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Minimum structure requirement of immunomodulatory glycolipids for predominant Th2 cytokine induction and the discovery of non-linear phytosphingosine analogs

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Abstract—Analogs of immunomodulatory glycolipid OCH (**2**) were prepared and minimum structure requirement to exhibit equivalent profiles was disclosed. Analogs bearing non-linear hydrocarbon chain in the phytosphingosine moiety (**18**, **19**) were shown for the first time to possess comparable cytokine inducing profile to **2**. Molecular modeling of **2**/hCD1d complex based on the crystal structure of α-GalCer (**1**)/hCD1d complex is also described.

Natural killer T (NKT) cells are potent producers of immunoregulatory cytokines, and are restricted to glycolipid antigens presented by CD1d, a glycoprotein strucurally and functionally related to non-classical major histocompatibility complex (MHC) class I.[1] The glycolipids, an α-galactosylceramide named KRN7000 **1**[2] and an altered analog termed OCH **2** possessing a shorter phytosphingosine side chain,[3] have been identified as NKT cell ligands (Fig. 1). It was recently shown that a isoglobotrihexosylceramide (iGb3 **3**), though not yet purified and characterized in all mammalian species, may be an endogenous ligand of CD1d.[4] The X-ray crystallographic structures of mouse (m) CD1d[5] and human (h) CD1d in complex with **1**[6] indicated that the complete occupation of the binding groove of CD1d by **1** contributes to the sustained stimulation of NKT cells to induce robust immunological response, while altered analogs such as **2** with short phytosphingosine chain may result in short duration of stimulation and cause differential polarization of NKT cells.[7] Compound **2** was shown to induce a predominant production of a key immunomodulatory Th2 cytokine interleukin-4 (IL-4) over proinflammatory Th1 cytokine interferon-γ (IFN-γ), while **1** induced both Th1/Th2 cytokines. Only compound **2** but not **1** is significantly effective in animal models of Th1-mediated autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA).[3,8]

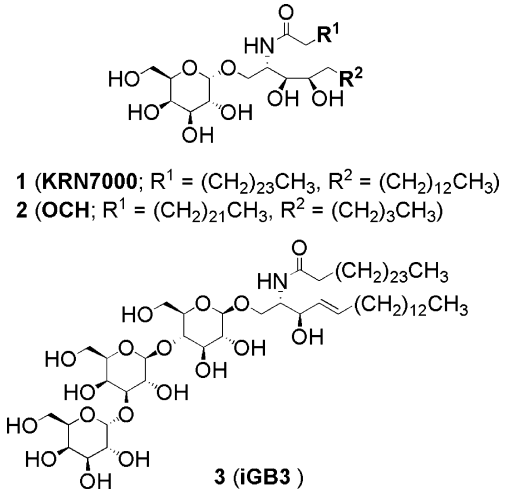

Figure 1. Known glycocylceramides as ligands of CD1d.

Keywords: OCH; Th2 cytokine; Non-linear phytosphingosine analogs.
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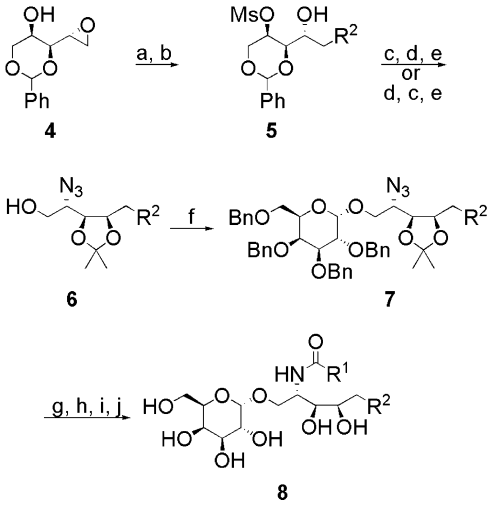
Savage et al. have reported the influence of the chain lengths on the cytokine releasing profile in the context of IL-4/IFN-γ ratio, which presented three compounds of short phytosphingosine chain including **2**.[9] Quite recently, Wong et al. have reported a structure–activity relationship (SAR) of fatty acyl chain analogs of **1** in which some showed more potent Th1 cytokine response than **1**.[10] As part of our efforts to obtain more potent ligand for the enhancement of Th2 response, a series of analogs based on **2** with altered ceramide moiety was prepared and assayed in vitro for cytokine production.[11] In this letter, the SAR of derivatives of **2** including non-linear hydrocarbon chain analogs is disclosed and minimum structure requirement to exhibit equivalent profiles is discussed.

The analogs were prepared by the versatile method developed by our group[12] (Scheme 1). The moieties of sphingosine base substituents were introduced to the known epoxide **4** by means of nucleophilic addition of alkyl or aryl lithium reagents or corresponding magnesium bromides. After regioselective mesylation of the axial hydroxyl group, compound **5** was subjected to benzylidene cleavage and azidation, after which secondary hydroxyl groups were protected to provide isopropylidene acetal **6**. Glycosidation with tetra-*O*-benzyl-α-D-galactosyl bromide in the presence of tetrabutylammonium bromide gave **7**, whose azido group was reduced to an amine and acylated with suitable carboxylic acids. Finally, all the protective groups were removed to give the desired analogs.

The analogs were evaluated for their ability to induce IL-4 and IFN-γ relative to **2**. IL-4 and IFN-γ secretion were assessed with spleen cells prepared from C57BL/6 mice, which were incubated with 100 ng/ml of glycolipids for 72 h and the cytokines in the culture supernatant were measured by ELISA.[13]

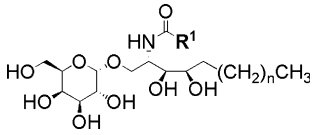


Scheme 1. Reagents and conditions: (a) R^2Li or R^2MgBr, CuI, THF, −40 °C, 93–98%; (b) MsCl, pyridine, −40 °C, 34–93%; (c) H_2, cat. $Pd(OH)_2$, EtOH, rt, or 6 N HCl, MeOH, rt, 69–100%; (d) NaN_3, DMF, 95 °C, 27–66%; (e) cat. *p*-TsOH, 2,2-dimethoxypropane, rt, 65–75%; (f) tetra-*O*-benzyl-α-D-galactosyl bromide, *n*-Bu_4NBr, MS4A, DMF–toluene (1:2.5), rt, 21–68%; (g) H_2, Lindlar catalyst, EtOH, rt; (h) R^1CO_2H, EDCI·HCl, HOBt, *i*-Pr_2NEt, DMF–CH_2Cl_2 (1:3.5), 40 °C, 51–100% (2 steps); (i) HCl–dioxane, rt, or 80% AcOH, 80 °C; (j) H_2, cat. $Pd(OH)_2$, MeOH–$CHCl_3$ (3:1), rt, 40 °C, 52–91% (2 steps).

Influence of the chain length of the fatty acid was examined first ($\mathbf{R}^1$, Table 1). The chain length of the phytosphingosine moiety was fixed to that of **2**. For unambiguous understanding, the length of the alkyl chain will be given as the number of carbon atoms (C_n) in the R moiety. Acyl chains shorter than C_{19} (**9**) showed only a weak cytokine production. As the chain became longer the cytokine release increased and for chains longer than C_{25} there was a marked increase in IFN-γ prduction that predominated IL-4 (**13**, **14**). For reference, **1** showed 128% release of IL-4 and 569% of IFN-γ relative to **2** in this assay.

Our interest was next focused on the phytosphingosine moiety. This is where it makes **2**, a mere truncated analog of **1**, a completely different switch of the NKT cell signal. We have prepared analogs altered in the phytosphingosine chain ($\mathbf{R}^2$, Table 2). In our hands chain length of C_1–C_4 (**15**–**17** and **2**) showed similar profiles.

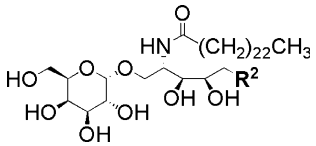
Table 1. Dependency of cytokine production on fatty acyl chain length[a]



Compound	R^1	*n*	IL-4 (%)[b]	IFN-γ (%)[b]
9	$-(CH_2)_{18}CH_3$	3	6	9
10	$-(CH_2)_{20}CH_3$	3	51	46
11	$-(CH_2)_{21}CH_3$	3	103	93
2	$-(CH_2)_{22}CH_3$	3	100	100
12	$-(CH_2)_{23}CH_3$	3	154	103
13	$-(CH_2)_{24}CH_3$	3	129	504
14	$-(CH_2)_{26}CH_3$	3	178	761
1	$-(CH_2)_{24}CH_3$	12	128	569

[a] At 100 ng/ml.
[b] Compared to **2** at 100 ng/ml.

Table 2. Dependency of cytokine production on phytosphingosine chain modification[a]



Compound	R^2	IL-4 (%)[b]	IFN-γ (%)[b]
15	$-CH_3$	102	81
16	$-CH_2CH_3$	105	96
17	$-(CH_2)_2CH_3$	115	112
2	$-(CH_2)_3CH_3$	100	100
18		98	74
19		211	284

[a] At 100 ng/ml.
[b] Compared to **2** at 100 ng/ml.

Although the mechanism of action at the molecular level is unclear,[14] this result is inconsistent with Savage's report in which compound **15** showed about twofold increase in IL-4 production without a change in IFN-γ.[9] Analogs bearing cyclic chain (**18**) or aromatic ring (**19**) were also prepared. To our knowledge, these are the first examples of non-linear hydrocarbon chain analogs of the phytosphingosine moiety that show similar cytokine inducing profile to **2**. As can be seen from the side view of the X-ray structure of **1**/hCD1d complex[6], the phytosphingosine chain of **1** is bent and enters the C′ pocket beyond ~C_6. There is a wide space before entering the C′ pocket, and we assume analogs **15**–**19**, which in length do not reach the C′ pocket, showed similar profiles to **2**.[15] Compound **19** with an aromatic ring appears to show slight increase in both cytokines compared to aliphatic analogs **2** and **15**–**18**. This is suggestive of aromatic interaction(s) with residues such as Phe77 and Trp131 in juxtaposition, although the possible interaction of the phenyl ring with the T cell receptor of NKT cells should not be omitted in this three-component interaction.

In order to explain the above results of short phytosphingosine analogs, we performed a molecular modeling of **2**/hCD1d complex based on the crystal structure of **1**/hCD1d complex[6] utilizing Maestro[16] program. Both acyl and phytosphingosine chains of **1** bound to hCD1d in the crystal structure were truncated in silico to correspond to **2**, and the complex thus obtained was further optimized[17]. Contrary to our expectation, the optimized structure of **2** in the complex had only a subtle, insignificant difference from **1** (Fig. 2).

As can be seen from Figure 3, the phytosphingosine chain of **2** has the precise length to reach the entrance of the C′ pocket. It can well be assumed from the model that C_1–C_5 segment of the phytosphingosine chain contributes little to the interaction with CD1d, and this might result in the short duration of **2** in complex with CD1d.[7] We have calculated the binding energy of CD1d and the ligands, whose acyl chain length was fixed to that of **2** and phytosphingosine chain altered incrementally from C_2 to C_9.[19] The binding energy increased linearly as the number of the methylene unit increased (data not shown), and no clear changing point was observed. It may be assumed that "static" energy calculation does not reflect the dynamics and the probability of the phytosphingosine chain to reside outside of the pocket. Dynamic simulation regarding the movement of the short chain is now in progress.

Several analogs related to **1** have been prepared to date, and many of them are equipotent to or even more potent than **1** in the aspect of IFN-γ secretion. In this study, the minimum structure requirement to exhibit equivalent profiles to **2** was disclosed, and also first examples of non-linear hydrocarbon chain phytosphingosine analogs that show similar cytokine inducing profile to **2** were discovered. The possibility of this alteration allows for synthetic access to the future analogs improved in their pharmacological and physicochemical properties.

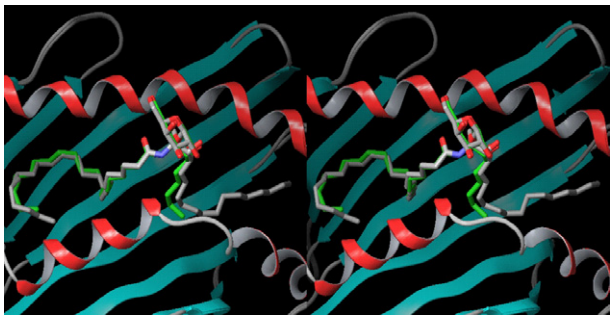


Figure 2. Optimized result of **2** (green)/hCD1d complex (stereo view). X-ray structure of **1** (gray) is superimposed.

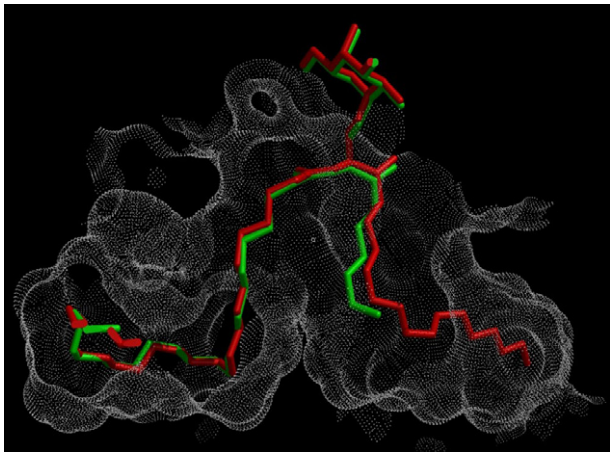


Figure 3. Side view of the optimized structure of **2** (green)/hCD1d complex. X-ray structure of **1** (red) is superimposed.

Acknowledgments

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13. Splenocytes were prepared from the spleens of C57BL/6 mice (6–8 weeks old, female) and suspended in a RPMI1640 medium (purchased from Nacalai) containing 10% fetal bovine serum (purchased from GIBCO), 5×10^{-5} M 2-mercaptoehtanol (purchased from GIBCO), 1 mM pyruvate (purchased from SIGMA), and 25 mM HEPES (purchased from SIGMA). The cells (5×10^5 cells/well) were stimulated with glycolipid derivatives at a concentration of 100 ng/ml for 72 h at 37 °C in a 96-well flat bottomed plate (purchased from IWAKI), and the concentration of IL-4 and IFN-γ in the culture supernatant was measured by ELISA (BD Pharmingen EIA Kit). OCH **2** was always included in the assay as a control and the cytokine release were expressed as relative to that of **2** for the mean of at least three experiments.
14. From the disclosed information in Ref. 9, the only difference in the experimental condition is the stimulation time, 72 h for us versus 60 h for Savage's group.
15. An analog bearing $-(CH_2)_4CH_3$ for R^2 was prepared but with an acyl chain shorter by one methylene unit [$-NHCO(CH_2)_{21}CH_3$]. The cytokine induction by this compound was 97% for IL-4 and 113% for IFN-γ, relative to **2**.
16. Maestro Ver. 7.5.112; Schrödinger, LLC: New York.
17. Optimization of the complex was performed stepwise as follows, utilizing Macromodel Ver. 9.0[18] [force field OPLS2005/solv.Water] (convergence threshold .05 kJ/mol/Å): (i) main chain, Asp80, Asp151, Thr154 and ligand fixed, (ii) main chain, Asp80($O^{\delta 1}$, $O^{\delta 2}$), Asp151($O^{\delta 1}$, $O^{\delta 2}$), Thr154(O^{γ}) and oxygen atoms of the ligand fixed, (iii) main chain, distances among selected ligand atoms and Asp80, Asp151, Thr154 fixed, (iv) main chain fixed, (v) optimization of all the atoms.
18. Macromodel Ver. 9.0 (Schrödinger, LLC); Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskcamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.
19. The optimization was performed for each analog according to the same procedure as for **2** described in Ref. 17.