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Synthesis of diglycosylceramides and evaluation of their *i*NKT cell stimulatory properties

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Abstract—Stimulation of *i*NKT cells is highly dependent on the structures of the glycolipids presented by CD1d. Furthermore, antigen processing and CD1d loading in lysosomes play central roles in controlling the stimulatory properties of glycolipid antigens. Previously, we determined that substitution at C6″ on α-galactosylceramides did not significantly impact stimulatory properties; however, it was not known if substitution at this position influenced lysosomal processing of oligoglycosylceramides. We have prepared a series of mono- and di-galactosylceramides to observe the impact of C6″ substitution on glycosidase truncation of these glycolipids. We found that substitution did not significantly impact glycosidase activity or loading into CD1d.

Natural killer T (NKT) cells are a subset of T cells and have been characterized as regulatory T cells.[1] The most prevalent and best studied subset of NKT cells expresses an invariant T cell Vα14/24 (mouse/human) receptor and is referred to as invariant NKT cells (*i*NKT) cells. Through this invariant T cell receptor, *i*NKT cells recognize bacterial and endogenous glycolipid antigens presented by CD1d, an antigen presentation protein related to major histocompatibility complex proteins. The hallmark response of *i*NKT cells is the rapid production and release of large quantities of cytokines, including proinflammatory cytokines (e.g., IFN-γ, IL-2) and immuno-modulatory cytokines (e.g., IL-4). These cytokines play critical roles in inducing a series of events leading to the activation of innate and adaptive immune cells. The most well-studied ligand for *i*NKT cells is a glycolipid, KRN7000 (Fig. 1), developed through anti-tumor structure–activity studies of glycolipids from the marine sponge *Agelas mauritianus*.[2] In further structure activity studies using cytokine release from NKT cells as an end point, we have determined that shortening the acyl chain in KRN7000 to eight carbons (from 26) yields a compound (**1** in Fig. 1) that loads effectively into CD1d without dependence on lipid-transfer proteins that are required for loading most glycolipids into CD1d.[3,4] We have also found that replacement of the hydroxyl group at C6″ (see Fig. 1 for glycosylceramide numbering) with an acetylamide gives a compound, **2**, that is a more potent stimulator of NKT cells than KRN7000 (both in vitro and in vivo).[5]

An important aspect of glycolipid presentation by CD1d and recognition by *i*NKT cells is lysosomal processing of glycolipids. Lysosomes in antigen-presenting cells contain an array of glycosidases that can truncate oligoglycosylceramides to monoglycosylceramides. Work by Prigozy et al.[6] demonstrated that diglycosylceramides with 1–2 or 1–3 glycosidic linkages (e.g., **3** in Fig. 1) require lysosomal processing and truncation to KRN7000 for presentation by CD1d and stimulation of *i*NKT cells. CD1d is cycled between lysosomes, where it is loaded with glycolipids, and the cell surface, where it presents bound glycolipids to *i*NKT cells. Deletion of the peptide sequence required for this cycling yields 'tail-deleted' CD1d (tdCD1d), which does not effectively cycle between the cell surface and lysosomes.[7] Consequently, glycolipids that are processed in the lysosome are not loaded into

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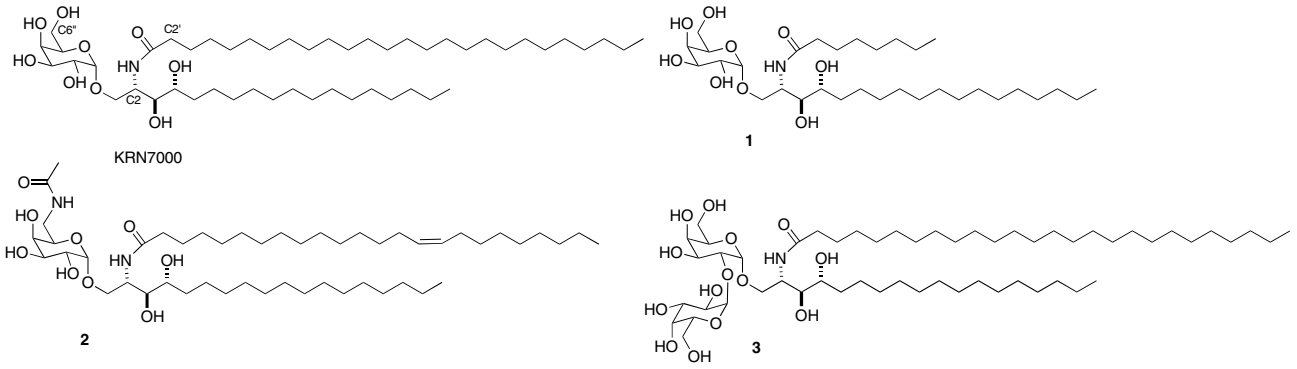


Figure 1. Structure of glycolipids KRN7000, **1**, **2**, and **3**.

CD1d, and tdCD1d is useful for observing dependence on lysosomal glycolipid processing.[6,7]

The rationale for preparing glycolipids substituted at C6″ was to use groups appended at this position to monitor trafficking, processing, and presentation of glycolipids.[8] We have found that fluorophores appended at C6″ do not significantly alter stimulation of *i*NKT cells.[9] However, we have not determined if C6″ substitution affects lysosomal processing. To this end, we prepared a series of diglycosylceramides including one with C6″ substitution. For this series, we used the C_8 acyl chain in **1** to remove possible influences of lipid-transfer proteins[10,11] and thereby only focus on activities of lysosomal glycosidases.

The glycolipids prepared to determine the effects of C6″ substitution on lysosomal processing are shown in Figure 2. Comparison of *i*NKT cell stimulation of **1** and **4** with wild-type CD1d and tdCD1d provides a measure of the influences of lysosomal processing on these glycolipids. Glycolipid **1** would be expected to load into CD1d well and stimulate *i*NKT cells comparably with wild-type and tdCD1d. However, the requirement for processing would make **4** less stimulatory with tdCD1d. If the substitution at C6″ does not significantly influence lysosomal trafficking, then the same trend would be expected. That is, **6** should lose stimulatory properties with tdCD1d.

The synthesis of disaccharide **4** is described in Scheme 1. A Schmidt coupling[12] of **7** and **8** gave the corresponding disaccharide, and the anomeric hydroxyl group was revealed after deacylation with the mono-acetate salt of ethylene diamine. The disaccharide was coupled with the appropriately protected ceramide using Gin's method,[13] and deprotection gave **4**.

The preparation of C6″ modified galactosylceramide **5** is detailed in Scheme 2. The synthesis borrows from work with fluorophore labeled galactosylceramides.[9] Coupling of C6 azido galactose **12** with ceramide gave **13**. Dissolving metal deprotection also caused reduction of the azide to the amine, and subsequent acylation gave **5**.

For the preparation of **6** (Scheme 3), C6 azido acceptor **15** was used, and a pathway similar to that used for the synthesis of **4** was followed, except that deprotection was achieved using dissolving metal conditions. The acyl group was installed as described in the preparation of **5**.

The *i*NKT cell stimulatory properties of glycolipids **1**, **4**, **5**, and **6** were determined using *i*NKT cell hybridoma

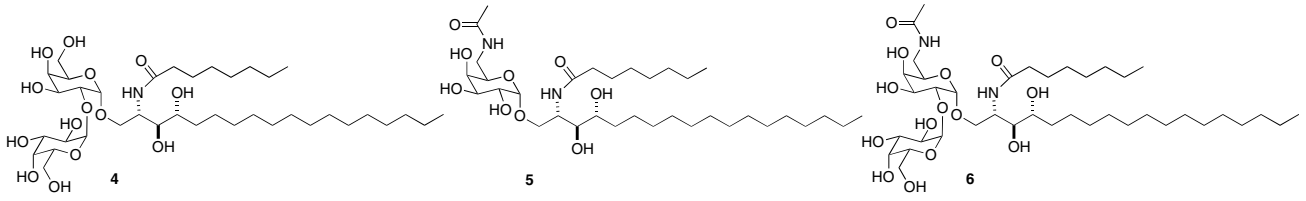
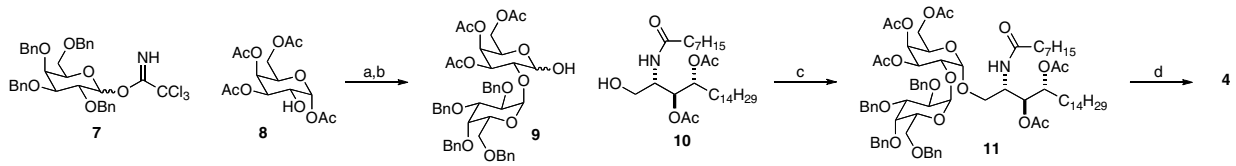
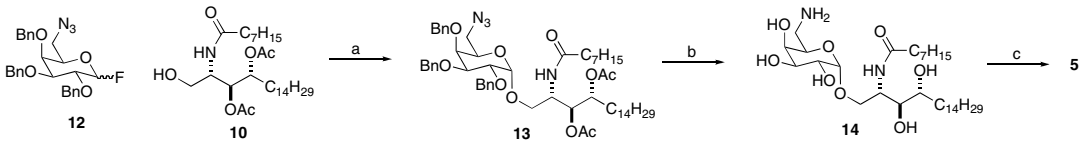


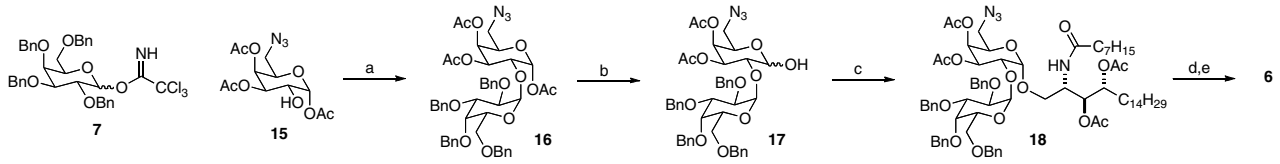
Figure 2. Structures of diglycosyl and C6″ appended glycolipids used in the study of lysosomal processing of C6″ substituted glycolipids.



Scheme 1. Preparation of diglycosylceramide **4**. Reagents (yields in parentheses) (a) TMSOTf, CH_2Cl_2 (40%); (b) elthylene diamine, AcOH, THF (75%); (c) diphenylsulfoxide, Tf_2O, tri-*t*-butylpyrimidine, CH_2Cl_2 (71%); (d) H_2, Pd/C, THF, methanol; MeONa, methanol (56%).



Scheme 2. Preparation of C6″ *N*-acyl amino substituted galactosylceramide **5**. Reagents (yields in parentheses) (a) $AgClO_4$, $SnCl_2$ CH_2Cl_2 (54%); (b) Na°, NH_3 (40%); (c) AcOH, HOBt, DCC (90%).



Scheme 3. Preparation of C6″ *N*-acyl amino substituted di-galactosylceramide **6**. Reagents (yields in parentheses) (a) TMSOTf, CH_2Cl_2 (37%); (b) ethylene diamine, AcOH, THF (64%); (c) diphenylsulfoxide, Tf_2O, tri-*t*-butylpyrimidine, CH_2Cl_2 (53%); (d) Na/NH_3, (27%); (e) AcOH, DCC, HOBt (22%).

and dendritic cells as antigen-presenting cells. Dendritic cells generate a well-established lysosome and effectively truncate many oligoglycosylceramides to stimulatory compounds. As shown in Figure 3A, all four of the glycolipids stimulated cytokine release. The monoglycosylceramides are not dependent on processing and can load into CD1d directly, and as expected they proved to be more potent than the diglycosylceramides. Notably, C6″ did not play a significant role in stimulatory properties. To further establish the dependency of the diglycosylceramides for lysosomal processing, dendritic cells with tdCD1d were used as antigen-presenting cells. Because tdCD1d does not effectively sample the lipids in lysosomes, it was expected that stimulation with **4** and **6** would be decreased with tdCD1d. As shown in Figure 3B, this trend was observed, while the monoglycosylceramides were less dependent on CD1d sampling of lysosomes.

Comparisons of the *i*NKT cell stimulatory properties of mono- and diglycosylceramides with and without substitution at C6″ indicate that substitution at this position does not play a significant role in stimulatory properties of these glycolipids or their abilities to act as substrates for lysosomal glycosidases. Small changes in glycosylceramide structure can change *i*NKT cell stimulatory properties dramatically: α-galactosylceramides (e.g., KRN7000 and **1**) are potent stimulators while β-galactosylceramide is only very weakly active or inactive; α-glucosylceramide is much less potent than α-galactosylceramide. However, the C6″ position appears to be well suited for manipulation without significantly impacting glycolipid processing and presentation. We have shown that fluorophores of modest size (e.g., a danyl group) can be appended at C6″ without significantly altering NKT cell responses,[9] and the acetamide at C6″ in **6** does not interfere with processing. It is anticipated that larger groups, such as fluorophores of modest size, appended at this position with no impact processing; however, this has not yet been demonstrated. Furthermore, it is not yet well understood how substitution at this position affects glycolipid trafficking and lipid-transfer protein interactions,

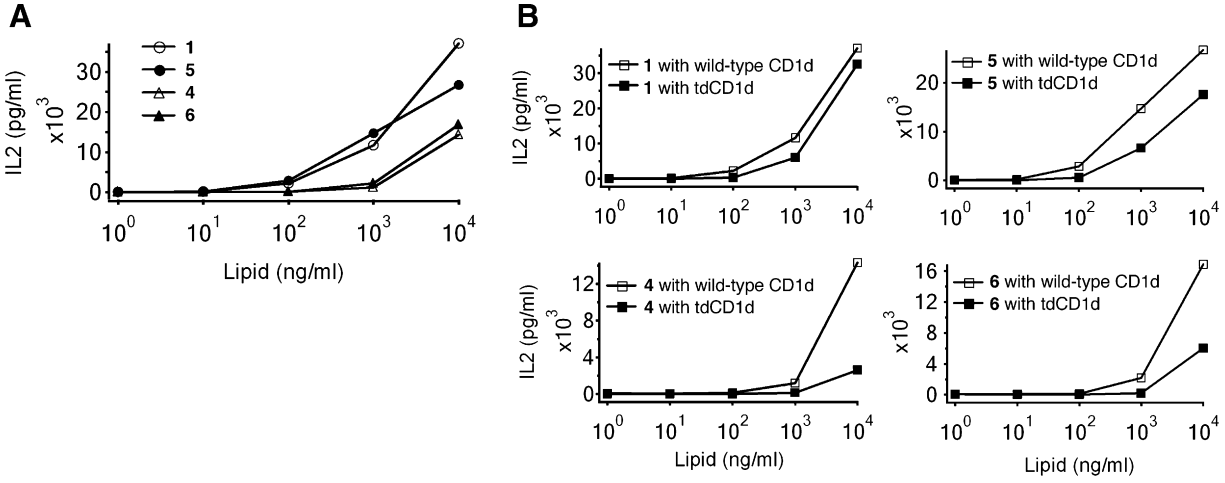


Figure 3. Comparison of stimulatory activity on mouse Vα14 *i*NKT hybridoma DN3.2.D3 by murine bone marrow-derived dendritic cells in the presence of different concentrations of glycosylceramides **1**, **4**, **5**, and **6**. Indicated are the mean release amounts of IL-2 (pg/mL) in cell culture supernatants determined by ELISA. (A) Wild-type CD1d. (B) Comparison of stimulation with wild-type CD1d and tdCD1d.

and studies are ongoing to determine impacts on these interactions.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.067.

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