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Heteroaromatic Moieties in the Sphingosine Backbone of α-Galactosylceramides for Noncovalent Interactions with CD1d

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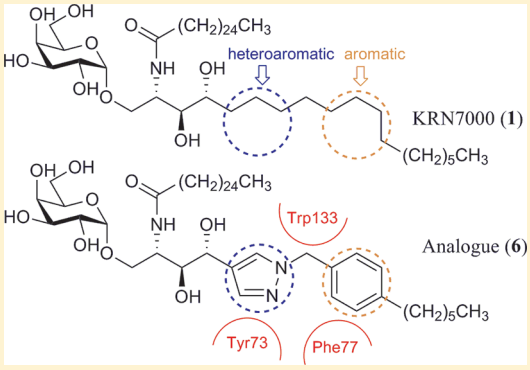
Supporting Information

ABSTRACT: A series of α-GalCer analogues containing heterocyclic and aromatic moieties in the sphingosine backbone were synthesized to improve the selectivity in the Th1/Th2 cytokine profile via noncovalent interaction with three aromatic residues at the binding pocket of CD1d. *In vitro* and *in vivo* biological evaluations revealed the treatment of α-GalCer analogue (**6**) induced the selective stimulation of natural killer T cells to facilitate the secretion of Th2 cytokines.



KEYWORDS: *α-Galactosylceramide, CD1d, selectivity, cytokine secretion, noncovalent interaction*

α-Galactosylceramide (α-GalCer) is a typical antigen for invariant natural killer T (iNKT) cells that are a subset of T cells and play a critical role in regulating immune responses.[1−5] After α-GalCer is loaded onto the CD1d protein in antigen-presenting cells (APCs), the CD1d-α-GalCer bimolecular complex is recognized by the T cell receptor (TCR) and the complex activates iNKT cells.[6−8] Subsequently, the activated iNKT cells release many cytokines, including pro-inflammatory T helper 1 (Th1) cytokines [interferon-γ(IFN-γ), tumor necrosis factor-α (TNF-α), etc.] and anti-inflammatory T helper 2 (Th2) cytokines [interleukin-4 (IL-4), interleukin-10 (IL-10), etc.].[1−5,9,10] The secreted cytokines are recognized by other cells in the immune system, which affect a wide variety of immune responses, including the host response to parasites and bacteria, antitumor responses, and the protection against autoimmune diseases.[1−5]

KRN7000 (**1**) is a well-known molecule of the α-GalCer family (Figure 1), and its therapeutic effects have been extensively studied in various disease models.[11−15] However, the *in vivo* efficacy of **1** has been somewhat limited because of its dual antagonizing effects on both Th1 and Th2 cytokines, and these dual effects are known to be polar opposites in immune response.[1] In other words, **1** can stimulate the secretion of both pro- and anti-inflammatory cytokines and can result in no net changes in immunity. Thus, many recent studies have focused on the synthesis of KRN7000 analogues that can selectively activate iNKT cells to secrete certain cytokines.[1] For example, the structural modification of the sugar moiety in α-GalCer revealed that the α-anomeric galactose is considerably better than other sugars, such as glucose, mannose, and β-galactose without selectivity.[12] However, the structural modification at the C6 position of the galactosyl moiety in α-GalCer with a 4Cl-3CF_3-benzamide substituent (**2**) showed a promising

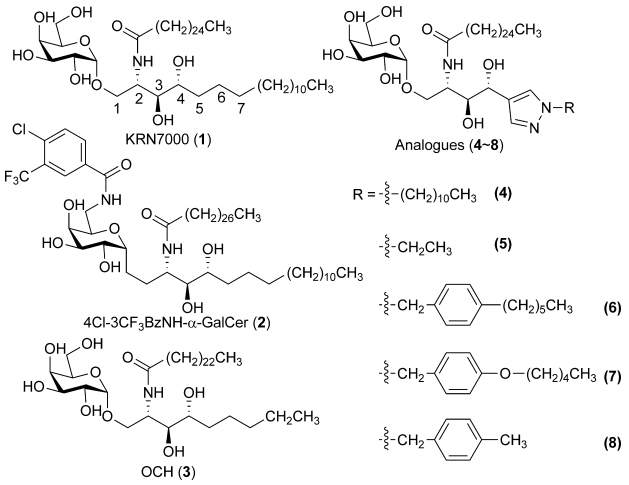


Figure 1. Chemical structures of representative α-GalCers (**1**−**3**) and proposed α-GalCer analogues (**4**−**8**) containing heterocyclic and aromatic moieties in the sphingosine backbone.

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Th1-based polarizing effect.[16,17] α-GalCer analogues containing aromatic groups at the end of the *N*-acyl side chain also induced a Th1 response.[18] In those papers, the enhanced stability of the CD1d−α-GalCer complex via additional noncovalent interactions is claimed to be responsible for the Th1-biased response. In contrast, α-GalCer analogues with a short aliphatic chain or multiple cis-double bonds in the *N*-acyl side chain induced a Th2-biased cytokine response.[19,20] The representative α-GalCer analogue for the Th2-biased immune response is OCH (**3**), with the truncation of the lipid chain of the sphingosine backbone in **1**.[9,10] These examples suggest that the decreased stability of the CD1d−α-GalCer complex leads to a Th2 response as a result of to their short retention time at the surface of APCs.[21]

Even though the structure−activity relationships of α-GalCer analogues are substantially difficult to predict,[1] we recognized the importance of the binding affinity in the CD1d−α-GalCer complex for the selective activation of either the Th1 or the Th2 response. Unlike the cases of sugar and *N*-acyl moieties, the extensive modifications of sphingosine backbones have not yet been actively pursued. Therefore, we focused on the perturbation of the complex stability via the structural diversities of sphingosine backbones. In addition, the crystal structure of CD1d revealed that the *N*-acyl and phytosphingosine lipid chains of α-GalCer fit tightly into the two hydrophobic pockets of the CD1d-A′ pocket and the -F′ pocket, respectively.[6,22−24] Through our structural investigation of the F′ pocket, we discovered a series of aromatic amino acids such as Tyr73, Phe77, and Trp133 located at the contact area with sphingosine backbones in the CD1d F′ pocket. Thus, we hypothesized that the introduction of a heteroaromatic moiety into sphingosine backbones might significantly influence the noncovalent interaction between α-GalCer analogues and the CD1d protein, which might induce the selective activation of a Th1 or Th2 response in iNKT cells.

Hence, we designed a series of α-GalCer analogues **4**−**8** with new sphingosine backbones that contain an aromatic heterocycle such as pyrazole at the C5−C7 positions. α-GalCer analogues **4** and **5** were designed to mimic KRN7000 (**1**) and OCH (**3**), respectively. In addition, we introduced benzene into the sphingosine backbone as another aromatic residue (α-GalCer analogues **6**, **7**, and **8**) for inducing an additional noncovalent interaction at the binding pocket of the CD1d protein. As shown in Figure 2, α-GalCer analogue **6** fits nicely

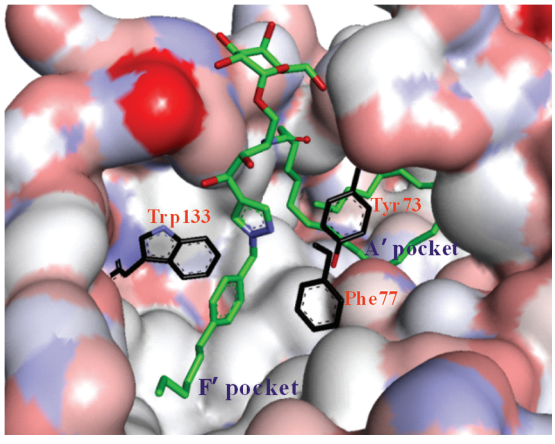
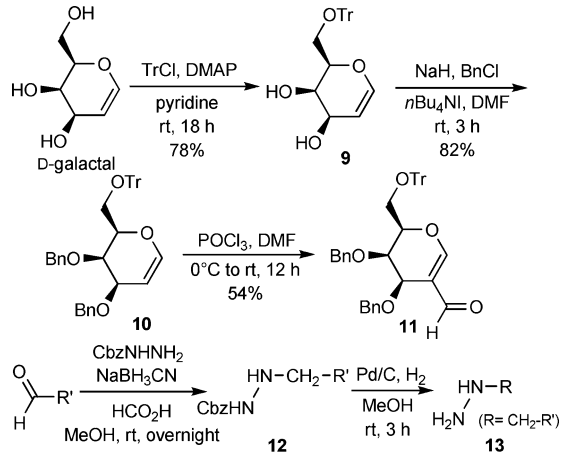


Figure 2. Schematic docking simulation of α-GalCer analogue **6** in the binding pocket of the mCD1d protein (PDB: 3HE6).

at the binding pocket of the CD1d; this was confirmed by our docking simulation study conducted using CD1d protein cocrystallized with **1** (PDB: 3HE6). All α-GalCer analogues, except **5**, were docked successfully and scored on the basis of an *in silico* binding study (see Table S1); this suggests that these analogues might influence the stability of the CD1d−α-GalCer complex through noncovalent interactions with three aromatic residues in the F′ pocket of CD1d and potentially induce the selective activation of the immune system.

We initiated our synthetic procedure with D-galactal, which was already equipped with the desired stereochemistry of all chiral centers required for α-GalCer analogues. As shown in Scheme 1, the primary alcohol of D-galactal was selectively

Scheme 1. Synthetic Routes of D-Galactalcarbaldehyde (11) and Alkyl Hydrazine Building Blocks (13)



tritylated to yield **9**, and the two remaining secondary alcohols were subjected to benzyl protection. The resulting product **10** was formylated via Vilsmeier−Haack reaction to yield D-galactal carbaldehyde **11**. Simultaneously, Cbz-protected alkylhydrazine **12** was produced by the reductive amination of the corresponding aldehyde with Cbz-hydrazine, and the subsequent deprotection of the Cbz group via catalytic hydrogenation produced the alkylhydrazine (**13**). Unlike the general alkylamines, alkylhydrazines were not readily obtained under typical reductive amination conditions ($NaBH_3CN$, cat. AcOH). After the systematic screening of various acids (AcOH, *p*TsOH, HCl, and HCO_2H), reductants ($NaBH_3CN$, BH_3, $LiAlH_4$, and $NaBH_4$), and solvents (MeOH, THF, and DCM), we decided to use the combination of $NaBH_3CN$, HCO_2H, and MeOH as the optimal condition for reductive amination in moderate isolated yields (data not shown).

The dielectrophilic α,β-unsaturated aldehyde moiety of **11** was cyclized with dinucleophilic alkylhydrazine **13** in MeOH to yield a sphingosine backbone **14** embedded with an aromatic pyrazole ring in high yields (see Scheme 2 and Supporting Information). By altering alkylhydrazines in this step, we easily obtained five different sphingosine-type compounds. Then, the C2-OH position of **14** was mesylated, and the resulting mesylate **15** was converted to azido compound **16** via a microwave-assisted substitution reaction of **15** with NaN_3. Detritylation at the C1-OH of **16** in methanolic *p*TsOH produced the suitably protected sphingosine derivatives **17** for the subsequent galactosidation. The anhydrous combination of a highly reactive galactosyl iodide donor with its acceptor **17** afforded an α-selective glycosidic bond with 61−88% yields.[25] The azido

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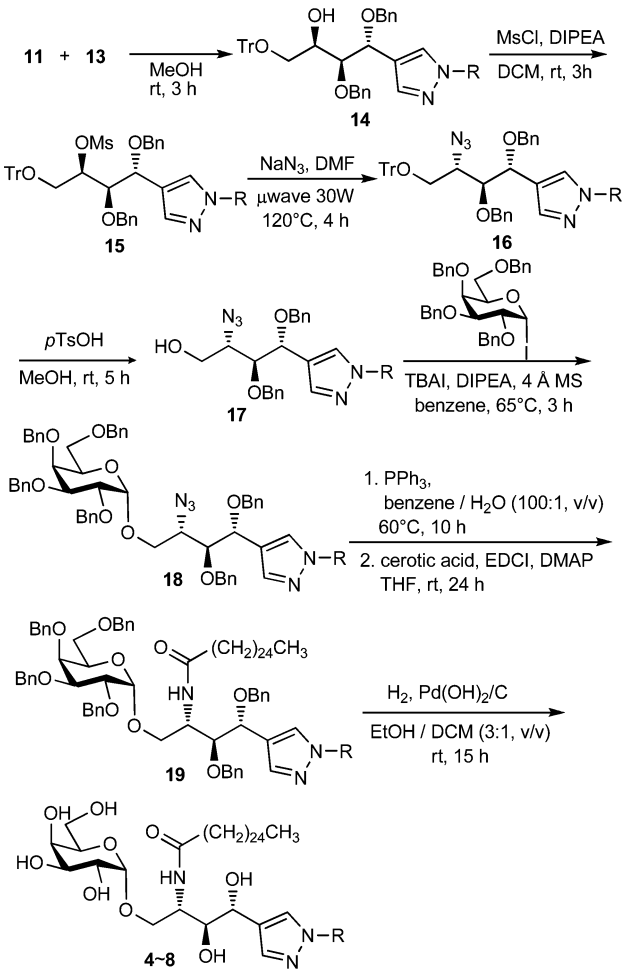
Scheme 2. General Synthetic Method for α-GalCer Analogues



group in **18** was reduced to an amino group via a Staudinger reaction, and the subsequent EDC-mediated amide coupling with cerotic acid produced the galactosylceramide derivatives **19**. Finally, six benzyl protecting groups in **19** were removed via catalytic hydrogenation with $Pd(OH)_2$ on carbon in EtOH–DCM cosolvent at atmospheric pressure of hydrogen to afford the final compounds (**4**–**8**) as white solids.

The biological activities of α-GalCer analogues (**4**–**8**) containing heteroaromatic rings in the sphingosine backbone were monitored using ELISA for the selective induction of cytokines such as IFN-γ and IL-4. Upon the treatment of α-GalCer analogues, the relative amounts of these cytokines released by the murine hepatic mononuclear cells (HMNC) containing iNKT cells were measured in comparison with KRN7000 (**1**). As shown in Figure 3a, the iNKT cells were not sufficiently activated in the case of analogues **4**/**5** and **8**, having long/short aliphatic chains and a short aryl chain, respectively, at the R position. However, analogues **6** and **7** containing C14-equivalent alkyl chains, which are an identical length to the sphingosine backbone of KRN7000, could activate primary iNKT cells toward the selective secretion of IL-4 instead of that of IFN-γ. The dose-dependent stimulation by analogue **6** was also monitored (Figure 3b). This observation was quite surprising because we expected the Th1-biased response due to its additional noncovalent interactions at the binding pocket of the CD1d protein. As stated earlier, it is difficult to predict the

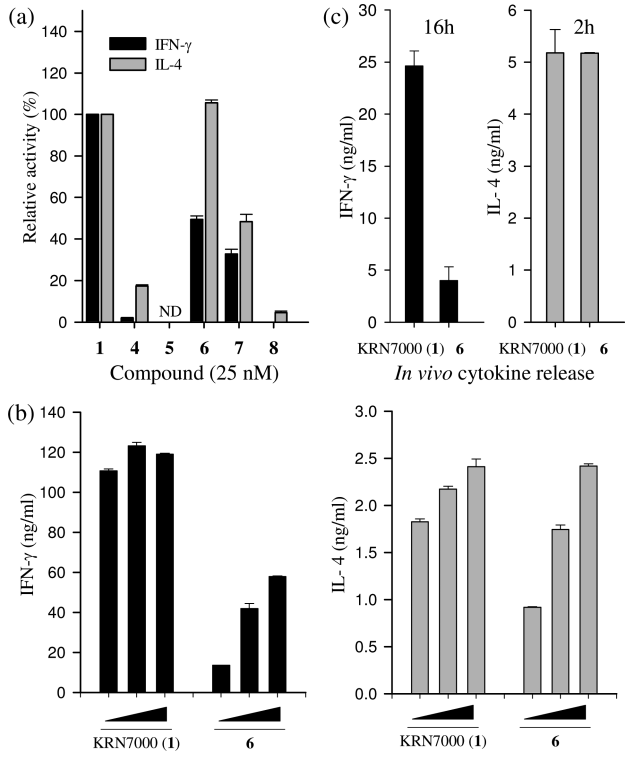


Figure 3. Biological evaluations. (a) IFN-γ and IL-4 secretion upon the treatment of individual α-GalCer analogues measured after 72 h in hepatic mononuclear cells (HMNC) (ND: not detected). (b) Dose dependent secretion of IFN-γ and IL-4 after 72 h in HMNC stimulated by analogue **6** (5 nM, 25 nM, and 125 nM). (c) Serum IFN-γ and IL-4 levels in mice after intravenous injection of KRN7000 (**1**) and analogue **6**.

structure–activity relationships of α-GalCer analogues for the selective immune response. In addition, the cytokine polarization can be significantly influenced by minor changes in various elements, including the binding stability of α-GalCer analogues with CD1d, the loading mechanism of glycolipid, conformational changes of CD1d, and the binding affinity of the CD1d–α-GalCer complex with TCR.[1,26] Even though we have not clearly addressed the biophysical mechanism of Th2-based polarization, the *in vivo* administration of analogue **6** via a single intravenous (i.v.) injection clearly enhanced the selective secretion of IL-4 in mice serum, compared to KRN7000, which is consistent with *in vitro* data (Figure 3). From these results, we concluded that our novel α-GalCer analogue **6** containing both pyrazole and benzene moieties in the sphingosine backbone can stimulate primary iNKT cells to secrete the Th2-type cytokine, IL-4, with high selectivity.

In conclusion, we reported the development of a synthetic strategy for a new series of α-GalCer analogues containing heteroaromatic groups in the sphingosine backbone through a rational design for introducing additional noncovalent interactions with aromatic residues such as Tyr73, Phe77, and Trp133 at the binding pocket of the CD1d protein. An ELISA-based biological evaluation of the resulting analogues in primary murine iNKT cells led to the identification of a new type of α-GalCer analogue, **6**, for the selective secretion of Th2-biased cytokines, which was confirmed by an *in vivo* study. Therefore, this new strategy can be adopted for the systematic discovery of α-GalCer-based analogues to selectively stimulate the secretion of anti-inflammatory cytokines in iNKT cells.

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■ ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedure, spectroscopic data, and full characterizations of all new compounds, and procedures for biological experiments including ELISA assay and *in vivo* study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

α-GalCer, α-galactosylceramide; iNKT cell, invariant natural killer T cell; APC, antigen-presenting cell; TCR, T cell receptor; IFN-γ, interferon-γ; IL-4, interleukin-4; ELISA, enzyme-linked immunosorbent assay

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