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6'-Derivatised α-GalCer Analogues Capable of Inducing Strong CD1d-Mediated Th1-biased NKT Cell Responses in Mice

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During the past years the use of glycolipids as immunostimulating agents has become increasingly important.[1,2] When presented by the major histocompatibility complex (MHC) class I-like molecule CD1d, certain glycolipids are recognized by the semi-invariant T cell receptors (TCR) of natural killer T (NKT) cells.[3] The prototypical antigen for NKT cells is α-galactosyl ceramide (α-GalCer; **1**).[4] Upon recognition of the CD1d-α-GalCer bimolecular complex by their TCR, NKT cells are activated, resulting in the rapid release of T helper 1 (Th1) and T helper 2 (Th2) cytokines.[5]

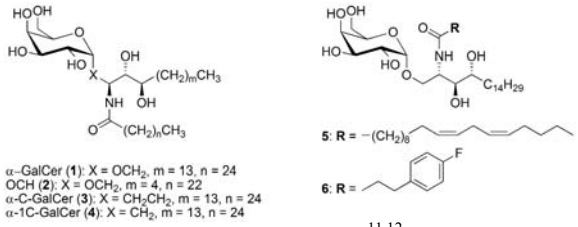
As both Th1 and Th2 cytokines influence the outcome of different immune responses, disruption of the carefully controlled Th1/Th2 balance can lead to disease induction and progression. While certain autoimmune diseases are characteristic of hyporesponsiveness to Th2 and overactivation of pathogenic Th1 cells, the opposite is true for many types of cancer that have a predominant Th2 response. Hence, α-GalCer analogues that induce a biased Th1/Th2 response are highly awaited.

Attempts to selectively control the rapid secretion of cytokines by NKT cells have led to the development of several α-GalCer analogs with interesting immunomodulatory properties (Chart 1). With the synthesis of OCH (**2**), a direct relationship has been shown between the shortening of lipid tail lengths and biasing of the cytokine release profile toward a Th2 response.[6] Porcelli reported that substituting the *N*-acyl chain of α-GalCer with shorter, unsaturated fatty acids modifies the outcome of Vα14i NKT cell activation.[7] Analogues containing multiple *cis*-double bonds in the acyl chain (e.g., **5**) potently induced a Th2-biased cytokine response. Conversely, Wong et al. have found that introduction of terminal aromatic groups into the fatty acyl tail (as in **6**) biases the profile toward a Th1 response.[8]

A proof-of-principle that α-GalCer analogues capable of skewing the cytokine release profile may translate in an improved therapeutical outcome was established with α-C-GalCer (**3**). Characterized by a marked Th1 response, this compound exhibits markedly improved activity against melanoma metastases and malaria compared with α-GalCer.[9] Bittman recently reported a truncated non-isosteric C-glycoside, termed α-1C-GalCer (**4**), which produced less IFN-γ than α-GalCer, but showed higher IFN-γ/IL-4 and IFN-γ/IL-13 ratios, typical for a Th1-immune response.[10]

In 2005, the crystal structures of human[11] and mouse[12] CD1d complexed with α-GalCer or a short-chain variant were elucidated, revealing that the galactose ring is well ordered and extends above the surface of a lipid-binding groove. Different intermolecular hydrogen bonds are assumed to anchor α-GalCer in a proper orientation for recognition by the TCR of NKT cells.

Chart 1. Structures of selected α-galactosyl ceramides

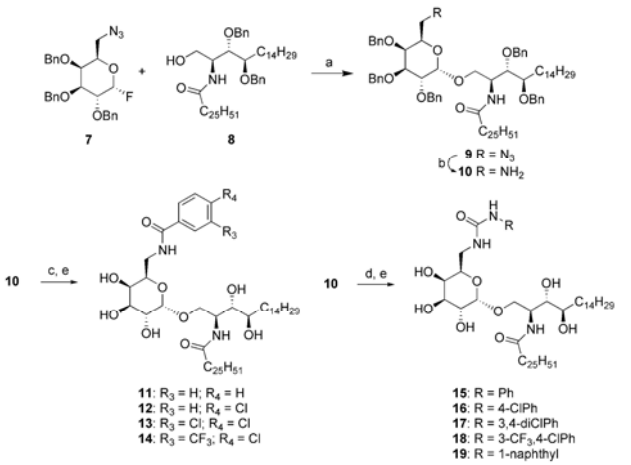


Recently, Borg et al. reported the structure of a human NKT TCR in complex with CD1d bound to α-GalCer.[13] Consistent with the previously proposed structures,[11,12] α-GalCer protrudes minimally from the CD1d cleft with only the galactosyl head group exposed for recognition by the NKT TCR and interacting solely with its CDR1α and CDR3α loops. The galactose ring is sandwiched between Trp-153 of CD1d and the aliphatic moiety of Arg-95α. The 2'-OH, 3'-OH and 4'-OH of the galactose ring form hydrogen bonds with amino acid residues of the invariant TCR α-chain. This mode of binding is consistent with the specificity the NKT TCR exhibits for α-GalCer versus closely related analogues modified at the sugar part.[14]

Interestingly, the Gal 6'-OH is the only sugar alcohol not involved in H-bond formation, suggesting the possibility of introducing modifications at that position. In this respect, we were attracted to a report from Savage et al. who attached small fluorophores at that position, resulting in modified α-GalCers that retained the capacity to stimulate NKT cells.[15] As it was envisioned that extra interactions might be established between CD1d and a 6'-OH modified α-GalCer, it was decided to investigate such analogues.

The synthetic strategy was similar to that used by Savage,[15] however, we chose to protect the secondary hydroxyl groups of the phytoceramide aglycon as benzyl ethers to facilitate the final deprotection (Scheme 1). Furthermore, to avoid solubility problems, the final debenzylation step was performed after modification of the 6'-amino group. The synthesis of 6'-azido-6'-deoxygalactosylceramide **9** started from the D-*ribo*-phytosphingosine building block **8**.[16] Mukaiyama glycosidation[17] involving **7**[15] as the donor afforded the desired α-glycoside **9** in 46% yield. Reaction of the crude amine **10**, obtained by Staudinger reduction of **9**, with the appropriate acid and EDC as the coupling reagent, followed by hydrogenolysis of the benzyl groups, gave the desired amide analogues **11-14**. Treatment of **10** with the appropriate isocyanates afforded, after final deprotection, the 6'-ureido-6'-deoxygalactosylceramide analogues **15-19**.

Scheme 1. Synthesis of NKT cell agonists **11-19**



Scheme I. Reagents (yields in parentheses): a) $SnCl_2$, $AgClO_4$, THF, 4 Å MS, -10 °C to rt, 2h (46%); b) (i) PMe_3, THF, rt, 4h; (ii) NaOH 1M, rt, 2h (quant.); c) R-COOH, EDC, DMF, 4h, rt; d) R-NCO, DMF, 0 °C to rt, 2h; d) H_2, Pd black, $CHCl_3$/EtOH: 1/3, 5h (33-65% over 2 steps).

All analogues were tested by measuring the serum cytokine levels after injection into C57Bl/6 mice. Based on their ability to induce significantly reduced IL-4 production and comparable levels of IFN-γ compared to α-GalCer (Figure 1), **12-19** were identified as Th1-skewing compounds.

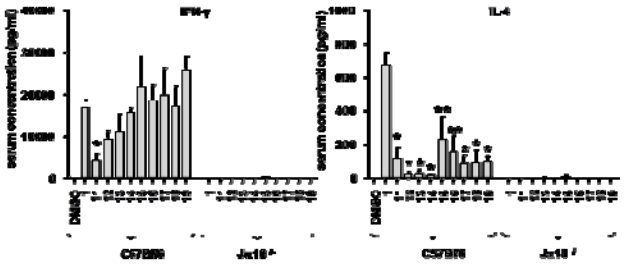


Figure 1. INF-γ and IL-4 secretion after intraperitoneal injection of α-GalCer and **11-19** in mice (** $P < 0.05$ and * $P < 0.01$ vs α-GalCer).

Considerably reduced IL-4 production was especially observed for the amides **12-14**, which still induced reasonable levels of IFN-γ. Remarkably, the unsubstituted benzamide **11** failed to induce a strong Th1 response, which could be restored by the introduction of electron-witdrawing substituents on the aromate. In general, levels of both cytokines were higher for the urea derivates compared to the amide derivatives, although the latter showed a more pronounced Th-1 bias. Compound **14**, featuring a 3-CF_3,4-Cl-benzamide substituent, emerged as the most promising Th1 polarizing agent, since it induced IFN-γ levels comparable to α-GalCer, and only marginal levels of IL-4. No cytokine induction was observed when **11-19** were injected into J$\alpha 18^{-/-}$ mice, indicating a TCR-dependent activation of NKT cells.

Although numerous factors likely play a role in shifting the cytokine profile, stability of the CD1d/glycolipid complex is believed to be a contributing cause.[18] Affirmatively, most of the known α-GalCer analogues able to induce polarized cytokine responses are characterized by modifications of the phytosphingosine or fatty acyl chains, expected to alter the affinity for CD1d. For the first time a series of α-GalCer analogues has been identified with an intact phytoceramide moiety, which are capable to skew the cytokine release profile to Th1 and possess a comparable ability to induce INF-γ secretion as α-GalCer. In contrast to modifications of other Gal OH-groups,[14a-c] these analogues clearly retain antigenic activity. Possibly the 6'-derivatives enjoy additional hydrophobic interactions, that increase binding with CD1d, resulting in the cytokine polarization. It is interesting to observe that at least in the amide series, electron withdrawing substituents on the aryl ring tend to induce the most promising Th1 cytokine profile. Interestingly, in the proximity of the 6'-position hCD1d structurally differs from mCD1d in that it contains a Trp-153 instead of Gly-155.[19] Hence, π–π interaction with the electron rich indole ring could lead to additional effect on the cytokine polarisation.[20] In vitro assays to investigate the effect of our analogues using human antigen presenting cells are in progress.

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Supporting Information Available: Experimental procedures for the preparation of **9-19** and for the *in vivo* stimulation with α-GalCer-analogues. This material is available free of charge via the Internet at htpp://pubs.acs.org.

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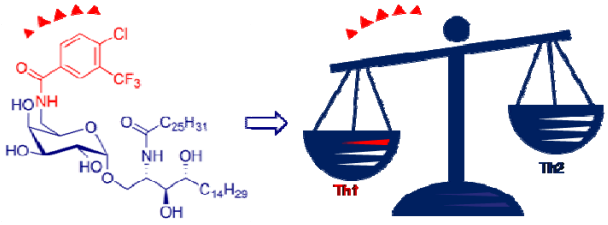

α-Galactosyl ceramide (α-GalCer, also known as KRN 7000) is known as the prototypical antigen for invariant natural killer T (NKT) cells. Stimulation of NKT cells by CD1d-mediated α-GalCer presentation leads to rapid release of Th1 and Th2 cytokines. Since Th1 and Th2 cytokines antagonize each other's effects, α-GalCer analogues that induce a biased Th1/Th2 response are highly awaited. With the exception of a C-glycoside (α-C-GalCer), most of the known α-GalCer analogues able to induce polarized cytokine responses are characterized by modifications of the phytosphingosine or fatty acyl chains, expected to alter the affinity for CD1d.

Herein we describe the synthesis of 6'-modified α-GalCer analogues with an intact phytoceramide moiety that are capable to skew the cytokine release profile to Th1, while maintaining strong antigenic activity. These analogues are characterized by the presence of an aromatic moiety that is connected via an amide or an urea linkage to C'-6 of the galactopyranose ring.