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New CD1d agonists: Synthesis and biological activity of 6″-triazole-substituted α-galactosyl ceramides

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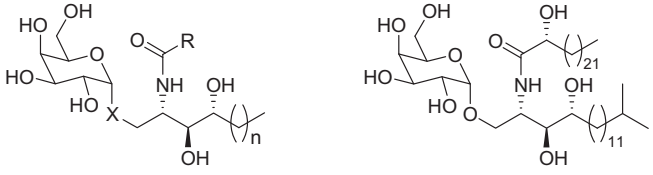
ABSTRACT

Huisgen [3+2] dipolar cycloaddition of 6″-azido-6″-deoxy-α-galactosyl ceramide **11** with a range of alkynes (or a benzyne precursor) yielded a series of triazole-containing α-galactosyl ceramide (α-GalCer) analogues in high yield. These α-GalCer analogues and the precursor azide **11** were tested for their ability to activate *i*NKT cells and stimulate IL-2 cytokine secretion in vitro, and IFN-γ and IL-4 cytokine secretion in vivo. Some of these analogues, specifically **11**, **12b**, **12f** and **13**, were more potent IL-2 stimulators than the prototypical CD1d agonist, α-GalCer **1**. In terms of any cytokine bias, most of the triazole-containing analogues exhibited a small Th2 cytokine-biasing response relative to that shown by α-GalCer **1**. In contrast, the cycloaddition precursor, namely azide **11**, provided a small Th1 cytokine-biasing response.

α-GalCer (KRN7000, **1**, Fig. 1) is a simplified synthetic analogue of the naturally occurring agelasphins (including AGL-9b, **2**, Fig. 1), which were isolated from the marine sponge *Agelas mauritianus*.[1] α-GalCer has become the prototypical ligand for studying the CD1d-restricted activation of invariant Natural Killer T cells (*i*NKT cells).[2] CD1d is an MHC-like protein located on the surface of various antigen-presenting cells, including dendritic cells and macrophages.[3] α-GalCer (**1**) binds through its two long lipid chains into two deep hydrophobic pockets of the CD1d molecule, to form an α-GalCer–CD1d complex.[4] CD1d then presents this glycolipid to T-cell receptors (TCRs) located on *i*NKT cells, an event which elicits an immune response through the release of both pro-inflammatory (Th1 (IFN-γ)) and regulatory (Th2 (IL-4)) cytokines.[5,6] The release of Th1 cytokines is associated with antitumour and antimicrobial functions,[7] whilst the release of Th2 cytokines is implicated in alleviation of autoimmune diseases[8–10] such as multiple sclerosis[11] and arthritis.[12] When both Th1 and Th2 cytokines are released together, however, their effects are counteractive, providing unpredictable biological effects.[13] The absence of a Th1/Th2 cytokine bias has hindered the therapeutic application of α-GalCer and encouraged the search for analogues of this CD1d agonist, which induce a more biased Th1/Th2 response.[14] Most modifications to α-GalCer have been to the ceramide portion of the molecule and some important examples are shown in Figure 1. For example, truncation of either the fatty acid chain (**3**)[15] or the sphingosine chain (OCH, **4**)[16] produces a Th2 cytokine-biasing response. A Th2 cytokine-biasing response is also observed when unsaturation is incorporated into the acyl chain (α-GalCer C20:2, **5**).[17] Switching the anomeric oxygen atom for a methylene group (α-*C*-GalCer, **6**) provides an example of a Th1-skewing analogue.[18] Despite the efforts of many laboratories, the factors which determine the nature and extent of any cytokine bias remain only partially understood.[19]



AGL-9b **2**

1 R = $(CH_2)_{24}CH_3$, n = 13, X = O (KRN7000)
3 R = $(CH_2)_6CH_3$, n =13, X= O
4 R = $(CH_2)_{22}CH_3$, n = 4, X = O (OCH)
5 R = (*Z*,*Z*)-$(CH_2)_9(CH{=}CHCH_2)_2(CH_2)_3CH_3$ n =13, X = O
6 R = $(CH_2)_{24}CH_3$, n = 13, X = CH_2 (α-*C*-GalCer)

Figure 1. Prototypical KRN7000 (**1**), naturally occurring AGL-9b (**2**) and biologically active analogues **3**–**6**.

The design of many glycolipid analogues has been guided by the published crystal structures of the CD1d–KRN7000 complex and

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the TCR–KRN7000–CD1d complex.[20,21] These crystal structures reveal the hydroxyl group at the 6-position of the sugar head-group is the only hydroxyl group that is not directly involved in hydrogen bonding to the CD1d protein or the TCR of the *i*NKT cell. As such, α-galactosyl ceramides in which the hydroxyl group at the 6-position of the sugar head group has been modified, have become attractive synthetic targets. Indeed, many groups have shown that the TCR–glycolipid–CD1d interaction is tolerant to derivatisation at this position of the molecule.[22,23] For example, Tashiro et al. found that methyl ether **7** (RCAI-61) skews the cytokine profile towards a Th1 response.[22a] The activity of **7** has been rationalised by the methylation disrupting a hydrogen bond between the oxygen atom of the 4″-hydroxyl group and the hydrogen atom of the 6″-hydroxyl group, thus rendering the 4″-hydroxyl group more available for TCR recognition.[22a]

The incorporation of nitrogen-containing functionalities into the 6-position of the sugar has also proven to be worthwhile.[23] From a practical point of view, incorporating a nitrogen functionality into this position of the glycolipid serves to increase substrate solubility (α-GalCer is very poorly soluble in most organic solvents and water), facilitating easier synthesis, purification, handling and biological administration.[23a] More importantly, this type of structural change has also been shown to produce desirable biological effects. For example, Trappeniers et al. have shown that analogues containing aryl amides and ureas at the 6″-position (e.g., **8** and **9**) can skew the cytokine profile in favour of a Th1 cytokine response.[23b] A crystal structure of the CD1d–**9**–TCR complex reveals the urea substituent residing in a hydrophobic pocket, effectively providing an extra site for binding. In addition, the naphthyl ring of **9** is likely to be involved in π–π stacking with the electron-rich indole ring of the proximal Trp153.[23c] Interestingly, when the 6-amido group is linked to a poly(ethylene glycol) chain, as in amide analogue **10**, the cytokine profile is reversed such that a Th2-biasing cytokine response is now observed (Fig. 2).[23c]

We have recently developed a synthesis of a 6″-azido-6″-deoxy-α-galactosyl ceramide (**11**), which is a useful precursor for the synthesis of 6″-*N*-derivatised α-GalCer analogues (Fig. 3).[24] Reducing the azide functionality provides the corresponding amine, potentially allowing ready access to amides, sulfonamides, ureas, thioureas and secondary amines. Azide **11** is also primed for more direct modification utilising click chemistry.[25–27] Alkyne–azide [3+2] dipolar cycloaddition reactions are highly

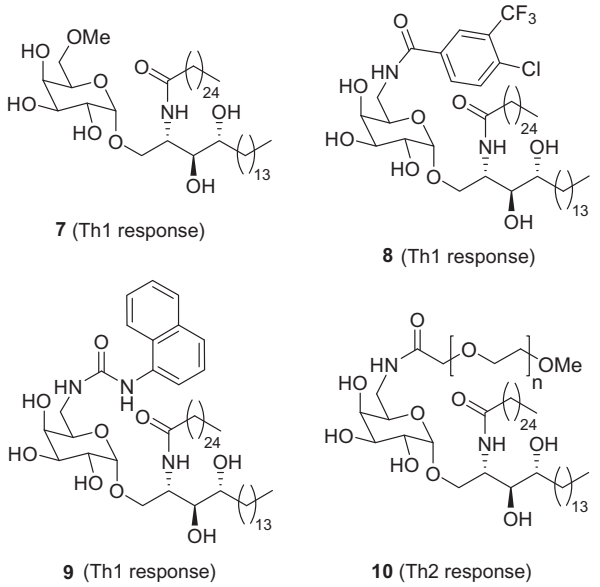

Figure 2. Biologically active 6″-derivatised α-galactosyl ceramide analogues **7**–**10**.

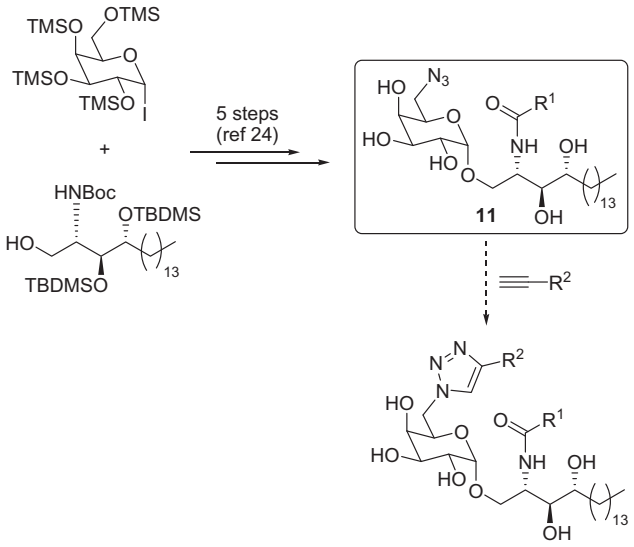

Figure 3. A route to 6-*N*-derivatised α-GalCer analogues, which allows late-stage modification of both the 6-*N*-substituent and the fatty acid group of the ceramide.

chemoselective, and we predicted that azide **11** should react readily without the need to protect the hydroxyl groups, providing ready access to a library of 1,2,3-triazole-containing KRN7000 derivatives from the large range of inexpensive, terminal alkynes that are available.

1,2,3-Triazoles are considered to be non-hydrolysable bioisosteres of the amide bond.[28a,29] In terms of atom positioning, the 1,4-disubstituted 1,2,3-triazole mimics the s-*cis* amide rotamer, whilst the 1,5-disubstituted analogue mimics the s-*trans* amide rotamer.[28b] The similarities and differences in hydrogen bonding of these isosteres have been described recently by Tron et al.[28b] Both 1,4- and 1,5-disubstituted triazoles can be accessed by judicious choice of reagents and reaction conditions.[25–27]

As part of a wider programme directed towards generating CD1d agonists,[30] and to test the scope of azide **11** as a cycloaddition partner, we embarked on the preparation of a range of 6″-triazole-substituted α-GalCer analogues. In the first instance, we focused on the 1,4-disubstitution pattern of the triazole, which was accessed via copper-catalysed click chemistry.[25,26] Heating equimolar quantities of azide **11** with different acetylenes of varying steric demand and hydrophilicity, in the presence of $CuSO_4$ and sodium ascorbate, provided the desired 1,4-disubstituted triazoles **12a–f** in excellent yields. In order to assess the effect of regiochemistry on the biological activity, we were also keen to access the alternative regioisomer of triazole **12a**.[27] To this end, heating azide **11** with phenyl acetylene in the presence of 5 mol % $Cp_*Ru(PPh_3)_2Cl$, provided 1,5-disubstituted triazole **13** in 78% yield. This ruthenium catalyst is also reported to mediate the reaction with internal alkynes and indeed, employing diphenyl acetylene yielded the 1,4,5-trisubstituted triazole **14** in 72% yield.[27] Finally, benzotriazole-containing analogue **15** was also prepared in 69% yield from an in situ-generated benzyne intermediate, by treating azide **11** with 2-(trimethylsilyl)phenyl trifluoromethanesulfonate in the presence of TBAF (Scheme 1).[31]

α-GalCer analogues **11**, **12a–f** and **13–15** were initially tested in vitro for their ability to stimulate IL-2 production by murine *i*NKT hybridoma cells (Fig. 4).[19] Analogues **11**, **12b**, **12f** and **13** were all more active than α-GalCer **1**, whilst the remaining analogues tested displayed significantly reduced activity relative to α-GalCer. There were interesting differences in activity between the benzene ring-containing analogues, in that triazole **13**, which contains the 1,5-disubstitution pattern on the triazole ring, was far more active