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Synthesis and biological evaluation of lisofylline (LSF) analogs as a potential treatment for Type 1 diabetes

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Abstract—Lisofylline (LSF, 1-(5-*R*-hydroxyhexyl)-3,7-dimethylxanthine) is an anti-inflammatory agent that protects β-cells from Th1 cytokine-induced dysfunction and reduces the onset of Type 1 diabetes in non-obese diabetic (NOD) mice. Due to its low potency, poor oral bioavailability, and short half-life, the widespread clinical utility of LSF may be limited. Our goal has been to develop new agents based on the LSF structural motif that resolve the potency and pharmacokinetic liabilities of LSF. In this study, we have generated a focused library of LSF analogs that maintain the side chain (5-*R*-hydroxyhexyl) constant, while substituting a variety of nitrogen-containing heterocyclic substructures for the xanthine moiety of LSF. This library includes the xanthine-like (5-aza-7-deazaxanthine), as well as non-xanthine-like skeletons. The LSF analogs were evaluated in a pancreatic β-cell line for the effects on apoptosis protection and insulin release. The metabolic stability of selected compounds was also tested.

Type 1 diabetes, a common and widespread disease occurring in every part of the world, is an autoimmune disorder that results from the immune-mediated inflammatory destruction of insulin-producing β-cells in pancreatic islets. There are an estimated 500,000 to 1 million people with Type 1 diabetes in the US today. Although the specific pathogenic mechanisms in Type 1 diabetes are not defined, it is clear that activated T cells and macrophages are required for the initiation. Once activated, macrophages secrete several inflammatory cytokines, such as interleukin 1β (IL-1β), interleukin 12 (IL-12), and tumor necrosis factor α (TNF-α), and trigger interferon-γ (IFN-γ) production from activated T cells.[1] These cytokines are cytotoxic to β-cells by inducing the formation of oxygen free radicals, nitric oxide, and lipid peroxides within β-cells and enhance Th1-cell-mediated inflammatory responses, which are responsible for β-cell destruction. [2]

Lisofylline (LSF) (Fig. 1), 1-(5-*R*-hydroxyhexyl)-3,7-dimethylxanthine, is a novel anti-inflammatory compound that was originally used to reduce the incidence of graft vs. host disease and to prevent the onset of experimental autoimmune encephalomyelitis by blocking IL-12-induced T helper 1 differentiation.[3,4] It abrogated release of inflammatory cytokines during oxidative lung injury and reduced inflammatory cytokine release in response to cytotoxic cancer chemotherapy.[5] LSF also showed beneficial effects in several inflammatory disorders such as sepsis, hypoxia, and hemorrhagic organ injury.[6,7] LSF has also been shown to protect β-cells from multiple inflammatory cytokine-mediated injuries by its ability to maintain insulin secretory capability and cell viability.[2] Although not established, the mechanism of LSF-induced protection may be due to promotion of β-cell mitochondrial metabolism, normalizing mitochondrial membrane potential and stimulating energy production. This unique spectrum of activity suggests that agents such as LSF could have clinical utility in preventing β-cell damage during the development of Type 1 diabetes or after islet cell transplantation. This hypothesis was supported by studies that showed LSF could signifi-

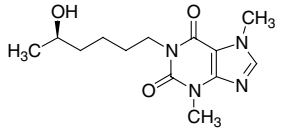

Figure 1. Structure of Lisofylline (LSF, (1-(5-*R*-hydroxyhexyl)-3,7-dimethylxanthine)).

Keywords: Type 1 diabetes; Lisofylline; LSF; LSF analogs; 5-Aza-7-deazaxanthine.
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cantly reduce spontaneous Type 1 diabetes development in the non-obese diabetic (NOD) mouse.[1]

LSF has disadvantages that may limit its clinical development: LSF exhibits low (to non-existent) oral bioavailability, has an extremely short half-life, requiring constant intravenous infusion in humans, and has relatively low potency for direct islet protective effects, requiring concentrations of 20 μM in vitro and at least 25 mg/kg body weight in rodents to see beneficial effects. In efforts directed at enhancing the potency of LSF, Klein et al.[8] prepared a library of LSF analogs based on the xanthine framework and studied the SAR for suppression of Th1 differentiation through IL-12 blockade. Despite considerable structural variation, only relatively small differences in activity were observed among the analogs studied. Importantly, it was found that the enantiomeric *S*-LSF (which possesses the *S*-alcohol configuration) was inactive, implicating a highly specific interaction of the *R* isomer with a binding point on the target site.

The aim of our investigation was to synthesize a focused library of small molecules based on the LSF structure to elucidate the SAR for potency and pharmacokinetic parameters. In one approach, we synthesized analogs of the 5-aza-7-deazaxanthine family (Fig. 2). The 5-aza-7-deazaxanthine structure is isosteric with xanthine, but has a distinct electronic character as evidenced by its lower basicity and its photophysical properties.[9] In a second approach, we substituted a series of nitrogen-containing heterocyclic substructures for the xanthine moiety of LSF.

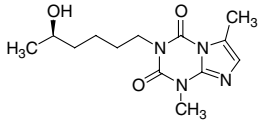
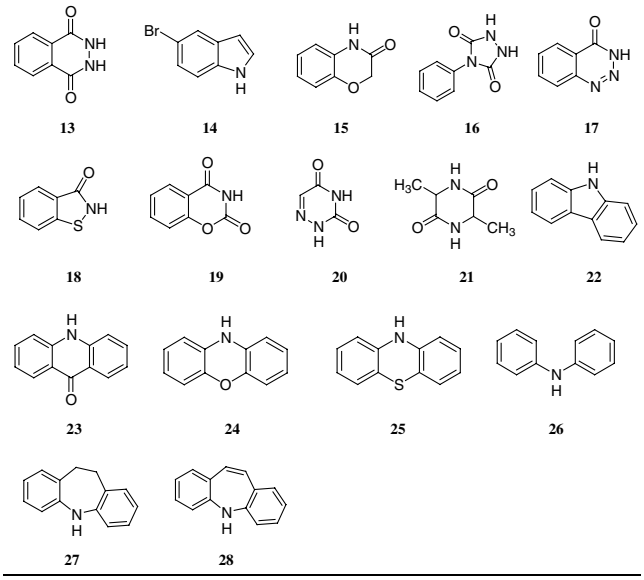
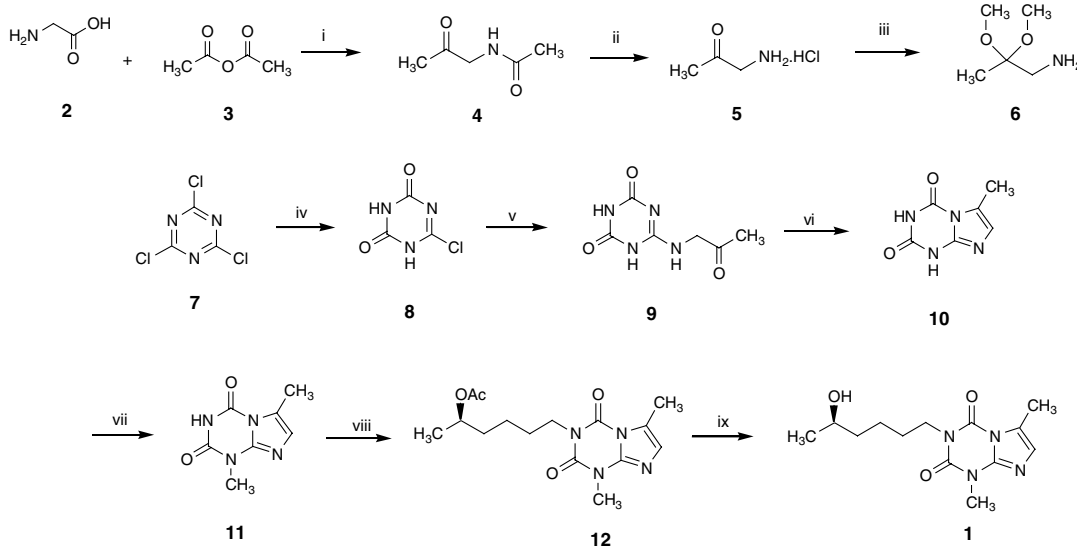


Figure 2. 5-Aza-7-deazaxanthine LSF (5,7-isoLSF) **1**.

The synthetic route to 5-aza-7-deazaxanthine-LSF **1** (Scheme 1) commenced with coupling of glycine **2** and acetic anhydride **3** in presence of pyridine affording acetamidoacetone **4**,[10] which was hydrolyzed in HCl to form aminoacetone hydrochloride **5**. The ketone was then transformed to its acetal by treatment with a mixture of trimethyl orthoformate and methanol in the presence of *p*-toluenesulfonic acid. The free amine **6** was obtained by treatment with sodium hydroxide.[11] Compound **6** was ready for later reactions with a protected carbonyl group and a free

Table 1. The selected heterocyclic compounds being investigated



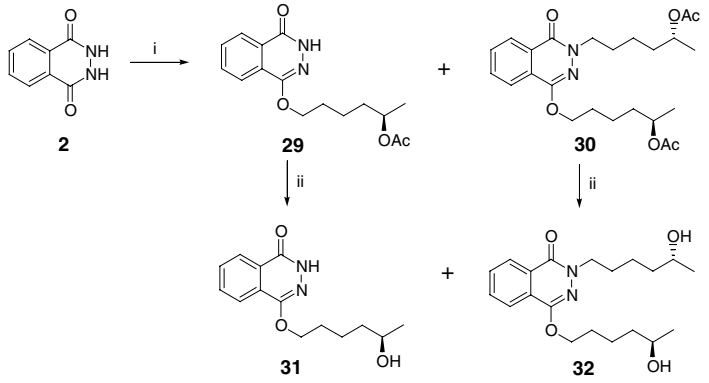


Scheme 1. Reagents and conditions: (i) pyridine, reflux, 6 h, 78%; (ii) HCl, H_2O, reflux, 6 h, 60%; (iii) trimethylorthoformate, MeOH, *p*-toluenesulfonic acid monohydrate, reflux 24 h, then 3 N NaOH, 49%; (iv) NaOH, H_2O; (v) **6**, H_2O, reflux, 3 h, 17%; (vi) H_2SO_4, 95 °C, 1.5 h, 35%; (vii) NaOH, CH_3I, H_2O, acetone, rt, 24 h, 34%; (viii) 5-(*R*)-acetoxy-1-chlorohexane, NaH, DMSO, 70–80 °C, overnight; (ix) 1 M HCl in ether, MeOH, rt, 12 h, 60% two steps.

amine. Compound **8** was prepared by controlled hydrolysis of cyanuric chloride **7** with sodium hydroxide in aqueous solution.[12] Condensation of compound **8** with compound **6** in aqueous media at reflux temperature furnished compound **9** and the hydrolysis of the protecting acetal groups was achieved in situ. The ring annulation was accomplished by heating with concentrated sulfuric acid.[13] Compound **10** was methylated with methyl iodide in basic solution to give **11**, which was then coupled with 5-(*R*)-acetoxy-1-chlorohexane[14] followed by removing the acetate group to afford compound **1**.

In our second study, the side-chain moiety (5-*R*-hydroxyhexyl) was held constant, while a series of nitrogen-containing heterocyclic scaffolds were substituted for the xanthine substructure (Table 1). The LSF analogs were synthesized by coupling the heterocyclic compounds with 5-(*R*)-acetoxy-1-chlorohexane, followed by hydrolysis of the resulting acetates. For the synthesis of LSF analogs based on phthalhydrazide **13** (Scheme 2), the byproduct **32** was also isolated. This compound proved valuable in elucidating the structure of **31** by demonstrating that alkylation occurred on the oxygen.

We investigated the effects of LSF analogs on β-cells in the mouse insulin-secreting INS-1 cell line.[15] Evidence showed that LSF and its analogs could protect β-cells from cytokine-induced cell death. INS-1 cells were treated with a combination of recombinant IL-1β, INFγ, and TNFα with 10^{-5}–10^{3} μM LSF and its analogs. At a concentration of 10^{-2} μM, LSF showed a maximum protective effect (Fig. 3a). There was no further increase in β-cell protection when the LSF concentration was increased from 10^{-2} to 10^{2} μM. Most of the LSF



Scheme 2. Reagents and conditions: (i) NaH, DMF, then 5-(*R*)-acetoxy-1-chlorohexane, tetrabutylammonium iodide, 70–80 °C, overnight; (ii) HCl (1 M in ether), MeOH, rt, overnight, **31**, 22%, two steps.

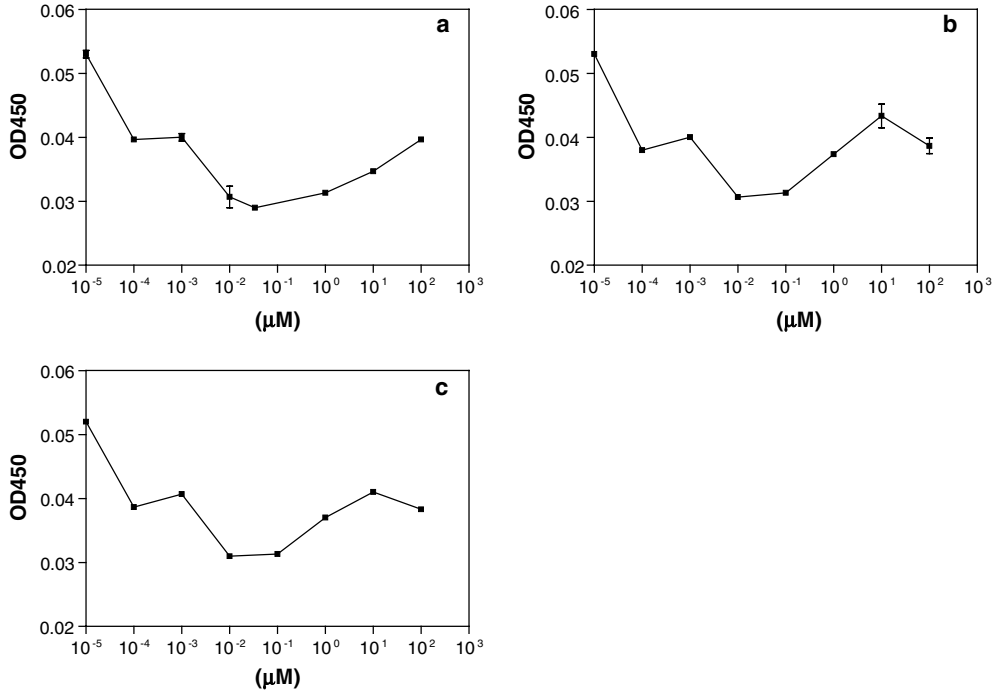


Figure 3. β-cell protection in INS-1 cells: (a) LSF, (b) compound **1**, and (c) compound **31**.

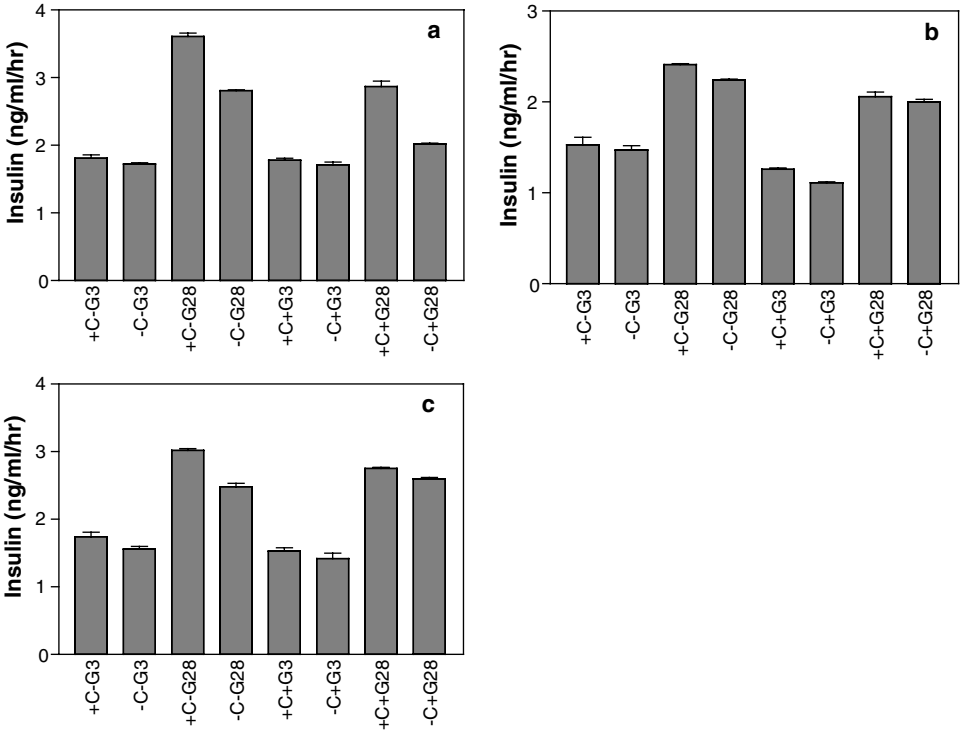


Figure 4. Insulin secretion in INS-1 cells: (a) LSF, (b) compound **1**, and (c) compound **31**.(+C−G3 = w/compound, w/o cytokines, 3 mM glucose; −C−G3 = w/o compound, w/o cytokines, 3 mM glucose; +C−G28 = w/compound, w/o cytokine, 28 mM glucose; −C−G28 = w/o compound, w/o cytokine, 28 mM glucose; +C+G3 = w/ compound, w/cytokines, 3 mM glucose; −C+G3 = w/o compound, w/ cytokines, 3 mM glucose; +C+G28 = w/compound, w/cytokines, 28 mM glucose; −C+G28 = w/o compound, w/cytokines, 28 mM glucose).

analogs showed some protective effects on β-cells (data not shown), especially compounds **1** (Fig. 3b) and **31** (Fig. 3c), which proved effective at low concentrations. Both of these two compounds showed comparative activity with LSF at concentration of 10^{-2} μM. No further increases in β-cell protection were observed when analog concentrations were increased.[16]

We also examined the effects of LSF and selected analogs on insulin secretion in INS-1 cells with and without inflammatory cytokines.[17] A maintenance in basal (3 mM) and glucose-stimulated (28 mM) insulin release was observed by 20 μM of LSF and its analogs (Fig. 4).

Table 2 shows results of metabolic stability experiments for compounds **1** and **31**. This assay evaluated the stability of 1 M concentrations of **1** and **31** in human liver microsomes at pH 7.4. Compound levels were measured by LC/MS. Three reference drugs were used: warfarin (high stability), propranolol (lower stability), and testosterone (poor stability). Both of the compounds showed acceptable stability values under these in vitro conditions. These results are relevant to our search for longer lived LSF analogs, since LSF is completely metabolized by the liver with half-life of less than 3 min in vivo.

Table 2. Metabolic stability of LSF analogs

	Warfarin	Propranolol	Testosterone	**1**	**31**
Metabolic stability % remaining	105	34	9	82	75

In summary, we have synthesized a series of LSF analogs that maintain the 5(*R*)-hydroxyhexyl side chain, while varying the xanthine core substructure. Two lead compounds **1** and **31** have been identified that protect β-cells from cytokine-induced injury and maintain insulin secretory capability. In addition, these lead compounds showed metabolic stability in vitro. Ongoing studies of the pharmacokinetic profiles of these molecules will determine if the goal of generating an LSF analog with improved potency, metabolic stability, and oral bioavailability has been achieved.

Acknowledgment

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15. Cells from passages 10–30 were maintained in RPMI 1640 medium(LifeTechnologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated FBS, 10 mm HEPES, 200 μm L-glutamine, 1 mm sodium pyruvate, 5 nm 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin at pH 7.4. The cells were cultured at 37 °C in a humidified incubator supplied with 5% carbon dioxide. Fresh medium was replaced every 2 days. The cells were plated at a density of 105/cm^2. Culture vessels were coated with poly-D-lysine and gelatin (Sigma, St. Louis, MO) to retain detached and dead cells so that seeding cell numbers reflect the actual cell numbers after all treatment conditions. INS-1 cells were treated with the combination of recombinant mouse IL-1β (5 ng/ml), IFNγ (100 ng/ml), and TNFα (10 ng/ml; R&D Systems, Inc., Minneapolis, MN) suspended in complete RPMI medium. LSF (provided by Cell Therapeutics, Inc., Seattle, WA) and analogs were added simultaneously with the cytokines in complete RPMI medium. All treatments were performed for 18 h.
16. Beta cells were treated with apoptosis detecting dye for 2–3 h at room temperature. Apoptotic cells were recognized with purple-red color under a microscope. After washing to eliminate free dye and adding dye release reagent, color density was quantified by reading at OD 450 nM.
17. At the end of treatment, cells were washed with Krebs–Ringer-bicarbonate–HEPES buffer (KRB) containing 134 mm NaCl, 4.7 mm KCl, 1.2 mm KH_2PO_4, 1.2 mm $MgSO_4$, 1.0 mm $CaCl_2$, 10 mm HEPES, and 0.1% BSA at 37 °C, pH 7.4. They were preincubated in the same buffer for 30 min, followed by 60-min incubation in KRB supplemented with 15 mM D-glucose (J. T. Baker, Phillipsburg, NJ). The supernatant was harvested and subjected to centrifugation to eliminate residue cells. Insulin secreted into the supernatant was measured by RIA with mouse insulin as a standard.