1400 W within the islet β-cells. If this is the case, it could be that an even higher daily dose of 1400 W than used herein must be given in order to block NO at the islet β-cell level and perhaps prevent MLDS-induced diabetes. However, Garvey et al. showed that an intravenous dose of 50 mg/kg body weight 1400 W was lethal to mice and rats (13). On the other hand, when 1400 W was given as an intravenous infusion, a dose of 120 mg/day for a 7-day period was tolerated (13).

The role of NO in type 1 diabetes animal models has been debated. For instance, it has been proposed that iNOS inhibitors (including 1400 W). The inhibitors reported showed exceptional potency and high selectivity in cell-based assays, indicating superior cell permeability compared with other iNOS inhibitors (including 1400 W).

Concerning human β-cells, it should be noted that studies performed on human pancreatic islets have shown them to be less sensitive towards cytokine-induced NO than mouse and rat pancreatic islets (35). Furthermore, to induce NO production in human islets, they must be exposed to combinations of cytokines, such as IL-1β + IFN-γ (8). It is also likely that the time between the appearance of islet autoimmunity and the clinical onset of type 1 diabetes is much longer in humans than in mice and rats. Thus administration of an iNOS inhibitor over a prolonged period to individuals at risk of developing type 1 diabetes will require that the compound is very safe.

In conclusion, we have found that 1400 W was effective in preventing IL-1β-induced inhibition of rat islet function and also in counteracting LPS-induced elevation of plasma nitrite. However, MLDS-induced diabetes was not prevented. The latter might be due to a failure by 1400 W in vivo to inhibit NO formation at the level of the pancreatic islet.

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