Design and Evaluation of ω-Hydroxy Fatty Acids Containing α-GalCer Analogues for CD1d-Mediated NKT Cell Activation

Chaemin Lim,[†] Jae Hyun Kim,[†] Dong Jae Baek,[†] Joo-Youn Lee,[†] Minjae Cho,[†] Yoon-Sook Lee,[†] Chang-Yuil Kang,[†] Doo Hyun Chung,[‡] Won-Jae Cho,[§] and Sanghee Kim*,[†]

[†]College of Pharmacy, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-742, Korea
[‡]College of Medicine, Seoul National University, 28 Yongon, Chongno-gu, Seoul 110-799, Korea
[§]College of Pharmacy, Chonnam National University, Yongbong, Buk-gu, Kwangju 500-757, Korea

Ⓢ Supporting Information

ABSTRACT: CD1d molecules recognize glycolipid antigens with straight chain fatty acid moieties. Although most of the residues in the CD1d binding groove are hydrophobic, some of the amino acids can form hydrogen bonds. Consequently, we have designed ω-hydroxy fatty acid-containing glycolipid derivatives of the prototypical CD1d ligand α-GalCer. The potency of the ω-hydroxy analogues of the proper length is comparable to that of α-GalCer. We propose, based on the biological results and molecular modeling studies, that a hydrogen bonding interaction is involved between the ω-hydroxy group and a polar amino acid residue in the hydrophobic binding groove.
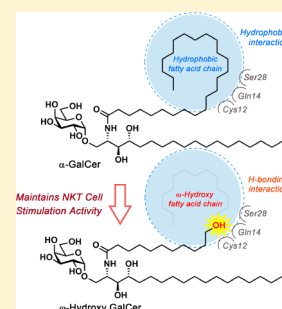
α-GalCer (also known as KRN7000, **1**, Figure 1) is a synthetic glycolipid composed of α-linked galactose, phytosphingosine,

α-GalCer, **1** (X = H, n = 22)
2 (X = OH, n = 7)
3 (X = OH, n = 8)
4 (X = OH, n = 12)
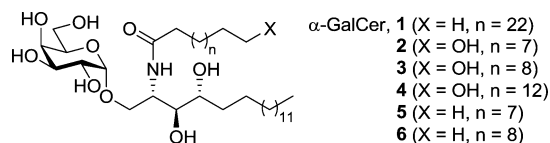**5** (X = H, n = 7)
6 (X = H, n = 8)

Figure 1. Structures of glycolipids **1**–**6**.

and hexacosanoic acid. This glycolipid is the first agonist discovered for natural killer T (NKT) cells[1,2] and has helped elucidate NKT cell biology.[3–5] In addition, its structure has served as a basic guide for the design and synthesis of various analogues.[6–8]

α-GalCer activates NKT cells in a CD1d-restricted manner. First, α-GalCer binds to the CD1d molecule of antigen-presenting cells to form a glycolipid/CD1d binary complex.[2,9] This complex is recognized by the T cell receptor (TCR) on the NKT cells, forming a ternary complex that eventually activates the NKT cells.[10,11] The activated NKT cells promptly secrete T helper 1 and 2 (Th1 and Th2) cytokines, such as interferon-γ (IFN-γ) and interleukin 4 (IL-4); these cytokines help induce a series of cellular activation events during immune response.[12,13] In this process, α-GalCer induces the secretion of Th1 and Th2 cytokines indiscriminately.[14–16]

The binding mode of α-GalCer within CD1d was clearly defined during X-ray crystallographic studies.[10,17–20] The phytosphingosine chain of α-GalCer fit into the F′ pocket of the binding groove, and the fatty acid chain filled the A′ pocket.

In this binding complex, the fatty acid chain circles around the central pole formed by residues Phe70 and Cys12 in the A′ pocket (Figure 2a). Various types of interactions hold α-GalCer in the correct position for recognition by the TCR. While hydrogen-bonding is the predominant interaction between the polar portion of α-GalCer and the surface residues of CD1d,[10] hydrophobic interactions are the principal factors generating the binding energy between the two lipid chains and the CD1d binding groove.[17]
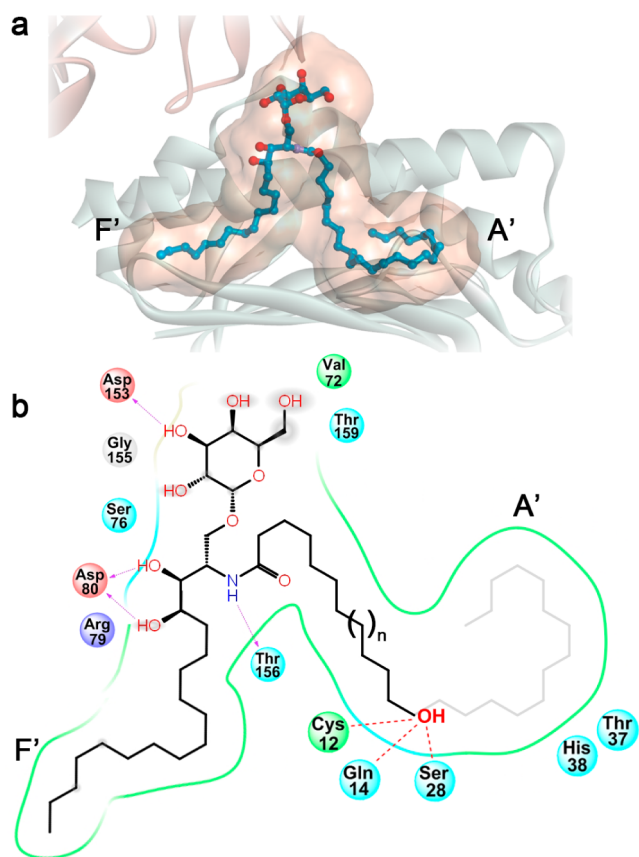
Most of the residues in the CD1d deep binding groove are hydrophobic amino acids. Interestingly, the binding groove also contains a few amino acids able to form hydrogen bonds, such as Cys12, Gln14, Ser28, Thr37, His38, and Arg74.[21] It was envisioned that these amino acids might be utilized to construct additional interactions and stabilize the glycolipid/CD1d binary complex.[22] Therefore, we introduced a heteroatom into the α-GalCer fatty acid chain with the hope of providing a new rational basis for the design of CD1-mediated immune modulating agents. We report here, as a part of our work on this subject, the synthesis and preliminary evaluation of the ω-hydroxy α-GalCer analogues.

Since the discovery of α-GalCer, various analogues have been designed and synthesized.[6–8,13,23] Previous structural alterations of the α-GalCer fatty acid chain have focused on modulating the hydrophobic interactions. Our ω-hydroxy α-GalCer analogue represents the first example that introduces a

Figure 3. Molecular docking simulation of ω-hydroxy α-GalCer analogue **3** within the mCD1d (PDB code 3GML) binding groove. H-bonds are displayed as green dotted lines. For clarity, only the key residues are visible in line model and are labeled using the 3-letter amino acid code.

Figure 2. (a) Overview of the binding mode of α-GalCer (**1**) within mCD1d (PDB code 3HE6). (b) Schematic representation of design concept for ω-hydroxyl group containing α-GalCer analogues. Red dotted arrow indicates their ability to form hydrogen bonds with designed ligands.

hydrogen bonding interaction within the binding groove of the A′ pocket.[24]

Using the CD1d X-ray crystal structure, we designed ω-hydroxy fatty acid-containing α-GalCer analogues. In this study, the mouse CD1d structure (Protein Data Bank code 3GML)[25] was used instead of the human analogue because our biological assay was based on the murine system. The optimal carbon chain length between the carbonyl carbon and the terminal hydroxyl group appears to be 10 to 11 atoms, placing the hydroxyl group close to the Cys12, Gln14, and Ser28 residues (Figure 2b). Therefore, ω-hydroxy α-GalCer analogues **2** and **3** (Figure 1) were designed. To probe the influence of the hydroxyl group on the interactions within the binding groove, additional analogues were designed. Analogue **4** contains an ω-hydroxy fatty acid with a 16 carbon atom chain that seemed too long to facilitate a good hydrogen bonding interaction with the desired residues. Analogues **5** and **6** have a saturated fatty acid with the same chain length as in analogues **2** and **3** but without the ω-hydroxy group.

To simulate our design, molecular modeling studies were performed on the interaction between the analogues and CD1d. In our docking model (Figure 3), the ω-hydroxy group of **3** was located close to the entrance of the A′ pocket and engaged in hydrogen bonding with the surrounding residues, such as Cys12, Gln14, and Ser28. The docking complex displaying the hydrogen bonding between the ω-hydroxy group and Gln14 had a better docking score than that with a
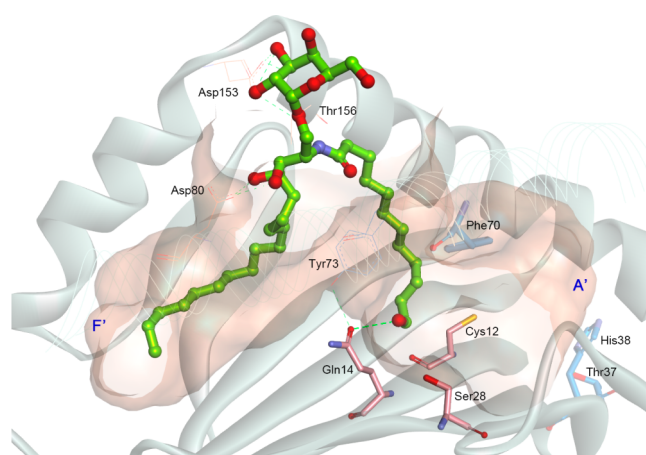
hydrogen bond at Cys12 or Ser28.[26] The ω-hydroxy-group-lacking analogue **6** adopted a docking conformation very similar to that of analogue **3**, but with a lower docking score (Figure S1 in the Supporting Information). These modeling studies suggest that the additional hydrogen bonding interaction caused by the ω-hydroxy group may increase the affinity for CD1d and strengthen the glycolipid/CD1d complex. To validate the docking results, we additionally performed docking simulation with a small set of virtual analogues. As expected, the docking scores of the analogues with hydrogen-bonding capability were higher than that of analogue **6** (Table S1 in the Supporting Information).

To synthesize the designed compounds, known α-galactosyl phytosphingosine **7**[27] was employed (Scheme 1). The amino

Scheme 1. Synthesis of the Designed Analogues 2−6[a]

2 (X = OH, n = 7)
3 (X = OH, n = 8)
4 (X = OH, n = 12)
5 (X = H, n = 7)
6 (X = H, n = 8)
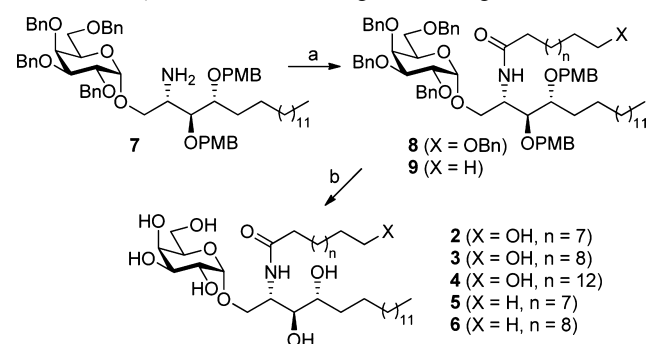
[a]Reagents and conditions: (a) ω-benzyloxy fatty acids or saturated fatty acids, EDCI, DMAP, CH_2Cl_2, rt, 69−80%. (b) H_2, Pd(OH)$_2$, EtOH/CH_2Cl_2 (3:1), rt, 2 d, 73−85%.

group of **7** was acylated with an ω-benzyloxy fatty acid followed by the global removal of the benzyl protecting groups in **8** via hydrogenolysis, affording the desired ω-hydroxy α-GalCer analogues **2−4**. The ω-hydroxy group-lacking analogues **5** and **6** were prepared in a similar manner using fatty acids with chain lengths of 11 and 12 carbon atoms.

To evaluate obtained analogues, initially we used mouse CD1d-specific NKT hybridoma cells (DN32.D3) that produce IL-2 upon stimulation. The parent α-GalCer **1** was also tested

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for comparison. As presented in Figure 4a, the rationally designed ω-hydroxy analogues **2** and **3** were as effective as α-
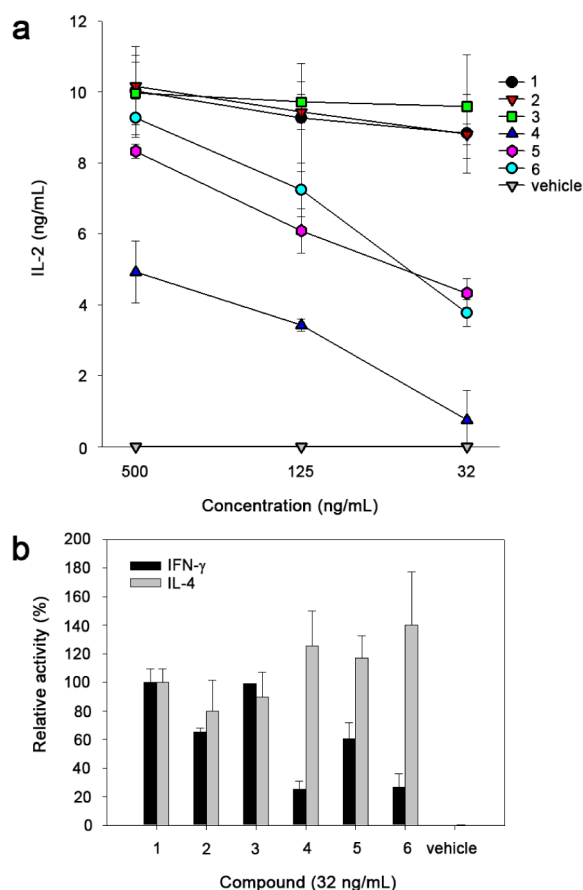

Figure 4. Biological evaluation of compounds **1–6**. (a) IL-2 secreted by the DN32.D3 NKT hybridoma cells. IL-2 production was measured from the cocultured supernatants of NKT hybridoma DN32.D3 and mouse CD1d transfected RBL cells after 16 h. The representative data of two individual experiments are expressed as the means ± SD of the duplicates. (b) IFN-γ and IL-4 secretion by mouse splenocytes after individual treatment with compounds **1–6**. Cytokine production was measured after 72 h of culture. The results are expressed as the relative activity. The representative data of two individual experiments are expressed as the means ± SD of the triplicates.

GalCer when promoting IL-2 production. Analogue **4** was less efficient than α-GalCer. The ω-hydroxy group lacking analogues **5** and **6** elicited the production of IL-2, but they were less efficient than α-GalCer or the corresponding ω-hydroxy analogues **2** and **3**.

IL-2 secretion is dependent on antigen-loaded CD1d in this assay system.[28,29] Therefore, the ω-hydroxy analogues with the proper chain lengths can be accommodated within the CD1d binding groove. By comparing the IL-2 production levels, we deduced that binding involves hydrogen bonding with the ω-group. If hydrogen bonding interactions of the ω-groups were not involved, relying only on the hydrophobic interactions in the binding groove, ω-hydroxy analogues **2** and **3** would not induce more IL-2 secretion than analogues **5** and **6**. This hydrogen bonding interaction might be strong enough to compensate for the loss of a full-length fatty acid chain from the α-GalCer **1** because the potencies of ω-hydroxy analogues **2** and **3** were maintained.

During a second biological evaluation, we measured the levels of the cytokines released from intact NKT cells. While NKT hybridoma cells tend to produce IL-2 when stimulated, primary NKT cells produce IFN-γ (Th1) and IL-4 (Th2) with various other cytokines. The IFN-γ and IL-4 levels were measured in the supernatant of mouse splenocytes cultured with 32 ng/mL analogues **2–6**. Figure 4b presents the relative IFN-γ and IL-4 production levels of **2–6** compared with those of **1**. The NKT stimulation activity for ω-hydroxy analogue **3** was very close to that of the parent α-GalCer **1**, while the activity of **2** was slightly lower than that of **1**. The cytokine release profiles for **2** and **3** were similar to that of α-GalCer. However, **5** and **6** biased cytokine secretion toward the Th2 response; these analogues demonstrated a stronger stimulatory effect on IL-4 production than α-GalCer **1** while promoting less IFN-γ production than **1**. The analogue with longer-alkyl chain (**4**) also induced NKT cell cytokine responses with a Th2 bias.

As an in vivo evaluation, the serum cytokine levels were measured after intravenous injection of analogue **2** (0.5 μg/mouse) into naïve C57BL/6 mice. The α-GalCer **1** was also tested for in vivo comparison. As shown in Figure 5, analogue **2**
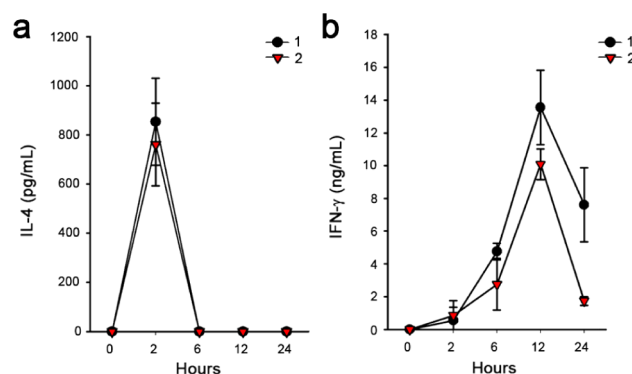
Figure 5. Serum (a) IL-4 and (b) IFN-γ levels from mice injected (i.v.) with α-GalCer (**1**) or ω-hydroxy α-GalCer analogue **2** (0.5 μg/mouse). Representative data of two individual experiments are shown as the means ± SD of three mice.

exhibited comparable activity to α-GalCer **1**. Both **1** and **2** induced a rapid rise of IL-4 with the peak value at 2 h and a delayed elevation of IFN-γ with the peak value at 12 h after treatment. Analogue **2** induced nearly an equal amount of IL-4 secretion as α-GalCer **1** did, while the effect of **2** on IFN-γ secretion was slightly lower than that of **1**. These in vivo cytokine release profiles for **2** were very similar to that in vitro.

Analogues **4–6**, which showed a weaker stimulatory effect on IL-2 production than α-GalCer **1** as well as analogues **2** and **3**, induced NKT cell cytokine responses with a Th2 bias. The Th1–Th2 balance is determined by numerous factors;[5,16,30] one of the important factors was the stability of the glycolipid/CD1d complex.[31] It has been generally believed that the less stable complex induces a biased Th2 response. In this respect, the Th2 biasing analogues **4–6** are expected to form less stable complexes than analogues **2** and **3**.[32] Conversely, ω-hydroxy analogues **2** and **3**, which provide responses that are less Th2-biased than analogues **4–6**, should form more stable glycolipid/CD1d complexes. The only structural difference between ω-hydroxy analogues **2** and **3** and truncated analogues **5** and **6** is the presence of a hydroxyl group at the end of each acyl chain. Thus, the hydroxyl group is thought to play an important role in strengthening the glycolipid/CD1d complex,

presumably via hydrogen bonding with an amino acid residue in the A′ pocket, as the docking experiments suggest. The structural difference between ω-hydroxy analogues **2/3** and **4** is that the latter has a longer space chain. Analogue **4** was designed to have a poor hydrogen-bonding interaction with the amino acid residue in the A′ pocket. Its pure Th2-like cytokine release profile suggests that it forms less stable complexes than analogues **2** and **3**, which may be due to the absence of hydrogen-bonding interactions. Together with the observed IL-2 production levels, these cytokine-polarizing properties support our hypothesis that ω-hydroxy analogues with proper chain lengths can bind to CD1d using hydrogen bonds.

In conclusion, we found that substituting the long fatty acid chain in α-GalCer with a shorter ω-hydroxy fatty acid chain maintains the NKT cell stimulation activity. After combining these results with our molecular modeling study, we suggest that hydrogen-bonding interactions occur between the ω-hydroxy group and a polar amino acid residue in the hydrophobic CD1d binding groove. This hydrogen bonding interaction may compensate for the loss of a hydrophobic interactions generated by a full-length fatty acid chain. This study will guide the design of novel immunotherapeutic agents that are structurally distinct from typical glycolipids. Further studies are in progress to design and synthesize therapeutically useful NKT cell activators by capitalizing on the polar interactions in the CD1d binding groove.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Additional figure displaying the molecular docking simulation of analogue **6** (Figure S1), Table S1, detailed information regarding the experimental procedures, characterization data, and copies of ^{1}H and ^{13}C spectra for final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(S.K.) Tel: 82-2-880-2487. Fax: 82-2-888-0649. E-mail: pennkim@snu.ac.kr.

Notes

The authors declare no competing financial interest.

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