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Nonglycosidic Agonists of Invariant NKT Cells for Use as Vaccine Adjuvants

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Invariant natural killer T (*i*NKT) cells are important for regulating a variety of microbial, allergic, autoimmune, and tumor conditions.[1,2] *i*NKT cells are restricted by CD1d, a non-polymorphic MHC class I-like molecule, express a semi-invariant T-cell receptor (TCR), and are activated by glycolipid ligands bound to CD1d expressed on antigen-presenting cells (APCs). A potent agonist of human and mouse *i*NKT cells is α-galactosylceramide (α-GalCer, Figure 1, **1**),[2,3] which is a structural derivative of the agelasphins, isolated from the marine sponge *Agelas mauritianus*.[4,5] Owing to the type of α-stereochemistry found in these sponge glycosphingolipids and the fact that phytosphingosines are rare in mammalian glycosphingolipids,[6] the natural or physiological ligands of CD1d are still under discussion.

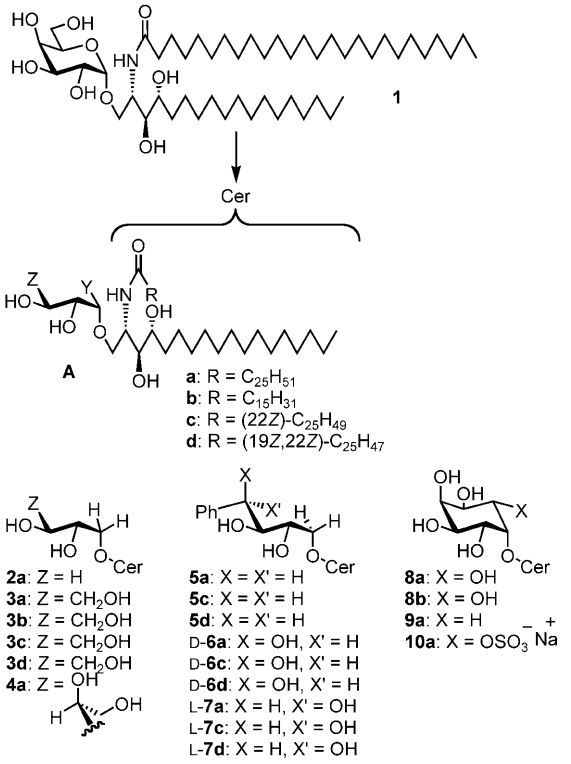

Figure 1. Nonglycosidic analogues of α-GalCer.

Activation of *i*NKT cells in vivo with α-GalCer leads to the release of both T helper type 1 (T_H1) and T helper type 2 (T_H2) cytokines. As a result, *i*NKT cells have the ability to either enhance or suppress T_H1 antigen-specific immune responses, such as virus- and tumor-specific immune responses.[7,8] Structural analogues of α-GalCer, with various affinities for CD1d or the invariant *i*NKT cell receptor TCR were shown to polarize *i*NKT cells differently, enhancing the response to pro-inflammatory bacterial, viral, and parasitic infections and some types of cancer, or suppressing autoimmune diseases in vitro and in vivo.[8–18] Of particular interest so far have been the *C*-glycoside[13] and a carbocyclic analogue[15] of α-GalCer, for which an enhanced T_H1 response has been reported.

Analysis of the crystal structure of mouse and human CD1d with and without α-GalCer has confirmed the binding architecture of the alkyl chains into the CD1d groove.[19,20] Additionally, the recently described co-crystal structure of human CD1d–α-GalCer with the human *i*NKT cell receptor TCR and ensuing studies have revealed new insight into the mode of recognition of CD1d-bound ligands by the *i*NKT cell receptor TCR.[21,22] As the polar head groups and the phytosphingosine chain occupying the F′ channel strongly influence the immunomodulatory effect of glycolipids,[18] fine-tuning of *i*NKT cell activation in vivo leading to a selective release of T_H1- or T_H2-type cytokines may be attainable by using analogues with specifically altered head groups. Another desirable feature of new analogues would be the ability to activate *i*NKT cells in such a way that therapeutic administration would not result in either the rapid loss of circulating *i*NKT cells, as found for α-GalCer,[23] or activation-induced anergy.[24]

Herein we describe the chemical synthesis and initial immunological characterization of a number of nonglycosidic α-GalCer analogues that result in the selective expansion and activation of *i*NKT cells, and that allow the identification of analogues with clinically more desirable features than α-GalCer. The crystal structure determination of mouse and human CD1d with and without α-GalCer revealed that hydrogen bonds with the anomeric oxygen atom and the hydroxy groups at positions 2 and 3 are important for the binding of the polar head group.[19,20] Therefore, compounds containing the L-*threo* configuration of the galactosyl residue at C2 and C3 (Figure 1, **A**) seemed to be ideal analogues of **1**. An increase in metabolic stability can be readily gained by having an ether linkage instead of an α-glycosidic linkage to the ceramide residue which consists of phytosphingosine and a C_{26}-fatty acyl

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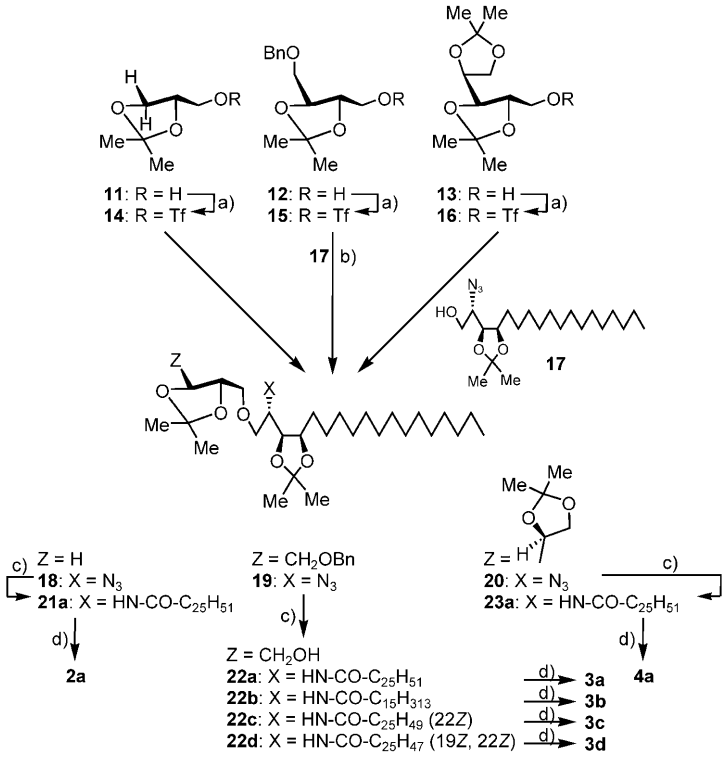
chain. The saturated C_{26}-fatty acyl chain fits quite well into the lipidic binding groove of CD1d (compounds **a**). However, the bound conformer has dihedral angles between carbon atoms C21–C24 and C18–C21 which indicate that C_{26}-fatty acyl chains with *Z*-configured C=C double bonds between C19–C20 and/or C22–C23 should also be accommodated in this groove (compounds **c** and **d**). For comparison, some truncated compounds with an *N*-palmitoyl residue were also prepared (compounds **b**). Based on these observations for binding of ceramides with hydrophilic head groups to CD1d and proper presentation of the polar head group to the T cell receptor (TCR), compounds **2**–**10** were designed as target molecules.

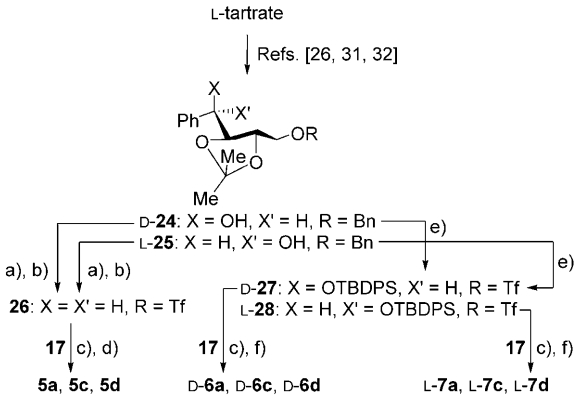
Synthesis of target molecules 2–7

First, target molecules **2**–**4** were prepared from **A** with Y = hydrogen, and Z = hydrogen, hydroxymethyl, or dihydroxyethyl, thus having been derived respectively from D-glycerol, L-threitol, and L-arabinitol (Scheme 1). To this end, known compounds **11**,[25] **12**,[26] and **13**[27–29] were transformed into the corresponding triflates **14**, **15**, and **16**, respectively. Reaction with azidophytosphingosine derivative **17**[30] with NaH as base in THF afforded the desired ether-linked intermediates **18**–**20** in excellent yields. Hydrogenolysis with palladium on carbon as the catalyst transformed the azido groups into amino groups; the benzyl ether of **19** was also cleaved, thus affording, after condensation with hexacosanoic acid, (22*Z*)-hexacosenoic acid, (19*Z*,22*Z*)-hexacosadienoic acid, and palmitic acid with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) in the presence of 1-hydroxybenotriazole (HOBt) in DMF at 45 °C, the *O*-isopropylidene-protected target molecules **21a**, **22a**–**d**, and **23a**. O-Deisopropylidenation with trifluoroacetic acid (TFA) as a catalyst in methanol/dichloromethane produced target molecules **2a**, **3a**–**d**, and **4a**, respectively, with good yields.

The crystal structure determination of the CD1d–α-GalCer complex, particularly with the *i*NK TCR, also revealed that aromatic amino acids are located close to C4 of the galactosyl residue.[19–21] Hence, in combination with a phenyl ring linked to C4, other binding characteristics could lead to a different presentation of the polar head group to the *i*NKTCR. Therefore, compounds **5**–**7** were designed as target molecules (Scheme 2). To this end, L-tartrate was transformed into D-*xylo*- and L-*arabino*-4-phenylbutanetetrols D-**24** and L-**25**.[26,31,32] Deoxygenation based on Barton's procedure,[33] hydrogenolytic debenzylation, and then introduction of the trifluoromethanesulfonyl (Tf) group afforded **26**. Ether bond formation with phytosphingosine derivative **17** and subsequent azido group reduction, attachment of the three different C_{26}-fatty acids, and cleavage of the *O*-isopropylidene groups afforded target molecules **5a**,**c** and **d**. 4-O-Silylation of D-**24** and L-**25** with *tert*-butyldiphenylsilyl (TBDPS) chloride and imidazole and following triflate formation as described above furnished intermediates D-**27** and L-**28**. Reaction with **17**, azide group reduction, introduction of the fatty acyl residues, and total deprotection led to target molecules D-**6a**,**c**,**d** and L-**7a**,**c**,**d**, respectively.



Scheme 1. Synthesis of target molecules **2a**, **3a**–**d** and **4a**. Reagents and conditions: a) 2,6-di-*tert*-butylpyridine, Tf_2O, CH_2Cl_2, −15 °C (90%); b) NaH, THF, 0 °C→RT (**18**: 90%, **19**: 94%, **20**: 95%); c) 1) Pd/C, H_2, MeOH, RT, 2) R-CO_2H, EDC, HOBt, DMF, 45 °C (**21a**: 70%, **22a**: 81%, **22b**: 80%, **22c**: 68%, **22d**: 71%, **23a**: 70%); d) TFA, MeOH/CH_2Cl_2 (10:1), RT (**2a**: 70%, **3a**: 71%, **3b**: 65%, **3c**: 81%, **3d**: 67%, **4a**: 60%).



Scheme 2. Synthesis of target molecules **5**–**7**. Reagents and conditions: a) 1) PhOC(S)Cl, Py, DMAP, CH_2Cl_2, RT, 2) Bn_3SnH, AIBN, toluene, reflux (95%); b) 1) Pd/C, H_2, MeOH/EtOAc (2:3), RT, 2) Tf_2O, DBP, CH_2Cl_2, −10 °C (76%); c) NaH, THF, 0 °C→RT (84–93%); d) 1) Pd/C, H_2, EtOAc, RT, 2) R-CO_2H, EDC, HOBt, NEt_3, DMF, 45 °C, 3) TFA, MeOH/CH_2Cl_2 (10:1), RT (yield over three steps: **5a**: 47%, **5c**: 43%, **5d**: 67%); e) 1) TBDPS-Cl, imidazole, RT, 2) Pd/C, H_2, EtOAc, 3) Tf_2O, 2,6-di-*tert*-butyl-4-methylpyridine, CH_2Cl_2, −10 °C (D-**27**: 92%, L-**28**: 94%); f) 1) Pd/C, H_2, EtOAc, RT, 2) R-CO_2H, EDC, HOBt, DMF, 45 °C, 3) TBAF, THF, RT, 4) TFA, MeOH/CH_2Cl_2 (10:1), RT (yield over four steps: D-**6a**: 41%, D-**6c**: 47%, D-**6d**: 53%, L-**7a**: 36%, L-**7c**: 48%, L-**7d**: 55%).

myo-Inositol with a *meso* structure is an ideal precursor for the design of α-GalCer analogues when the ceramide residue is linked to C5, thus leading to *neo*-inositol derivatives **8** (Figure 1) with enantiotopic L- or D-*arabino* and *threo* moieties. In addition, the C6 hydroxy group could be either replaced by hydrogen (compound **9**) or, more importantly, used for the attachment of a sulfate residue (compound **10**). The latter modification could lead to a different biological behavior because CD1d contains an arginine residue (Arg 79) as a binding partner ~6 Å from this position.[19,20] These compounds were readily obtained from *myo*-inositol[34] and their immunological properties are under investigation.

The nonglycosidic compounds are functional *i*NKT cell agonists in vitro

To assess whether the newly synthesized compounds are functional in activating *i*NKT cells, splenocytes from C57BL/6 mice were cultured in the presence of various concentrations of CD1d-binding ligands. The concentration of IFN-γ and IL-4 in the supernatant released after *i*NKT cell activation (Figure 2 A, B; shown for compounds **2a**, **3a**, and **4a**) was measured using cytokine-specific ELISAs.[35–38] The results of these experiments demonstrated that threitolceramide **3a** and arabinitolceramide **4a** induced the release of both IFN-γ and IL-4, though at slightly lower levels (for **3a**) than α-GalCer (**1**); this result is considered therapeutically desirable (see below). In contrast, glycerolceramide **2a** did not stimulate mouse *i*NKT cells. This outcome is in accordance with recently published data.[39]

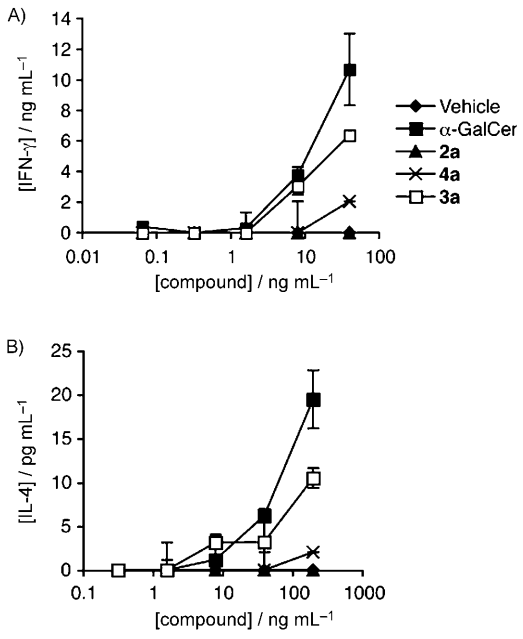


Figure 2. Nonglycosidic compounds differentially activate *i*NKT cells in vitro. Splenocytes from C57BL/6 mice were cultured for 48 h in the presence of various concentrations of vehicle, α-GalCer **1** or threitolceramide **3a**, arabinitolceramide **4a** or glycerolceramide **2a**. The supernatants were analyzed using ELISA for the presence of A) IFN-γ and B) IL-4. Both **3a** and **4a** induced the desired intermediate amounts of both IL-4 and IFN-γ in vitro, whereas **2a** was nonfunctional in these assays.

To further investigate the degree of substitution that could still allow recognition by the *i*NKTCR, compounds D-**6a** and L-**7a**, which contain a phenyl group at position 4 of the threitolceramide head group, were studied. Molecular modeling of both compounds on the existing structure of hCD1d–α-GalCer-TCR[21] suggested that the phenyl group should not prevent recognition by the TCR (data not shown). As a first step, binding affinities of phenyl threitolceramides D-**6a** and L-**7a** to the *i*NKTCR were assessed by Biacore analysis using hCD1d monomers loaded with the various ligands and soluble *i*NKTCR (Figure 3 A, B). The *i*NKTCR bound to D-**6a** and L-**7a**–CD1d monomers with affinities of 4.25 and 3.84 μM, respectively (Figure 3 A, B). These binding affinities were similar to unmodified threitolceramide, and as expected, were slightly lower than that of α-GalCer (1.3 μM).[39,40]

To confirm that phenyl threitolceramides D-**6a** and L-**7a** are recognized by *i*NKT cells in vitro, human *i*NKT cells were co-cul-

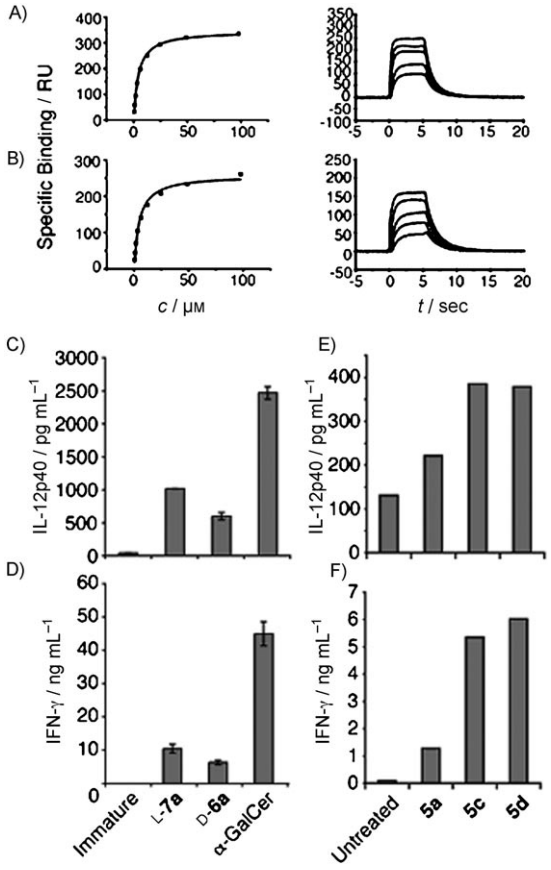


Figure 3. Human and murine *i*NKT cells can recognize phenyl derivatives of threitolceramide in vitro. Equilibrium binding and kinetics measurements of a soluble *i*NKT cell receptor for hCD1d molecules refolded with compounds were assessed for A) L-**7a** ($K_d = 3.84 \pm 0.29$ μM) and B) D-**6a** ($K_d = 4.25 \pm 0.76$ μM). K_d values were calculated from equilibrium binding. K_d and k_{off} (L-**7a**: $k_{off} = 0.96\ s^{-1}$; D-**6a**: $k_{off} = 0.69\ s^{-1}$) values indicated represent the mean of at least two independent experiments; k_{on} values were calculated from k_{off} and K_d (L-**7a**: $k_{on} = 2.68 \times 10^5\ M^{-1}\ s^{-1}$; D-**6a**: $k_{on} = 1.50 \times 10^5\ M^{-1}\ s^{-1}$). Human DCs were co-cultured with *i*NKT cells for 40 h in the presence of the compounds indicated (100 ng mL^{-1}), and the supernatants were analyzed for C) IL-12p40 and D) IFN-γ. Using similar assays, **5a**, **5c**, and **5d** (200 ng mL^{-1}) were tested and analyzed for E) IL-12p40 and F) IFN-γ.

tured for 40 h with dendritic cells (DC) that had been pulsed with 100 ng mL^{-1} vehicle, α-GalCer, D-**6a** or L-**7a**. Cytokine production was then assessed using ELISA. Both D-**6a** and L-**7a** were recognized by *i*NKT cells when presented by DC as indicated by the presence of IL-12p40 and IFN-γ (Figure 3C, D). Both compounds were recognized by a murine *i*NKT cell hybridoma when presented by cells expressing mouse CD1d (data not shown). Consistent with the lower affinity of the *i*NK TCR for D-**6a** and L-**7a** (Figure 3A, B) compared with α-GalCer, the amount of cytokines produced in response to either compound was also lower than the amount induced by α-GalCer. However, it is important to note that although the head groups of these compounds are significantly different from the head groups in α-GalCer, there is sufficient flexibility within the TCR–ligand–CD1d interface to allow their recognition. In a similar assay (Figure 3E, F) the 4-deoxy derivatives **5a**, **5c**, and **5d** were tested and also showed significant activity that could be further enhanced by introducing *cis* double bonds between C22–C23 (**5c**) and C19–C20, C22–C23 (**5d**), respectively, into the fatty acyl chain. However, as shown in Figure 4 the introduction of unsaturated *N*-fatty acyl chains at the sphingosine moiety does not enhance the functional efficacy of all ligands. For instance, in the threitolceramide series (**3a**, **3c**, and **3d**) compound **3a** with the saturated fatty acid showed the highest efficacy when tested against human *i*NKT cells (Figure 4A, B). The pattern was somewhat different with murine *i*NKT cells. The 4-deoxy derivatives (**5a**, **5b**, and **5c**) have lower efficacy toward murine *i*NKT cells than the lead ligand threitolceramide **3a** (Figure 4C), which was not improved by introducing *cis* double bonds. In contrast, whereas L-**7a** did not induce significant IL-2 production from the murine *i*NKT cell hybridoma, this series of compounds benefited from introduction of *cis* double bonds, as both L-**7c** and L-**7d** show activity on par with **3a** (Figure 4E). These data suggest that the profile of ligands that *i*NKT cells can respond to between humans and mice are different, as was previously reported.[39] It is also clear that the introduction of *cis* double bonds into the molecules does not automatically confer increased efficacy compared with compounds lacking such double bonds.

Summary of the functional data of *i*NKT cell agonists

It has become evident that the strength of the interaction between the *i*NK TCR and CD1d molecules controls the lymphokine repertoire secreted by *i*NKT cells, the activation status of *i*NKT cells, and DC maturation.[18,39] *i*NKT agonists with an affinity in the ~1 μM range, such as α-GalCer, induce large amounts of IFN-γ secretion by *i*NKT cells and DC maturation.[18] However, over-stimulation of *i*NKT cells has been shown to result in *i*NKT cell anergy and unresponsiveness to subsequent stimulation.[24,39] Although a slightly lower-affinity ligand such as threitolceramide **3a** does induce a degree of activation-induced *i*NKT cell anergy, recovery of the cells from anergy is more rapid than observed with α-GalCer.[39]

The novel ligands described herein induce lower levels of cytokines than does α-GalCer (Figure 2), while maintaining the ability to mature DCs. Using both in vitro and in vivo[40] assays, we found that 80–90% of DCs pulsed with α-GalCer are killed by *i*NKT cells, whereas in contrast, a significantly lower proportion of DCs are lysed when pulsed with threitolceramide **3a**.[39] In addition, we found that threitolceramide **3a** was an effective adjuvant for priming both T and B cell responses to a model antigen, and was useful in a therapeutic

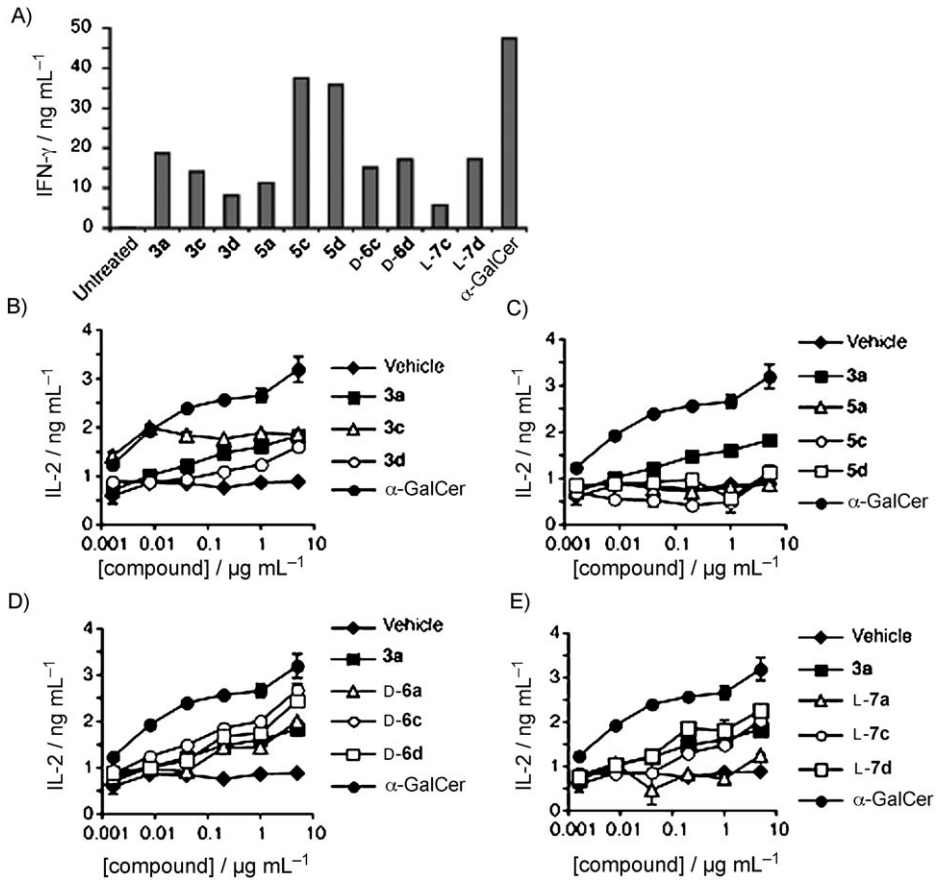


Figure 4. Influence of unsaturated fatty acyl chains and the sphingosine moiety. A) C1R-hCD1d cells were pulsed with a range of ligands (as indicated; each at 200 ng mL^{-1}) with or without unsaturated tail groups and used to stimulate human *i*NKT cells in vitro. The supernatants were tested for IFN-γ. B)–E): C1R-mCD1d cells were pulsed with various concentrations of the ligands as indicated and used to stimulate the DN32 hybridoma in vitro. The supernatants were tested for the presence of IL-2.

and to some extent a prophylactic mouse tumor model.[39] Together these data suggest that analogues of α-GalCer with a more fine-tuned binding affinity should be highly useful for clinical applications such as cancer immunotherapy by decreasing the over-activation of *i*NKT cells and subsequent off-target effects such as cytokine production and *i*NKT-cell-mediated DC lysis, while maintaining the ability to induce DC maturation and priming of antigen-specific immune responses. Hence, threitolceramide **3a** is now in development for clinical evaluation as adjuvant in vaccines against cancer and infectious diseases.

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