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Small-Molecule Inhibitors of Cytokine-Mediated STAT1 Signal Transduction In β-Cells With Improved Aqueous Solubility

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Abstract

We previously reported the discovery of BRD0476 (**1**), a small molecule generated by diversity-oriented synthesis that suppresses cytokine-induced β-cell apoptosis. Herein, we report the synthesis and biological evaluation of **1** and analogs with improved aqueous solubility. By replacing naphthyl with quinoline moieties, we prepared active analogs with up to a 1400-fold increase in solubility from **1**. In addition, we demonstrated that compound **1** and analogs inhibit STAT1 signal transduction induced by IFN-γ.

INTRODUCTION

Type-1 diabetes (T1D) is a chronic metabolic disorder that 78,000 children worldwide develop each year.[1] This disease is characterized by autoimmune destruction of pancreatic β-cells, resulting in decreased insulin production. The secretion of pro-inflammatory cytokines by macrophages in the pancreatic islets of Langerhans is believed to trigger intracellular signaling cascades leading to β-cell apoptosis.[2] Small molecules that restore β-cell viability in the presence of cytokines may have potential to augment insulin replacement therapy. However, relatively few small molecules are known to possess protective effects from cytokines.[3–6]

In a campaign to discover new therapies and probes for T1D, our group developed a phenotypic assay using the rat INS-1E β-cell line to screen for small-molecule suppressors of apoptosis in the presence of the cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ).[7] High-throughput screening identified several hits that increased β-cell viability. Subsequent medicinal-chemistry optimization of a screening hit belonging to a library derived from diversity-oriented synthesis (DOS)[8–10] led to the discovery of BRD0476 (**1**, ML187[11]) (Figure 1), a small molecule with sub-micromolar activity (EC_{50} = 0.78 μM).[12] **1** represents a novel chemotype with a highly functionalized and stereochemically rich medium-sized (8-membered) lactam ring.[13–14]

Compound **1** exhibits poor aqueous solubility, which would adversely affect the bioavailability of **1** in animals and limit its use *in vivo*. In this study, we describe our efforts

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Supporting Information. New compound characterization and procedures for compound synthesis, solubility determination, and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

toward the synthesis and biological evaluation of analogs of **1** with improved solubility. In addition, we show that the biological activity of **1** and analogs largely results from inhibiting cell-signaling pathways activated by IFN-γ.

RESULTS AND DISCUSSION

To rapidly access compound **1** and urea analogs, we developed an efficient, solution-phase synthesis of aniline **4** in four steps from **2**, a DOS-generated intermediate previously prepared using an aldol-based "build/couple/pair" strategy (Scheme 1).[12–13] Treatment of **2** with TBSOTf resulted in a protecting group switch from a *N*-Boc-carbamate to a *N*-silyl-carbamate intermediate, which was desilylated (TBAF) to afford a secondary crude amine. Direct cleavage of the Boc-carbamate to a free amine under acidic conditions was not feasible due to competing deprotection of the *p*-methoxybenzyl (PMB) ether. Capping the amine intermediate with 1,4-benzodioxan-6-sulfonyl chloride provided sulfonamide **3** in 74% isolated yield for three steps. Hydrogenation (H_2, Pd/C) of the nitro moiety generated **4** in gram quantities.

Two strategies were pursued to install the urea moiety for compound **1** and analogs. One strategy involved addition of isocyanates directly to aniline **4**. However, these reactions were sluggish and required microwave heating under basic conditions to achieve moderate yields at best. Furthermore, quinolyl isocyanates were not commericially available for the preparation of quinoline urea analogs, and their syntheses proved problematic using standard protocols for isocyanate formation. Instead, the urea moiety could be installed using a two-step protocol by subjecting **4** to diphosgene ($ClCO_2CCl_3$) to form isocyanate intermediate **5**,[15] followed by addition of various aryl and alkyl amines (Scheme 2). Isocyanate **5** did not require purification for further reactions and could be stored at −20 °C for relatively short periods of time (at least one week). Moreover, addition of amines to **5** was facile and proceeded at room temperature under neutral conditions to afford PMB-protected urea derivatives **6** in moderate to excellent yields (36–99%). Deprotection of the PMB ether with DDQ or TFA provided **1** and urea analogs **7** (see Table 1 for complete structures).

In our earlier report,[12] we determined the potency and maximum activity of compound **1** and analogs using the CellTiter-Glo viability assay with INS-1E β-cells.[16] In the present study, caspase-3/7 activity was measured as a more direct readout of apoptosis. **1** demonstrated a small protective effect with standard concentrations of cytokines in the caspase assay (condition A, Figure 2). Lowering the concentrations of IL-1β and TNF-α by 20- and 10-fold, respectively, as well as increasing IFN-γ 4-fold resulted in higher rescue efficacy of **1**, despite lowering caspase-3/7 activity for this mixture (condition B). Further increasing the concentration of IFN-γ to 500 ng/mL resulted in equal levels of cytokine-induced caspase-3/7 activation, but elicited the strongest protective effects from **1**, reducing caspase activity by 74% (condition C). Using these cytokine concentrations, **1** generated a dose-response with an IC_{50} value of 1.66 μM and a maximum activity of 71% (Table 1). We used this assay for the biological evaluation of additional analogs.

Using a thermodynamic solubility assay, the aqueous solubility of compound **1** was determined to be 0.1 μM in phosphate buffered saline (pH 7.4) (Table 1). This low solubility may be attributed to the lipophilic naphthyl moiety. In previous SAR studies, this group was found to be a crucial structural requirement in suppressing cytokine-induced β-cell apoptosis.[12] An approach to increase aqueous solubility while retaining the naphthyl moiety involved disrupting molecular planarity between the naphthyl and urea groups to lower crystal packing energy of solutes.[17] 1-Naphthylmethyl urea **7a** was designed to disrupt planarity by interrupting delocalization of the naphthyl- and urea-conjugated pi system with a methylene spacer. However, **7a** did not show enhanced solubility and was inactive.

N-Methyl-*N*-1-naphthyl urea **7b** was also designed to increase aqueous solubility by disrupting molecular planarity. The addition of the methyl group to the urea nitrogen may prevent access to planar conformations, which should be hindered by a steric interaction between the *N*-methyl and C(2)- or C(8)-hydrogen of the quinoline moiety. In fact, **7b** exhibited a 110-fold increase in solubility (11.2 μM), which correlated to a decreased melting point for **7b** (144–146 °C) compared to **1** (170–172 °C). However, **7b** was less efficacious in inhibiting apoptosis (maximum activity = 15%)

Alternatively, quinoline analogs **7c**–**i** should retain the steric properties of the naphthyl urea moiety crucial for biological activity, while increasing aqueous solubility. Introduction of nitrogen atoms would decrease the lipophilicity of the naphthyl component and provide an additional hydrogen bond acceptor that should enhance solubility.[18–19] Indeed, quinoline analogs **7c**–**i** demonstrated an increase in solubility, in which 5-quinolyl urea **7f** showed a remarkable 1400-fold increase in solubility compared to **1. 7f** was equally potent (1.62 μM) to **1** in the caspase assay but had a lower maximum activity (41%). Likewise, the exceptionally soluble 4-isoquinolyl (**7d**) and 5-isoquinolyl (**7g**) ureas exhibited similar biological activity. 1-Isoquinolyl (**7c**) and 8-quinolyl (**7i**) ureas were less soluble (0.7 and 1.8 μM, respectively) than other quinoline analogs, which may be due to hydrogen bonding of the urea hydrogen (N-H) and quinoline nitrogen. However, **7i** possessed the best activity among the quinoline analogs, inhibiting apoptosis similarly to **1** with a favorable maximum activity of 69%.

As indicated in the optimization of cytokine concentrations for the caspase-3/7 assay, compound **1** and active analogs may directly perturb cell-signaling pathways induced by IFN-γ. This cytokine activates the JAK/STAT signaling pathway,[20–21] and STAT1 has previously been shown to regulate apoptosis in INS-1E β-cells.[22–23] Binding of IFN-γ to cell surface receptors leads to activation of JAK1/JAK2, which subsequently phosphorylate cytoplasmic STAT1 at Tyr701. Upon phosphorylation, STAT1 dimerizes and translocates to the nucleus to regulate gene transcription.

When INS-1E β-cells were treated with IFN-γ, rapid phosphorylation of STAT1 at 30 min was observed by Western blotting (Figure 3). In addition to Tyr701, phosphorylation was also observed at Ser727.[20–21] Co-treatment with IFN-γ and compound **1** led to a complete reduction of STAT1 phosphorylation, while inactive analog **7a** did not result in inhibition. Further, total STAT1 levels were slightly increased by IFN-γ alone or in combination with **1** and analogs. Taken together, these results suggest that STAT1 phosphorylation may be an excellent biomarker for compound activity. To this end, the 8-quinolyl urea analog **7i** with improved aqueous solubility also significantly decreased levels of pSTAT1 in an analogous manner to **1**.

To examine the effect of inhibiting STAT1 phosphorylation on subsequent nuclear translocation, cells were treated with IFN-γ in the presence or absence of compounds, fixed, and immunofluorescence was performed to monitor subcellular localization of STAT1 (Figure 4).[24] STAT1 was located in both the cytoplasm and nucleus of untreated cells, while treatment with IFN-γ induced a substantial accumulation of STAT1 in the nucleus. Remarkably, compounds **1** and **7i** nearly abolished this nuclear localization of STAT1. As a negative control, compound **7a** did not inhibit nuclear translocation of STAT1 in the presence of IFN-γ.

CONCLUSION

In summary, we developed an efficient synthesis of compound **1** and analogs, which suppress cytokine-induced β-cell apoptosis. Quinoline-containing analogs showed improved

aqueous solubility from **1**, of which 8-quinolyl urea **7i** retained optimal biological activity. In addition, we have demonstrated that these small molecules inhibit the JAK-STAT signaling pathway by inhibiting IFN-γ-induced STAT1 phosphorylation and nuclear translocation. Studies to improve the pharmacokinetic properties of **1** and **7i**, as well as develop a quantitative immunofluorescence assay for STAT1 nuclear translocation, are ongoing and will be described in subsequent reports.

EXPERIMENTAL SECTION

Chemistry

The purity of all compounds was >95% as determined by UPLC-MS. The synthesis of **3**, **4**, and **7i** is described below. See Supporting Information for detailed syntheses and spectral characterization of all compounds.

N-(((2*R*,3*R*)-5-((*S*)-1-((4-methoxybenzyl)oxy)propan-2-yl)-3-methyl-10-nitro-6-oxo-3,4,5,6-tetrahydro-2*H*-benzo[*b*][1,5]-oxazocin-2-yl)methyl)-*N*-methyl-2,3-dihydrobenzo[*b*][1,4]dioxine-6-sulfonamide (3)—TBSOTf (1.98 mL, 8.61 mmol) was added dropwise to a solution of *N*-Boc-protected amine **2**[12] (1.6 g, 2.87 mmol) and 2,6-lutidine (1.35 mL, 11.59 mmol) in CH_2Cl_2 (29 mL) at rt. The reaction mixture was stirred at rt for 1h, diluted with sat. NH_4Cl, and extracted into CH_2Cl_2. The organic layers were dried over $MgSO_4$, filtered, and concentrated *in vacuo* to provide a *N*-silyl carbamate intermediate. LCMS (ESI+) m/z: 616.39 (M+H). This crude intermediate was dissolved in THF (57 mL) and cooled to 0 °C. To the resulting solution was added AcOH (0.181 mL, 3.16 mmol) followed by dropwise addition of TBAF (1.0 M in THF, 3.16 mL, 3.16 mmol). The reaction mixture was further stirred for 30 min at 0 °C, diluted with sat. NH_4Cl, and extracted into EtOAc. The organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to provide a secondary amine. LCMS (ESI+) m/z: 458.41 (M+H). This crude intermediate and 2,6-lutidine (1.67 mL, 14.35 mmol) were dissolved in CH_2Cl_2 (29 mL), and 1,4-benzodioxan-6-sulfonyl chloride (808 mg, 3.44 mmol) was added to the resulting solution at rt. The reaction mixture was further stirred for 20 h at rt, diluted with sat. NH_4Cl, and extracted into CH_2Cl_2. The organic layers were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. Purification on silica gel (gradient of 10–50% EtOAc in hexanes) provided 1.39 g (2.12 mmol, 74% yield for 3 steps) of **3** as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.86 (dd, *J* = 8.1, 1.2 Hz, 1H), 7.70 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.30-7.21 (ovrlp m, 5H), 6.95 (d, *J* = 8.4 Hz, 1H), 6.84 (d, *J* = 8.7 Hz, 2H), 4.55 (d, *J* = 11.7 Hz, 1H), 4.47 (d, *J* = 11.7 Hz, 1H), 4.39 (m, 1H), 4.29 (m, 4H), 3.90-3.77 (ovrlp m, 3H), 3.77 (ovrlp s, 3H), 3.66 (dd, *J* = 9.9, 4.5 Hz, 1H), 3.38-3.20 (ovrlp m, 2H), 3.11 (d, *J* = 12.6 Hz, 1H), 2.81 (s, 3H) 2.70 (m, 1H), 1.38 (d, *J* = 6.9 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 3H); LCMS (ESI+) m/z: 656.38 (M+H).

***N*-(((2*R*,3*R*)-10-amino-5-((*S*)-1-((4-methoxybenzyl)oxy)propan-2-yl)-3-methyl-6-oxo-3,4,5,6-tetrahydro-2*H*-benzo[*b*][1,5]oxazocin-2-yl)methyl)-*N*-methyl-2,3-dihydrobenzo[*b*][1,4]dioxine-6-sulfonamide (4)—**Under hydrogen (H_2) atmosphere, a solution of **3** (1.39 g, 2.12 mmol) and palladium (10% on activated carbon, 226 mg, 0.212 mmol) in EtOH (42 mL) was stirred at 35°C for 3.5 h. The reaction mixture was cooled, filtered, and washed through a pad of celite, and concentrated *in vacuo*. Purification on silica gel (gradient of 10–60% EtOAc in hexanes) provided 1.21 g (1.93 mmol, 91% yield) of **4** as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34-7.26 (ovrlp m, 4H), 7.02 (d, *J* = 8.4 Hz, 1H), 6.97 (d, *J* = 7.5 Hz, 1H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.79 (d, *J* = 7.8 Hz, 2H), 4.67 (m, 1H), 4.54 (d, *J* = 11.4 Hz, 1H), 4.47-4.43 (ovrlp m, 3H), 4.36-4.33 (ovrlp m, 4H), 3.81-3.74 (ovrlp m, 2H), 3.79 (ovrlp s, 3H), 3.67 (dd, *J* = 10.2, 4.5 Hz, 1H), 3.49 (dd, *J* = 15.6, 10.5 Hz, 1H), 3.30 (d, *J* = 13.8 Hz, 1H), 3.06 (d, *J* = 15.0 Hz, 1H), 2.94-2.86 (ovrlp m, 1H), 2.90

(ovrlp s, 3H), 2.01 (m, 1H), 1.32 (d, *J* = 6.9 Hz, 3H), 0.83 (d, *J* = 6.9 Hz, 3H); HRMS (ESI^+) m/z calculated for $C_{32}H_{39}N_3O_8SNa$ (M+Na): 648.2356, found 648.2352.

***N*-(((2*R*,3*R*)-5-((*S*)-1-hydroxypropan-2-yl)-3-methyl-6-oxo-10-(3-(quinolin-8-yl)ureido)-3,4,5,6-tetrahydro-2*H*-benzo[*b*][1,5]oxazocin-2-yl)methyl)-*N*-methyl-2,3-dihydrobenzo[*b*][1,4]dioxine-6-sulfonamide (7i)—**To a solution of disphosgene (11 μL, 0.095 mmol) in CH_2Cl_2 (0.5 mL) at 0°C was added dropwise a solution of aniline **4** (100 mg, 0.160 mmol) and proton sponge (67 mg, 0.314 mmol) in CH_2Cl_2 (0.5 mL). The reaction mixture was warmed to rt, stirred for 15 min, and concentrated *in vacuo*. The residue was redissolved in CH_2Cl_2, washed with 1M HCl and 1M NaOH, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to provide 120 mg (0.184 mmol, 96% yield) of isocyanate **5**. LCMS (ESI^+) m/z: 652.25 (M+H). Crude **5** (80 mg, 0.123 mmol) and 8-aminoquinoline (89 mg, 0.615 mmol) were dissolved in toluene (2.1 mL) and stirred at rt for 2.5 h. The reaction mixture was concentrated *in vacuo* and directly purified on silica gel (gradient of 0–5% MeOH in CH_2Cl_2) to provide 88 mg (0.111 mmol, 90% yield) of quinolyl urea **6i**. LCMS (ESI^+) m/z: 796.37 (M+H). To a solution of **6i** (53 mg, 0.067 mmol) in CH_2Cl_2 (2.3 mL) was added TFA (1.1 mL) dropwise at rt. The reaction mixture was further stirred at rt for 15 min, concentrated *in vacuo*, and directly purified on silica gel (gradient of 0–5% MeOH in CH_2Cl_2) to provide the 39 mg of **7i** (0.058 mmol, 87% yield). ^1H NMR (300 MHz, $CDCl_3$) δ 9.63 (s, 1H), 8.86 (d, *J* = 2.7 Hz, 1H), 8.49 (d, *J* = 7.2 Hz, 1H), 8.27 (d, *J* = 8.1 Hz, 1H), 8.18 (s, 1H), 8.07 (dd, *J* = 6.6 Hz, 3.0 Hz, 1H) 7.53 (ovrlp m, 3H), 7.32 (d, *J* = 1.8 Hz, 1H), 7.26 (ovrlp dd, *J* = 8.1 Hz, 1.5 Hz, 1H), 7.16 (ovrlp m, 2H), 6.85 (d, *J* = 8.4 Hz, 1H), 5.65 (br s, 1H), 4.22 (ovrlp m, 4H), 4.10 (m, 1H), 3.78 (dd, *J* = 11.1 Hz, 2.1 Hz, 1H), 3.79-3.47 (ovrlp m, 4H), 3.31 (d, *J* = 13.5 Hz, 1H), 3.02 (ovrlp d, *J* = 15.6 Hz, 1H), 2.95 (ovrlp s, 3H), 2.26 (m, 1H), 1.41 (d, *J* = 6.9 Hz, 3H), 0.83 (d, *J* = 6.3 Hz, 3H); HRMS (ESI^+) m/z calculated for $C_{34}H_{38}N_5O_8S$ (M+H): 676.2441, found 676.2441.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

JAK	Janus kinase
STAT	signal transducer and activation of transcription
T1D	type-1 diabetes

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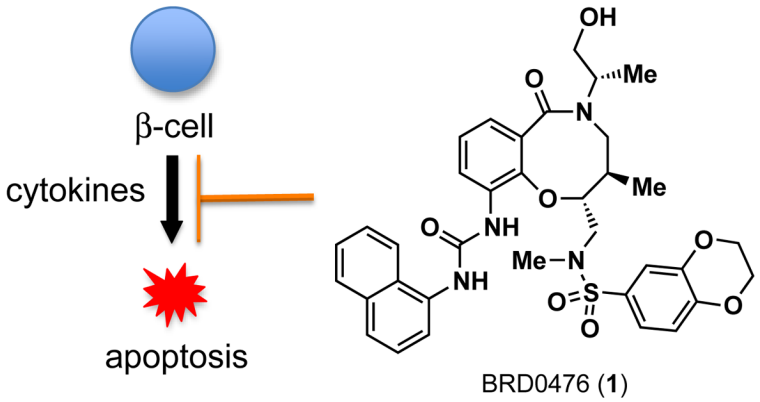


Figure 1.
DOS-generated BRD0476 (**1**) suppresses cytokine-induced β-cell apoptosis.

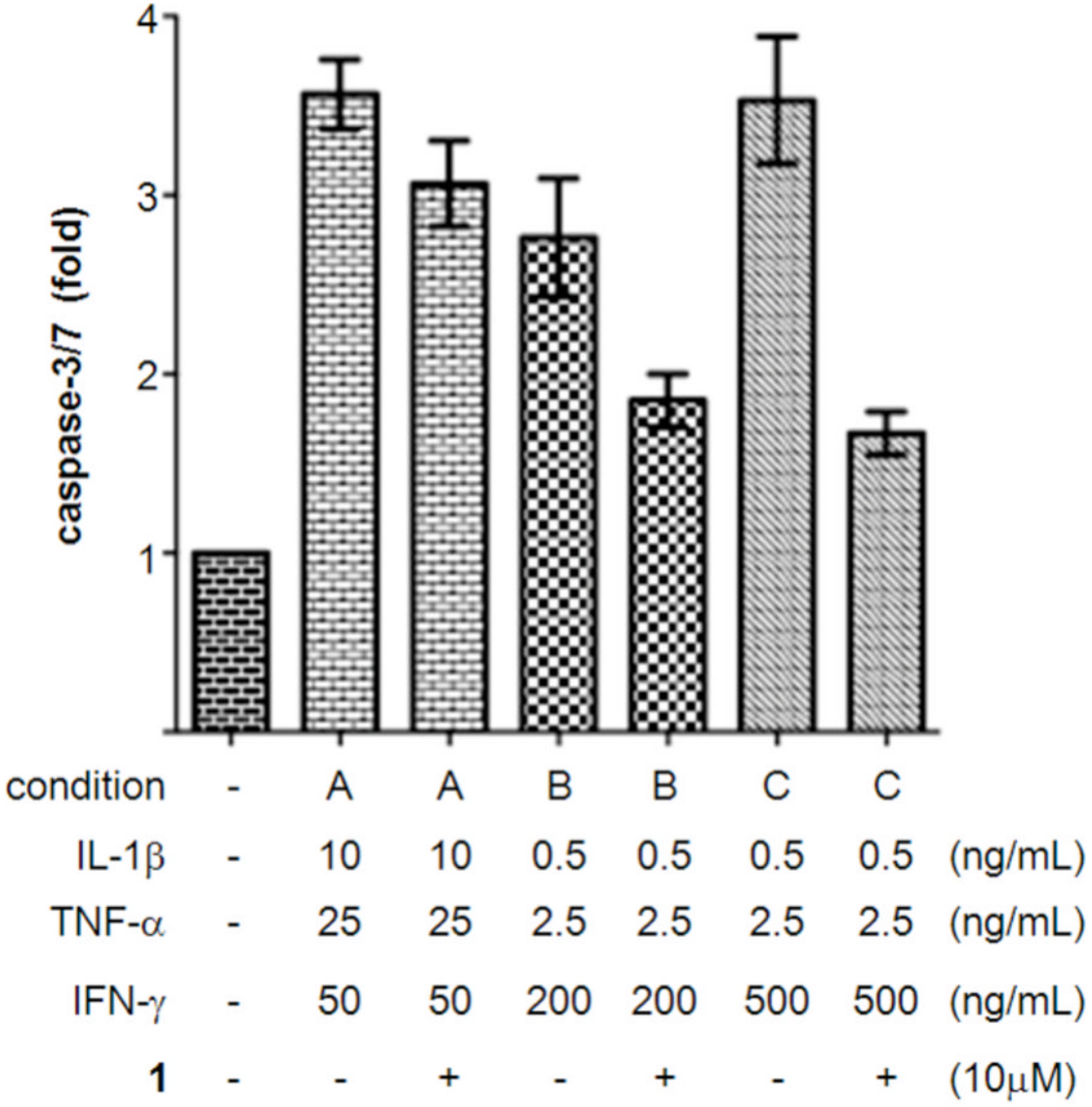


Figure 2.
Optimization of cytokine concentrations for the caspase-3/7 assay in INS-1E β-cells. Effects of cytokines and compounds were measured after 48 h. Data represent the mean ± standard error for three independent experiments with six replicates.

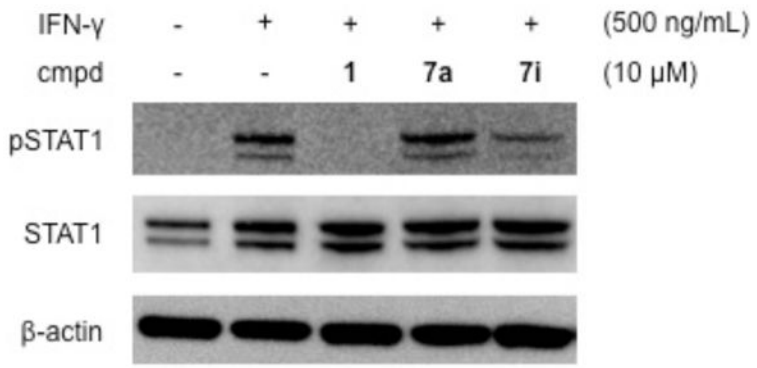


Figure 3.
Regulation of STAT1 phosphorylation by IFN-γ, **1**, and analogs in INS-1E β-cells. Effects of cytokines and compounds were analyzed by Western blot after 30 min.

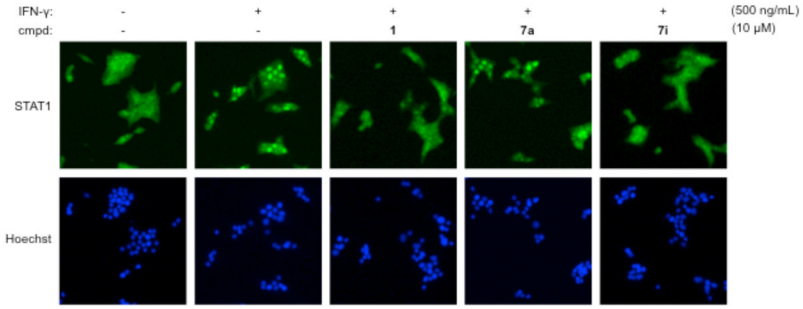
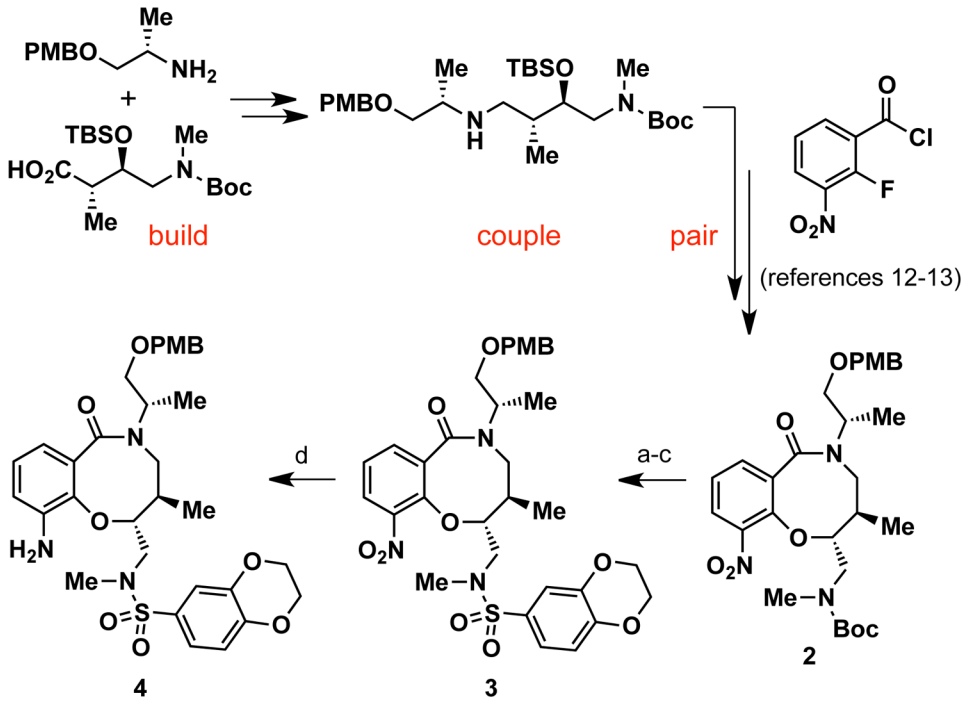


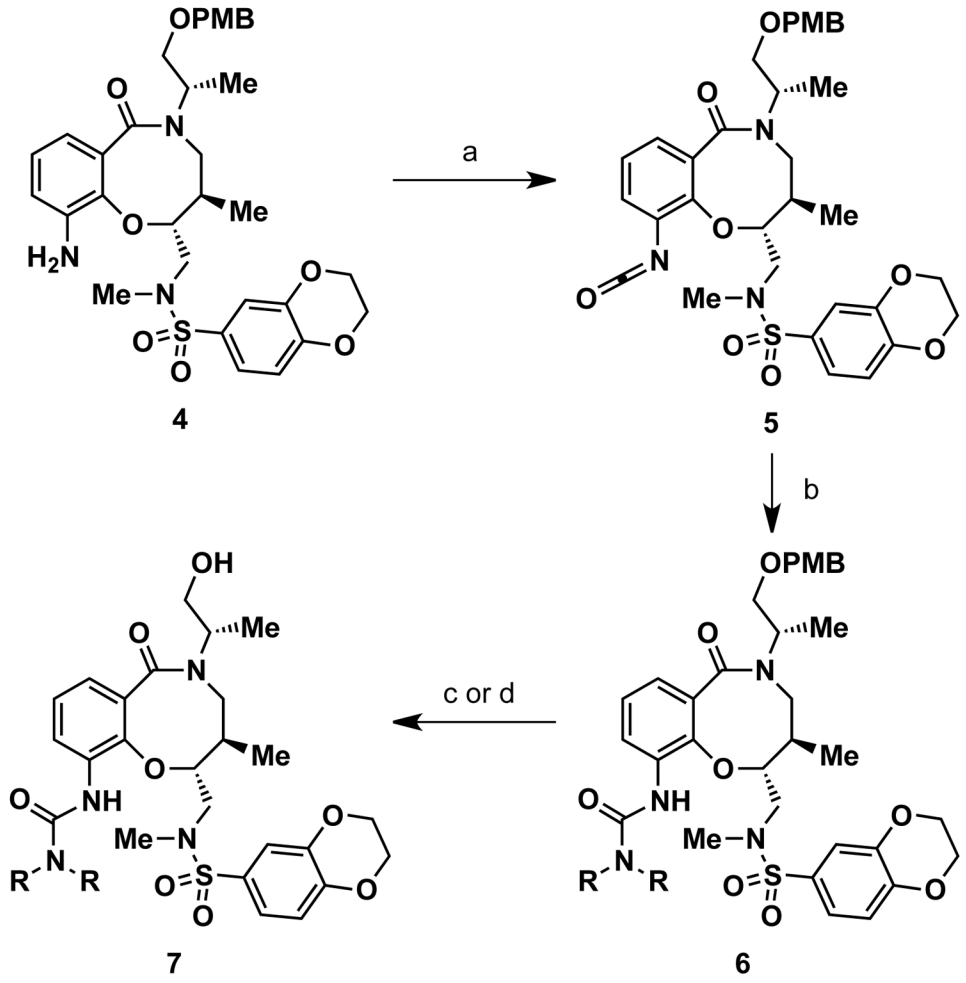
Figure 4.

Regulation of STAT1 nuclear translocation by IFN-γ, **1**, and analogs in INS-1E β-cells. Effects of cytokines and compounds were assessed after 30 min by immunofluorescence imaging.



Scheme 1. Synthesis of advanced aniline intermediate 4.[a]

[a]Reagents and conditions: (a) TBSOTf, 2,6-lutidine, CH_2Cl_2; (b) TBAF, AcOH, THF; (c) 1,4-benzodioxan-6-sulfonyl chloride, 2,6-lutidine, CH_2Cl_2, 74% yield (3 steps); (d) H_2, Pd/C, EtOH, 91% yield.

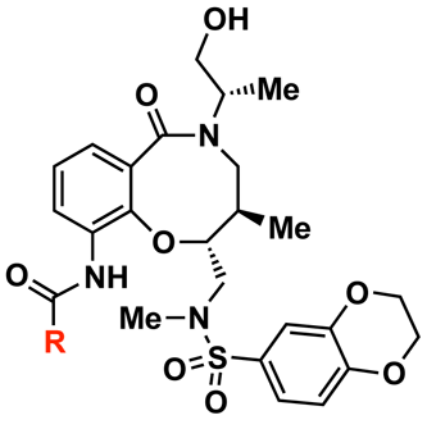


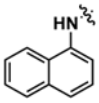
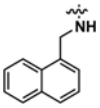
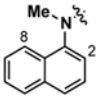
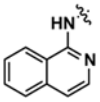
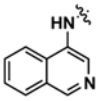
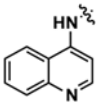
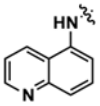
Scheme 2. General synthesis of compound 1 and urea analogs.[a]

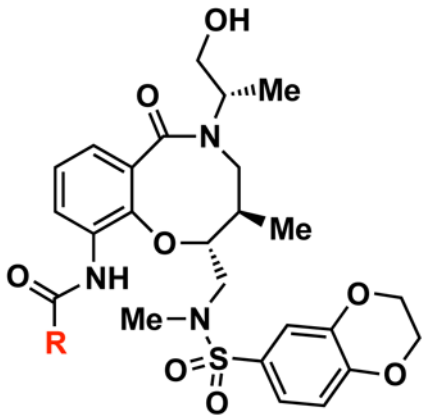
[a]Reagents and conditions: (a) diphosgene ($ClCO_2CCl_3$), proton sponge, CH_2Cl_2, 96% yield; (b) R_2NH, PhMe, 36–99% yield; (c) DDQ, pH 7.4 buffer, CH_2Cl_2, 63% and 50% yield (for **1** and **7b**, respectively); (d) TFA, CH_2Cl_2, 44–87% yield (for **7a**,**c**–**i**).

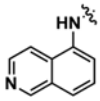
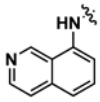
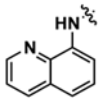
Table 1

SAR and solubility for compound 1 and analogs.



cmpd	R	IC_{50} (μM)[a]	maximum activity (%)[b]	solubility (μM)[c]
1		1.66	71	0.1
7a		>20	n.d.[d]	0.1
7b		4.74	15	11.2
7c		1.05	21	0.7
7d		4.10	31	84.9
7e		>20	n.d.	30.3
7f		1.62	41	138.5



cmpd	R	IC_{50} (μM)[a]	maximum activity (%)[b]	solubility (μM)[c]
7g		4.65	59	60.5
7h		n.d.	n.d.	101.3
7i		3.26	69	1.8

[a] IC_{50} values were determined using three replicates in the caspase-3/7 assay in INS-1E β-cells.

[b] Maximum activity is reported as % rescue from cytokine-induced caspase-3/7 activation.

[c] Solubility was measured in a phosphate buffered saline solution (pH 7.4).

[d] n.d., not determined.