Synthesis of 5-Thio- α -GalCer Analogues with Fluorinated Acyl Chain on Lipid Residue and Their Biological EvaluationPeng He,[†] Chuanfang Zhao,[‡] Jiao Lu,[§] Yang Zhang,[‡] Min Fang,[§] and Yuguo Du^{*,†,‡,#}[†]State Key Laboratory of Environmental Chemistry and Eco-toxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Science, Beijing 100085, China[‡]School of Chemical Sciences, University of Chinese Academy of Sciences, Beijing 100049, China[§]CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China[#]National Engineering Research Center for Carbohydrate Synthesis, Jiangxi Normal University, Nanchang 330022, Jiangxi, China

Supporting Information

ABSTRACT: Invariant natural killer T (iNKT) cells are a subclass of T cells that initiates the secretion of T helper 1 and 2 cytokines after recognizing CD1d protein presented glycolipid antigens. In this Letter, we designed and synthesized a novel series of CD1d ligand α -galactosylceramides (α -GalCers) in which the acyl chain backbone of the lipid was incorporated with fluorine atoms. The *in vivo* evaluation of immunostimulatory activities revealed that the synthesized α -5-thio-galactopyranosyl-*N*-perfluorooctanoyl phytosphingosine exhibited a remarkable potency toward selectively enhancing T_H1 cytokine production with the IFN γ /IL-4 ratio of 9/1, while its perfluorotetradecanoyl counterpart showed T_H2 profile with an IFN γ /IL-4 ratio of 0.59/1. The analogues synthesized here would be used as probes to study lipid–protein interactions in α -GalCer/CD1d complexes.

KEYWORDS: α -GalCer, iNKT cells, antitumor activity, fluorinated 5-thio- α -GalCers

α -Linked galactosylceramides (α -GalCers) exhibited intriguing and impressive immunostimulating and immunoregulatory properties toward the exploration of applicable treatments on tumors, microbial infections, or autoimmune diseases.^{1–3} Among the promising α -GalCers (Figure 1), the synthetic

Figure 1. Structures of some α -GalCers.

KRN7000 is a hot molecule that has presented convincing bioactivities and contributed better understanding to the related immuno-action mechanism.^{4–8} iNKT cell activation by α -GalCers occurs in district steps. For example, KRN7000 first combines with the CD1d protein on the surface of APCs (Antigen Presenting Cells) to form a glycolipid–protein complex, the semi-invariant TCR (T cell receptor) on the surface of invariant NKT (iNKT) cells recognize the glycolipid–protein complex to build a three-molecule conjugate, which could lead to the release cytokines rapidly by activating NKT cells.^{8,9} Unfortunately, the released pro-inflammatory T_H1 (e.g., IL-2, IFN- γ) and immunomodulatory T_H2 (e.g., IL-4/10) cytokines antagonize each other's effects.¹⁰

The discovery of new α -GalCer analogues that favor either of the two biased cytokine profile without severely interfering the immune response may have therapeutic potentials and therefore has long been an active research topic in this area.^{11–14}

To date, it has not been completely understood regarding the relationship between cytokine polarization and glycolipid structure, as it may be related to the stability of the glycolipid complex with CD1d.¹⁵ In the past decade, many efforts have been made to design and synthesize KRN7000 analogues through modifying sugar or ceramide parts. It was widely accepted that the property of the sugar moiety of KRN7000, D-galactose, was strongly preferred with very few structural exceptions.^{16,17} The α -anomeric configuration of the ceramide aglycone moiety was critical as the corresponding β -anomer displayed far lower agonist activities.^{18,19} On the ceramide part, it has been found that truncation of the sphingosine alkyl chain, as depicted by OCH²⁰ in Figure 1, decreased the ratio of IFN- γ /IL-4, favoring the T_H2 biased immune response, while the aromatic residues on either the acyl tail or sphingoid base could restore the T_H1 cytokine profile.

It is well recognized that the incorporation of fluorine atom could endow certain drugs with various properties through

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affecting metabolic stability and binding interactions. More recently, the fluorine atom has also been embedded into α -GalCer structures, which led to changes of cytokine releases. For example, sphingosine fluorinated 4-deoxy-4,4-difluoro-KRN7000 analogue²¹ showed reinforced hydrogen-bond donating capacity of 3-OH with CD1d, resulting in a comparable IFN- γ secretion comparing to KRN7000, and the results suggested that T_H1 polarization needs a tighter bonding of the 3-OH group with CD1d. Replacement of the 3-OH in α -GalCer structure²² with a fluorine atom eliminated CD1d Asp80 hydrogen bonding and thus drastically decreased cytokine release in mice iNKT cells. Introduction of the two neighboring difluorine atoms enhanced acidity of the amide NH group of the ceramide fragment and may reinforce the hydrogen bonding with mCD1d Thr156 to stabilize the CD1d/GalCer/TCR complex and favor the T_H1 bias. However, 2',2'-difluorinated KRN7000²³ exhibited surprisingly T_H2-biased immune response by murine NKT cells.

We have successfully prepared three 5-thio-galactopyranosyl substituted KRN7000 analogues,²⁴ namely, 5-thio-KRN7000, 5-thio- α -GalCer 566, and 5-thio-PBS-25. Compared to the original KRN7000, 5-thio-KRN7000 showed a similar ability and tendency in inducing IFN- γ and IL-4 both *in vitro* and *in vivo*. Interestingly, 5-thio- α -GalCer 566 containing an acyl chain with 14 carbon atoms on ceramide residue showed a compressed IL-4 release, but with a relatively high IFN- γ production, indicating a T_H1 bias property. In continuation with our efforts in exploring the pharmacological activity of 5-thio- α -GalCer analogues to modulate the response of iNKT, we were also curious about the cytokine secretion profile in the presence of fluorinated acyl chain. In order to compare the biological data with the reported results, we chose PBS-25 and α -GalCer 566 as template molecules during structural modification. Accordingly, α -GalCers with varied fluorinated lipid tails (8, 9, and 10) and their corresponding counterparts 5-thio- α -GalCers (11, 12, and 13) were designed and synthesized (Figure 2), and their biological activities to stimulate cytokine release were evaluated *in vivo*.

Figure 2. Fluorinated target α -GalCers analogues 8–13.

We started from the synthesis of 8,8,8-trifluorooctanoic acid 17a, which will be used as acyl residue in assembly of compounds 8 and 11. As shown in Scheme 1, Wittig reaction of aldehyde 14²⁵ with phosphonium salt 15²⁶ obtained olefin

Scheme 1. Synthesis of 8,8,8-Trifluorooctanoic Acid^a

^aReagents and conditions: (a) LHMDS, THF, −78 °C to rt, 76%; (b) Pd(OH)₂/C, H₂, 95%.

16, followed by Pd(OH)₂-catalyzed hydrogenation to afford 8,8,8-trifluorooctanoic acid 17a in a good overall yield.

With this trifluoromethylated octanoic acid 17a as well as the commercially available perfluorooctanoic acid (17b) and perfluorotetradecanoic acid (17c) in hand, we prepared three ceramide derivatives smoothly, as shown in Scheme 2.

Scheme 2. Synthesis of Fluorinated Lipid as Glycosyl Acceptor^a

^aReagents and conditions: (a) EDCI, HOBT, Et₃N, DMF, rt, 16 h; (b) TBDPSCI, Pyr., rt, 10 h, 73% for 20a, 64% for 20b, 65% for 20c (for two steps); (c) BzCl, Pyr., rt, 12 h, 89% for 21a, 94% for 21b, 93% for 21c; (d) HF-Pyr., CH₂Cl₂, rt, 10 h, 91% for 22a, 92% for 22b, 95% for 22c.

Chemoselective condensation of acids 17a–c with phytosphingosine 18 in the presence of EDCI, HOBT, and Et₃N in DMF at r.t. afforded amides 19a–c in good yields, which were then subjected to regioselective protection of primary hydroxyl group with TBDPSCI to generate silylated derivatives 20a–c. The remaining secondary hydroxyl groups were further masked with benzoyl using benzoyl chloride in pyridine to afford compounds 21a–c. Cleavage of the silyl group of 21a–c with HF-pyridine in CH₂Cl₂ furnished acceptors 22a–c in good yields over four steps.

To prepare α -GalCer analogues (8, 9, and 10) which contain natural D-galactose sugar head, compound 23²⁷ was performed as glycosyl donor by taking advantage of its predominant α stereo-outcomes during glycosylation. Coupling of 23 with lipid derivative 22 in the presence of NIS and TMSOTf in CH₂Cl₂ at 0 °C gave the desired α -anomer 24 as a major product (Scheme 3). Hydrolysis of 24 treated with catalytic amount of NaOMe in MeOH yielded compounds 25, followed by Pd(OH)₂/C catalyzed hydrogenation to achieve the target α -GalCer analogs 8, 9, and 10, respectively.

To prepare 5-thio- α -GalCer analogs (11, 12, and 13), our glycosyl donor 26²⁴ was employed. Thus, glycosylation of compound 26 with lipid acceptor 22 was carried out in anhydrous CH₂Cl₂ at 0 °C in the presence of TMSOTf (0.04 equiv). The reaction proceeded smoothly and gave exclusively α -linked product 27 in a moderate yield (Scheme 4). This α -selectivity could be interpreted by the greater thermodynamic stability of the axially oriented aglycon in 5-thio-sugar as compared to the 5-oxy-counterpart. Removal of all protecting groups of 27 furnished α -GalCer analogs 11, 12, and 13 in about 50–60% overall yields, respectively.

To investigate whether the synthetic compounds 8–13 could activate CD1d-dependent NKT cell *in vivo*, B6 mice intraperitoneally received glycolipids (100 μ g/kg) in 0.1 mL of vehicle, then kinetic releases of IL-4 and IFN- γ were measured

Scheme 3. Synthesis of α -GalCer Analogues^a

^aReagents and conditions: (a) TMSOTf, NIS, DCM, molecular sieves 4 Å, N₂, 0 °C, 5 h, 66% for 24a, 63% for 24b, 61% for 24c; (b) NaOMe, MeOH, rt, 5 h, quantitative; (c) 4 bar of H₂, Pd(OH)₂/C, MeOH/EtOAc (v/v, 4:1), rt, 14 h, 95% for 8, 91% for 9, 93% for 10.

Scheme 4. Synthesis of 5-Thio- α -GalCer Analogues^a

^aReagents and conditions: (a) TMSOTf, DCM, molecular sieves 4 Å, N₂, 0 °C, 2 h, 64% for 27a, 61% for 27b, 54% for 27c; (b) NaOMe, MeOH, rt, 6 h, quantitative.

and compared with that induced by KRN7000. As previously reported,²⁴ KRN7000 caused rapid IL-4 production peaked at 3 h and delayed but extended elevation of IFN- γ in B6 mice. This uniformity proved that our assay was convincing when comparing data with those of other reports. The area under curve (AUC) ratio and the maximum productivity ratio of IFN- γ /IL-4 were applied to judge the relative potency with regard to T_H1 and T_H2 selectivity for all these compounds.

When 8,8,8-trifluorooctanoyl substituted PBS-25 analogues (8 and 11) were injected, respectively, no desired cytokine secretion of IL-4 and IFN- γ was observed (Figure 3). It is quite interesting to note that PBS-25 itself was believed to be a strong T_H2 agonist in cytokine production,²⁸ while 5-thio-PBS-25 analog from our previous work showed a very weak ability to stimulate IFN- γ with no detectable IL-4 secretion.²⁴ Interestingly, injection of perfluorooctanoylated PBS-25

Figure 3. Biological activities of compounds 8–13 *in vivo*. (a,b) *In vivo* secretion of cytokine after treatment with KRN7000 or its analogues (100 μ g/kg). Each group has three mice, and the serum were examined individually. (c) AUC ratio of IFN- γ /IL-4 production.

analogues (9 and 12) resulted in notable higher levels of serum cytokine relative to KRN7000. Compound 9 expressed an identical IFN- γ /IL-4 profile with KRN7000 in mice testing but a better T_H1 polarization (IFN- γ /IL-4 maximum productivity ratio of 3.4 for 9 to 2.8 for 1, AUC ratio of 5.8 for 9 to 12.1 for 1). Surprisingly enough, perfluorooctanoylated 5-thio-PBS-25 analog 12 exhibited an enormous increase of IFN- γ secretion in B6 mice testing. The high ratios of both AUC (26.7/1) and the maximum productivity (9.0/1) of IFN- γ /IL-4 suggested that compound 12 was an excellent T_H1 stimulator to selectively induce IFN- γ release. The greater value of AUC ratio indicated an extended elevation of IFN- γ expression in mice after injection, but a compressed IL-4 secretion instead. Furthermore, when perfluorotetradecanoylated α -GalCer 566 analogue 10 was subjected to the bioassay, no stimulation on either IFN- γ or IL-4 were observed. This result is quite different from the reported bioactivity of α -GalCer 566, which exhibited good potency to stimulate B cells and induce IL-2 from mouse NKT cells.²⁹ In contrast, perfluorotetradecanoylated 5-thio- α -GalCer 566 analogue 13 caused a lower release of IFN- γ than KRN7000 by a factor of 9, thus favoring a T_H2 profile with IFN- γ /IL-4 AUC ratio of 0.58 and the maximum productivity ratio of 0.59. Apparently, the binding potency of 5-thio-sugar to TCR and fluorinated lipid to CD1d determined cytokine secretion profile. The T_H2 profile of 13 in mice could be ascribed to a destabilization of perfluorotetradecanoylated alkyl chain in the related binding groove.

In conclusion, based on our finding that 5-thio- α -GalCers stimulate stronger IFN- γ production of iNKT cells than that of KRN7000, we have synthesized six acyl-fluorinated α -GalCer analogues 8–13. The immunostimulatory activities of these synthetic compounds were investigated *in vivo* with KRN7000 as a positive control. Mice iNKT responses showed strong divergences after fluorine-modification on the acyl chain. 8,8,8-Trifluorooctanoyl-substituted α -GalCer and 5-thio- α -GalCer analogues (8 and 11) were inert to the stimulation, indicating that a trifluoromethylation of the acyl chain could not stabilize the ternary complex of CD1d/GalCer/TCR. To our delight, a strong T_H1 orientation was observed through changing octanoyl to perfluorooctanoyl in acyl residue. Perfluorooctanoylated α -GalCer 9 and 5-thio- α -GalCer 12 induced higher serum cytokine levels comparing to KRN7000, and expressly, the perfluorooctanoylated 5-thio- α -GalCer 12 exhibited a striking augment of IFN- γ secretion. When the acyl chain of ceramide was substituted by perfluorotetradecanoyl, the α -GalCer analog 10 showed no activity toward cytokine secretion, while the corresponding 5-thio- α -GalCer analog 13 presented a distinct behavior with a moderate level of IL-4 production, providing a T_H2-biased cytokine profile. The current results open a new area to develop effective and selective immunostimulating agents. More work should be done to get a better understanding for the cytokine profile shift with respect to the unusual 5-thio-galactose head and multifluorinated lipids in α -GalCer structures.

■ ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00640.

Experimental details and spectral characterization of key compounds and procedure of bioassays (PDF)

AUTHOR INFORMATION

Corresponding Author

*Tel: +86-010-62849126. Fax: +86-010-62923549. E-mail: duyuguo@rcees.ac.cn.

ORCID

Yuguo Du: 0000-0002-2081-9668

Author Contributions

Y.D. and P.H. conceived the project and designed the experiment. P.H. synthesized the compounds and analyzed the physicochemical properties. J.L. and M.F. have given approval to the *in vivo* assays. C.Z. and Y.Z. have given approval to high-resolution mass determination. P.H. and Y.D. performed the data analysis and the manuscript written.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

iNKT, invariant natural killer T cells; T_H1, T helper 1; IL-4, interleukin-4; T_H2, T helper 2; IL-2, interleukin-2; IFN- γ , interferon γ ; LHMDs, lithium bis(trimethylsilyl)amide; TCR, T cell receptor; IL-10, interleukin-10; α -GalCer, α -galactosylceramide; ELISA, enzyme-linked immunosorbent assay; DCM, dichloromethane; THF, tetrahydrofuran; TMSOTf, trimethylsilyl trifluoromethanesulfonate

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