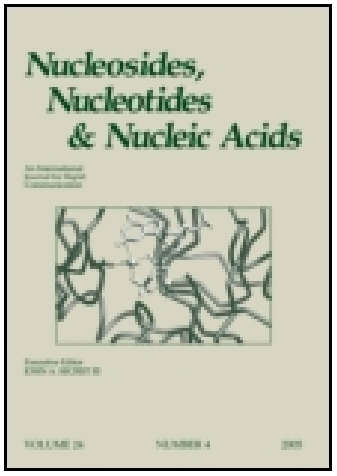

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GUANOSINE ANALOG IN THE PYRIDO[2,3-d]PYRIMIDINE RING SYSTEM AS A POTENTIAL TOLL-LIKE RECEPTOR AGONIST

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□ *The synthesis of a guanosine analog in the pyrido[2,3-d]pyrimidine ring system has been accomplished by glycosylation of the preformed aromatic heterocyclic base, which was prepared in 2 steps by condensation of methyl acrylate with guanidine carbonate and methyl cyanoacetate in the presence of sodium methoxide, followed by dehydrogenation. The analog was evaluated in vitro for its ability to modulate the innate immune response by acting as an agonist or as an antagonist of Toll-like receptor (TLR) signaling by measuring cytokine induction or inhibition of induction, respectively, in mouse bone marrow-derived macrophages. Despite its structural similarity to 7-thia-8-oxoguanosine, a known TLR7 agonist, the analog was found to antagonize TLR7-induced cytokine induction in this cell-based assay.*

Keywords Innate immunity; Guanosine analog; Pyrido[2,3-d]pyrimidine; Toll-like receptor agonist

INTRODUCTION

In the mid-1980s, our laboratory and others began studying a group of synthetic nucleosides that had the unique ability to activate the innate immune response. These compounds were mainly derivatives and analogs of guanosine. Structure-activity studies within this class showed that certain compounds in the thiazolo[4,5-d]pyrimidine,[1] pyrazolo[3,4-d]pyrimidine,[2] purine,[3] 7-deazapurine,[4] and 9-deazapurine[5] ring systems were all active. Examples in each group were found to be potent antiviral agents in mouse models due to their ability to rapidly induce the production of interferon.[4,6,7] Among these guanosine analogs, 7-thia-8-oxoguanosine (TOG, Figure 1) was studied in greatest detail and shown to exhibit broad spectrum antiviral activity and to activate natural killer cells, macrophages, and B-lymphocytes.[8] Other guanosine analogs

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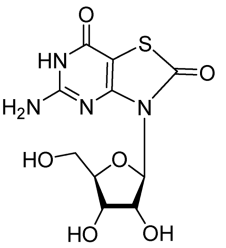
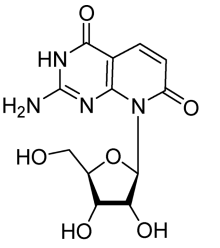


7-thia-8-oxoguanosine
TOG



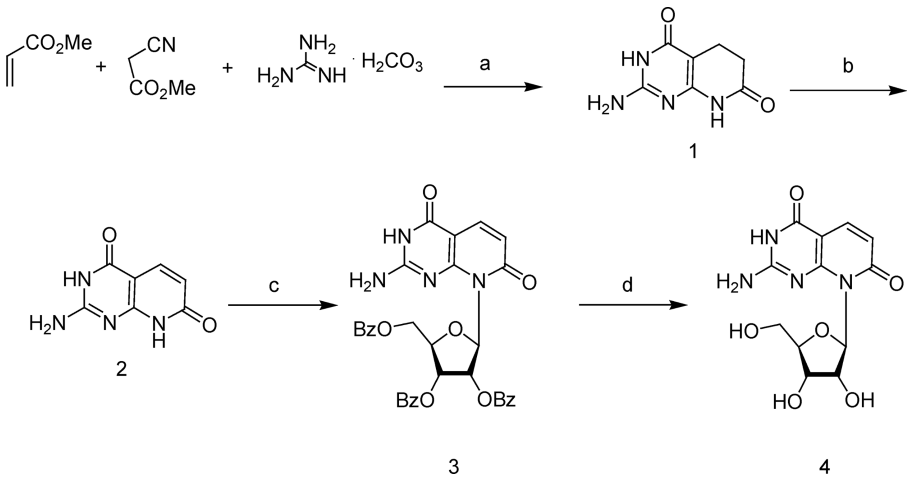
Pyrido[2,3-d]pyrimidine analog

FIGURE 1 Structural features of guanosine analog.

extensively studied were 7-deazaguanosine[9] and 7-allyl-8-oxoguanosine (loxoribine).[10] The exact mechanism of immune potentiation by these guanosine-like compounds had not been elucidated, and we were prompted to investigate the possibility that these small molecules could be acting via signaling through one or more of the known TLRs. As a result of those studies, we recently showed that TOG and certain other guanosine analogs activate immune cells via TLR7.[11] Thus, compounds of this type might be clinically useful in immune-based therapy or prophylaxis against viral diseases and other infectious diseases and cancer. Indeed, TOG, also known as isatoribine, recently has been reported to be effective at reducing the plasma virus concentration in patients with chronic hepatitis C virus (HCV) infections[12] with minimal side effects. Our own recent studies provide evidence that TLR7-mediated immunity against HCV involves at least 2 mechanisms: one depends on type 1 interferon production by leukocytes, and the other is mediated by TLR7 expressed by virally infected hepatocytes.[13]

As part of our ongoing studies in the design and preparation of agonists of TLR signaling, we investigated the guanosine analog in the pyrido[2,3-d]pyrimidin-7-one ring system, which may be considered bioisosteric with TOG, since a ring sulfur atom may be replaced by a carbon-carbon double bond.[14,15] A review of the literature revealed no reports of the fully aromatic guanine base analog or any nucleosides thereof. However, the preparation of this analog was facilitated by the recent report[16] of the one-pot preparation of the dihydro derivative 2-amino-5,6-dihydropyrido[2,3-*d*]pyrimidine-4,7(3*H*,8*H*)-dione (**1**) from guanidine carbonate, methyl acrylate and methyl cyanoacetate (Scheme 1).

Dehydrogenation of compound **1** using 10% palladium on carbon in acetic acid gave a good yield of the aromatic guanine analog (**2**). Glycosylation of **2** was accomplished by a Lewis acid-catalyzed Vorbruggen-type procedure using TMS triflate as the catalyst and 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribfuranose as the glycon to yield the protected ribonucleoside **3**. Deprotection of **3** using sodium methoxide in methanol gave the desired guanosine analog **4**. The structure assignment of **4** as the N-8 β isomer was confirmed by proton NMR and UV spectra. The NMR spectrum of **4** in



SCHEME 1 Reagents and conditions: a) NaOMe / MeOH, Reflux, 36 hours **b**) Pd/C (10%) / HOAc, Reflux, 30 hours **c**) 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose, HMDS/TMStriflate/CH_3CN, 78°C, 18 hours **d**) NaOMe/MeOH, RT, 36 hours.

deuterated methanol displayed a doublet at 7.00 ppm of 4.0 Hz coupling constant, characteristic of the anomeric proton for a nucleoside of this ring system. Similar downfield chemical shifts for the anomeric proton have been observed for the adenosine analog in this ring system.[17] The small coupling constant of this doublet is consistent with ribonucleosides of the β configuration.[18] In addition, the UV spectrum of **4** resembles that of the corresponding base **2**, especially considering the absorption maxima at longer wavelengths. It has been shown previously for 7-deazapurines,[19] purines, and 7-thia-8-oxopurines[1] that substitution at N-9 (purine numbering, analogous to **4**) results in a slight hypsochromic or no shift relative to the aglycon, whereas substitution at N-3 (purine numbering, analogous to N-1 of the pyridopyrimidine system) leads to a bathochromic shift relative to the aglycon. Thus, the site of glycosylation and configuration of the product (**4**) are N-8 and β, respectively.

The guanosine analog **4** was first evaluated in vitro for its ability to stimulate the innate immune response by acting as an agonist of Toll-like receptor (TLR) signaling by measuring cytokine induction in mouse bone marrow-derived macrophages. Cells were cultured in the presence or absence of compound **4** and then assayed for the production of certain cytokines (IL-6, TNFα, IL-12, interferon, etc.). In this assay, no significant increase was observed for any of the cytokines tested even at concentrations of **4** up to 100 μM, whereas TOG was active as the positive control (data not shown). Since compound **4** did not appear to be active as an agonist for TLR signaling, it also was tested for potential antagonist activity. Therefore, cells were stimulated with a known inducer of cytokine production, such as R848 (resiquimod),[20] in the presence or absence of compound **4** and cytokine levels were measured as before. Here, compound **4** did show

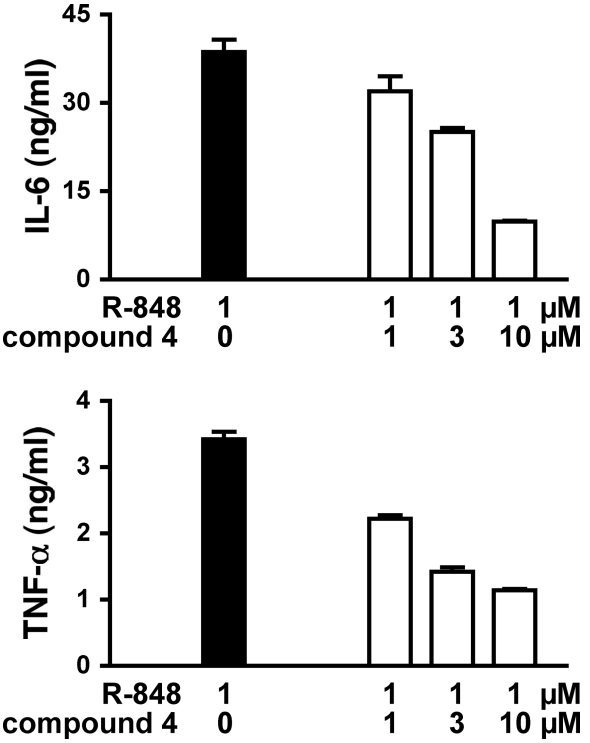


FIGURE 2 Inhibition of R848-induced cytokine induction by compound **4**.

modest inhibition of TNFα and IL-6 production, but had no significant effect on IL-12 production (data not shown) in this system (Figure 2). Thus, the analog was found to antagonize cytokine production resulting from TLR signaling.

The mechanism of this inhibition presently is unknown and may or may not be a result of direct inhibition of TLR signaling. Further studies to elucidate this mechanism are underway.

EXPERIMENTAL SECTION

Chemistry

Melting points were obtained on a Mel-temp II capillary melting point apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were obtained on a Varian Mercury (Electrothermal, Dubuque, IA, USA) at 400.06 MHz. The chemical shifts are expressed in δ values (parts per million) relative to tetramethylsilane (TMS) as internal standard. Mass spectrometric analyses were performed on a Finnigan MAT900XP mass spectrometer using electro- spray ionization. UV spectra were measured using a Kontron Uvikon 9330 spectrophotometer. Elemental analyses were performed by NuMega Resonance Labs (San Diego, CA, USA).

Thin-layer chromatography was performed on silica gel 60 F-254 plates (EM Reagents). E Merck silica gel (230–400 mesh) was used for flash column chromatography.

*2-Amino-5,6-dihydropyrido[2,3-d]pyrimidine-4,7(3*H*,8*H*)-dione* (***1***). A mixture of Guanidine carbonate (3.10 g, 17.3 mmol), methyl acrylate (1.31 g, 15.0 mmol) and methyl cyanoacetate (1.46 g, 15 mmol) in a fresh solution of NaOMe, prepared by addition of Na (1.14 g, 49.5 mmol) to 150 mL of dry MeOH, was heated at reflux for 36 hours. The reaction was cooled to RT, filtered, the resulting solid was washed with MeOH (3 × 10 mL), and dried to yield **1** (1.16 g) as a white powder. The filtrate was concentrated in vacuo and the precipitate was treated as above to obtain a second crop of **1** (0.327 g). Yield 55% (1.48 g). mp: >350°C. UV λ_{max} (pH 11): 220 nm, 291 nm; €12,600; 3,100. MS (ESI) 181 (MH^+). The spectral and analytical data were in agreement with literature.[21]

*2-Amino-pyrido[2,3-d]py- rimidine-4,7(3*H*,8*H*)-dione* (***2***). A mixture of **1** (0.90 g, 5 mmol) and palladium on carbon (10%) in acetic acid (250 mL) was heated at reflux for 30 hours. The reaction mixture was cooled to RT and filtered. The filtered solid was added to water (25 mL) and basified with 2N NaOH to pH 10, the catalyst was filtered out and the clear solution was acidfied to pH 5 with acetic acid, and cooled at 0°C for 2 hours. The precipitate was filtered and washed with water and dried to provide **2** (0.79 g) as a white solid. Yield 88%, mp > 300°C (dec); UV λ_{max} (pH 11): 211, 326 nm. €9,400; 11,000. ^{1}H-NMR (DMSO-d_6) δ 11.65 (s, 1H), 10.91 (s, 1H), 7.70 (d, J = 12 Hz, 1H), 6.96 (br., 2H), 5.95 (d, J = 12 Hz, 1H). MS (ESI) m/z: 179.19 (MH^+). Anal. Calcd. for $C_7H_6N_4O_2$: C 47.19, H 3.39, N 31.45. Found: C 46.80, H 3.72, N 31.05.

*2-Amino-8-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)pyrido[2,3-d]pyrimidine-4,7 (3*H*,8*H*)-di-one* (***3***). Compound (**2**) (60 mg, 0.34 mmol) and HMDS (162 mg, 1.0 mmol) were mixed in acetonitrile (6 mL). Trimethylsilyl-trifluromethanesulfonate (TMStriflate) (222 mg, 1.0 mmol) was added and the reaction mixture was heated at 78°C until a clear solution was obtained. 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (172 mg, 0.34 mmol) and TMStriflate (74 mg, 0.34 mmol) were then added to the reaction mixture. The reaction was heated at 78°C for 18 hours. The reaction was cooled to RT, solvent was removed in vacuo, the residue was dissolved in DCM (30 mL), washed with Sat. $NaHCO_3$, and dried with anhdra. Na_2SO_4. Solvent was distilled off and the crude product was separated by column chromatography on silica gel (30 g) (EtOAc) to get **3** (120.6 mg) as colorless oil, yield 57%. Compound **3** was used directly in the next reaction. ^{1}H-NMR ($CDCl_3$) δ 8.01 (m, 3H), 7.92 (d, J = 8.0 Hz, 1H), 7.86 (d, J = 8.0 Hz, 1H), 7.29–7.52 (m, 12H), 6.23 (d, J = 8.0 Hz, 1H), 5.72 (dd, J = 4.0 Hz, 1H), 5.06 (dd, J = 4.0 Hz, 1H), 4.62 (m, 2H), 4.48 (d, J = 8 Hz, 2H), 4.12 (m, 1H). MS (ESI) m/z: 645.10 (MNa^+).

*2-Amino-8-(β-D-ribofuranosyl)-3H,8H-pyrido[2,3-d]pyrimidine-4,7-dione (**4**).* Compound (**3**) (100 mg, 0.16 mmol) was dissolved in anhydrous methanol

(25 mL) and anhydrous $NaOCH_3$ (130 mg, 2.3 mmol) was added. The reaction mixture was stirred at RT for 36 hours. The pH of the reaction solution was adjusted to 4–5 using acetic acid and the solvent was removed in vacuo, the residue was separated by column chromatography on silica gel (20 g) (EtOAc-MeOH-Acetone-H_2O: 7:1:1:1) and then recrystallized from isopropanol, compound **4** (31 mg) was obtained as a white solid, yield 62%, mp: 256–260°C. UV λ_{max} (MeOH): 216 nm, 292 nm, 327 nm. €11,500; 5,000; 6,700. ^{1}H-NMR (CD_3OD) δ 7.91 (d, J = 12 Hz, 1H), 6.14 (d, J = 12 Hz, 1H), 4.79 (dd, J = 4 and 4 Hz, 1H), 4.49 (d, J = 12 Hz, 1H), 3.90 (dd, J = 4 Hz, 1H), 3.82 (m, 1H), 3.72 (d, J = 12 Hz, 2H). MS (ESI) m/z: 333.08 (MNa^+). Anal. Calcd. for $C_{12}H_{14}N_4O_6 \cdot 2.5H_2O$: C 40.53, H 5.35, N 15.76. Found: C 40.81, H 5.79, N 15.47.

Biological Evaluation

R848 was from GL Synthesis (Worchester, MA, USA). Bone marrow was isolated from the femur and tibia of C57BL/6 mice. Cells were plated on nontissue culture treated petri dishes and cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin (all from Invitrogen, San Diego, CA, USA) and 30% L929 cell conditioned medium. Cells were grown at 37°C, 5% CO_2 for 7 days without replacing the media. The 7-day-old bone marrow-derived macrophages (BMDM) were harvested with trypsin/EDTA, counted and replated at a density of 5×10^4 cells per well in 96-well plates and grown for another 3 days before stimulation with compounds. For cytokine induction study, compounds were prepared in medium at a final concentration ranging from 1 to 10 μM and cells were incubated in such medium for 24 hours. For antagonist study, cells were first incubated with compounds at 1, 3, or 10 μM in the medium for 30 minutes, R-848 was then added at 1 μM and incubation was continued for 24 hours. Cell supernatants were collected and IL-6, IL-12p40/p70, and TNF-alpha levels determined by ELISA. Duplicates were included in each experiment and at least 3 separate experiments were carried out. The data presented is representative of the experiments. Error bars indicate SEM.

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