Letter

Design, Synthesis and Immunological Evaluation of Benzyloxyalkyl substituted 1,2,3-Triazolyl #-GalCer Analogues

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Design, Synthesis and Immunological Evaluation of Benzyloxyalkyl-substituted 1,2,3-Triazolyl α-GalCer Analogues

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ABSTRACT: Replacement of the amide moiety in the structure of α-GalCer with a 1,2,3-triazole linker is known to elicit a response skewed towards Th2 immunity, and glycolipids containing an aromatic ring in the terminus of their acyl or phytosphingosine structural component exhibit an enhanced Th1 immune response. In the current study, synthesis and immunological screening of a focused library of benzyloxyalkyl-substituted 1,2,3-triazolyl α-GalCer analogues are reported. The novel α-GalCer analogues activate invariant natural killer T (*i*NKT) cells *via* CD1d mediated presentation, which was confirmed by *in vitro* tests performed on *i*NKT hybridomas incubated with CD1d proteins. When tested on isolated murine splenocytes, the T1204B and T1206B compounds stimulated higher levels of both IFN-γ and IL-4 cytokine expression *in vitro* compared to that of α-GalCer.

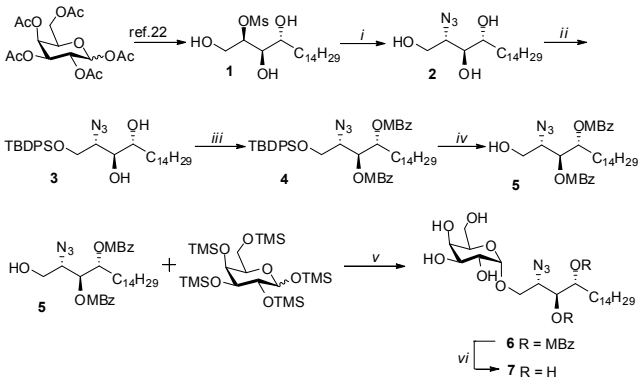
KEYWORDS: benzyloxyalkyl α-GalCer, Th1/Th2, IL-4, IFN-γ, kinetic release, *i*NKT hybridoma

α-Galactosylceramide (α-GalCer) was isolated from the extracts of the Japanese marine sponge *Agelas mauritianus* from the *Okinawan sea.*[1,2] In last two decades, α-GalCer and its derivatives have drawn considerable interest due to their versatile utility as *i*NKT activators, immunomodulators[3] and adjuvants in many diseases including malaria,[4] HIV,[5] tuberculosis[6] and tumor immunotherapy.[7] Natural killer T (NKT) cells are subsets of T lymphocytes that share common features of both NK cells and conventional T cells. NKT cells that express semi-invariant surface receptor (*i*NKT cells) specifically recognize α-GalCer presented by an MHC class-I like molecule (i.e., CD1d). Activation of *i*NKT cells stimulates production of both IFN-γ and IL-4.[8-10] Studies have revealed that production of Th1 cytokines may correlate with antiviral, antibacterial, antitumor and adjuvant activities. However, Th2 cytokine production may subdue autoimmune diseases. Therefore, the identification of compounds that are capable of inducing varying degrees of Th1/Th2 polarization as reflected by their cytokine responses is desirable. In the search for more effective glycolipids, several analogues have been synthesized through modifications at suitable positions on the α-GalCer structure, and as a result, more potent analogues, such as OCH, C-GalCer, 7DW8-5, RCAI-56, Nu-α-GalCer, SMC-124 and EF77, were prepared.[11-13] Among the large number of α-GalCer analogues that have been synthesized over the past several years, some analogues with a fascinating immune stimulatory nature have attracted our attention. The first variety of these analogues are α-GalCer derivatives that include a 1,2,3-triazole moiety as a replacement for the amide linkage in the structure.[14-16] These compounds exhibited comparable *i*NKT stimulatory effect with Th2 bias on mice splenocytes.[14] The triazole moiety served as a rigid linker that is more stable to hydrolysis and oxidative/reductive conditions in biological systems. In addition, two additional hydrogen bonds were reported between the Thr154 residue and the triazole moiety.[14] α-GalCer derivatives with different aryl moieties at the acyl chain terminus have exhibited remarkable activity.[17, 18] These analogues exhibited strong *i*NKT stimulation with Th1 bias.[18, 19] According to Wu *et al.,* their docking models revealed additional hydrogen bonding between the phenyl/aryl ring of the fatty acyl chain and the aromatic amino acid residues present on the wall of the A′ pocket in the CD1d hydrophobic groove.[20] One such analogue (7DW8-5) from the same structural class exhibited superior adjuvant activity compared to that of α-GalCer in HIV and malaria vaccines in mice.[18, 21]

To achieve a better stimulatory effect with an enhanced Th1 and Th2 response, we have designed novel α-GalCer analogues that combine both the previously discussed properties. A benzyl group is utilized to serve as an aryl terminus of the alkyl chain, which was attached to the azido-galactosylceramide moiety through a triazole linker. Additionally, this molecular design would enable the evaluation of the possible role of the benzyl and triazolyl moieties present in the same structural framework in modulating the immune response. To explore the immunomodulatory property of these glycolipid entities, we prepared a focused library of α-GalCer analogues where a 1,2,3-triazole replaced the amide linkage of α-GalCer along with lipid chains of varying lengths bearing a terminal benzyl group with some intervening oxygen atoms. These

structural modifications may enhance the hydrogen bonding involved during the *i*NKT cell activation cascade, which may result in a more pronounced Th1 and Th2 response. In continuation of our ongoing research program to develop novel immunomodulators, we present the synthesis and immunopharmacological studies of novel benzyloxyalkyl-substituted 1,2,3-triazolyl α-GalCer analogues.

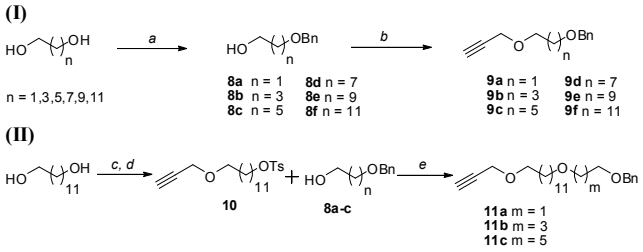
Scheme 1. Synthesis of azido α-GalCer.



Reagents and conditions: (i)- NaN_3, DMF, 100 °C, 6 h, 65%. (ii) TBDPSCl, imidazole, DCM, rt, 88%. (iii) *p*-Methoxybenzoyl chloride, Et_3N, rt, 1 h, 95%. (iv) TBAF, THF, –10 °C, 3 h, 75%. (v) TBAI, DIPEA, DCM, 4 Å MS, rt, 4 h, 85%. (vi) NaOMe, MeOH, 20 min, 94%.

The synthesis of α-GalCer analogues has been accomplished by following literature procedures with some minor modifications. To achieve the synthesis of the designed analogues, two key fragments (i.e., azido α-GalCer and acytelenic lipid spacers of varying chain lengths) were prepared, and their syntheses is briefly discussed below. The synthesis of mesylate **1** was accomplished by starting from D-galactose pentaacetate according to previously published methods (Scheme 1).[22, 23] Mesylate **1** was subjected to SN_2 substitution to achieve azido phytosphingosine **2**. Compound **2** was selectively protected as a silyl ether followed by the orthogonal protection of the secondary hydroxyls with *p*-methoxybenzoyl (MBz) protection to afford compound **4,** which was treated with a TBAF solution for silyl deprotection to prepare intermediate **5**. A *p*-methoxybenzoyl group was employed to minimize the benzoyl migration to the primary hydroxyl group under TBAF deprotection conditions, which has been reported as a hurdle encountered with conventional benzoyl protection strategies.[24] The glycosyl acceptor **5** was subjected to glycosidation with persilylated galactose under TBAI/DIPEA catalysis, as described by Hague *et al.* to afford protected GalCer **6** with a selective alpha configuration.[25] The high yields (>80%) of the glycosidation reaction with *p*-methoxybenzoyl protection are impressive because glycosidation yields are reported to be substantially reduced with changes in the orthogonal protection in the phytosphingosine acceptor.[26] Subsequent treatment of compound **6** with NaOMe/MeOH was performed to remove the *p*-methoxybenzoyl protection, and azido α-GalCer intermediate **7** was obtained as a white solid.

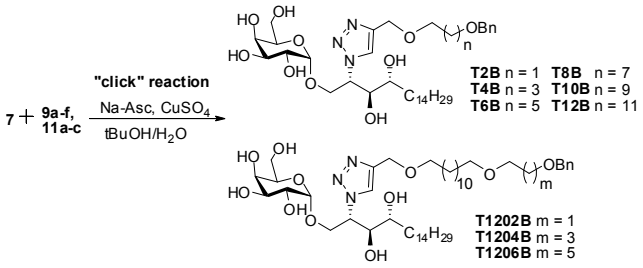
Scheme 2. Synthesis of acetylenic lipid chains.



Reagents and conditions: (a) BnBr, NaH, THF, rt, 12 h, >75%. (b) Propargyl bromide, NaH, THF, rt, 2 h, 80%. (c) Propargyl bromide, NaH, THF, rt, 2 h, 86%. (d) TsCl, DCM, Et_3N, rt, 1 h, 90%. (e) NaH, THF, reflux, 12 h, ≥ 65%.

The terminal benzylated acetylenic lipid intermediates with varying chain lengths that are required for click chemistry were generated by following standard aliphatic condensation protocols starting from the corresponding diols with different chain lengths (Scheme 2-I). The mono benzylation of diols were carried out using benzyl bromide/NaH in THF at rt to afford alcohols **8**a-f, which afforded propargylated intermediates **9**a-f upon condensation with propargyl bromide in the presence of sodium hydride at rt. Alternatively, propargyl intermediates **11**a-c with longer chain length lipids have been prepared by mono propargylation of 1,12-dodecanediol followed by tosylation to afford acetylenic tosylate **10,** which yielded the required benzylated acetylenic lipids **11**a-c (Scheme 2-II) upon substitution with benzylated alcohols **8**a-c.[27]

Scheme 3. "Click chemistry" assembly of 1,2,3-triazolyl α-GalCer analogues.



The synthesis of the final constructs was accomplished by applying a copper-mediated "click chemistry" protocol using sodium ascorbate in a ^{t}BuOH/water mixture (Scheme 3).[14] All of the products were purified by column chromatography with $CHCl_3$/methanol as eluents, and the products were obtained in quantitative yields.

CD1d–GalCer engagement with a T cell receptor of *i*NKT cells activates their co-stimulatory molecules followed by activation of bystander cells. This activation leads to the production of a large amount of various cytokines and chemokines, leading to Th1 and Th2 immune responses.[28] Based on previous results, the synthesized analogues were screened at three different concentrations (1000, 100 & 10 ng/ml).[29] All of the α-GalCer analogues exhibited good solubility in DMSO. The α-GalCer and its T2B, T4B, T6B, T8B, T10B, T12B, T1202B, T1204B and T1206B analogues were examined for their splenocyte proliferation potential and determine to be non-toxic up to 1000 ng/ml. In comparison to the untreated splenocytes, some analogues, such as T8B, T1204B & T1206B, exhibited satisfactory proliferative effect when incubated *in vitro* with splenocytes isolated from the spleen of BALB/c mice (figure 1).

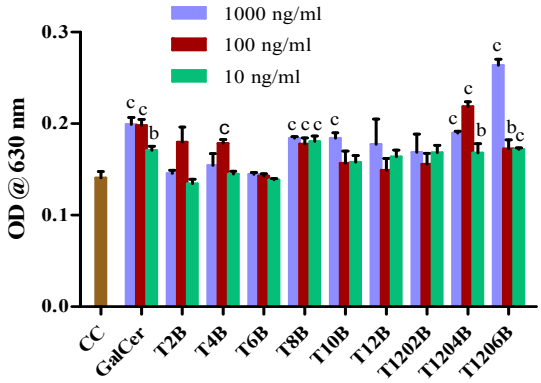


Figure 1. Effect of α-GalCer and its analogues on splenocyte proliferation. Splenocytes were treated with α-GalCer and its analogues for 48 h, and proliferation was estimated using the MTT method.

The effect of α-GalCer and its analogues on IL-2, IL-4 & IFN-γ expression in mouse splenocytes was determined *in vitro* by ELISA. Cytokine estimation after 48 h treatment with glycolipids revealed that all of the analogues exhibited T-cell stimulation in a dose-dependent manner.[30] All of the GalCer analogues exhibited uniform IL-2 expression with α-GalCer except for T2B bearing the shortest acyl lipid chain. However, the analogues with longer acyl chains, such as T8B, T12B, T1202B, T1204B and T1206B, exhibited a significant increase in IL-2 (figure 2a) production. Further analysis revealed that compounds T1204B & T1206B exhibited a notable increase in both IL-4 and IFN-γ (figures 2b, 2c) at all three concentrations compared to that of α-GalCer, and T6B and T1202B exhibited a slight increase in IFN-γ expression at 1000 ng/ml. The high level of IL-4 and IFN-γ expressed by T1204B and T1206B may influence Th1 and Th2 cells to modulate cellular immunity.

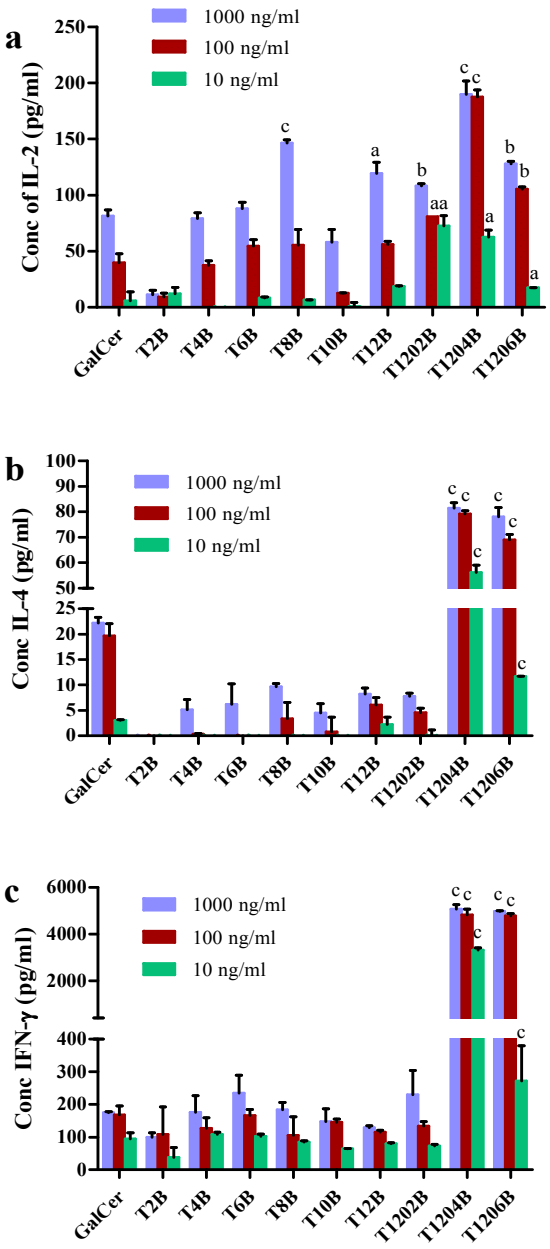


Figure 2. Cytokine secretion by mouse splenocytes when stimulated with α-GalCer and its analogues. IL-2, IFN-γ and IL-4 production was measured after 48 h treatment.

Earlier studies demonstrated that α-GalCer induced kinetic release of cytokines by *i*NKT cells in a signature fashion at longer periods of time after treatment with the glycolipid, but shorter periods of time indicate dominance of IL-4 followed by high IFN-γ production after a long period of time. The kinetic release of IL-4 and IFN-γ was studied over the 2nd, 12th and 24th hour after *in vitro* treatment of mice splenocytes with glycolipids at a 1000 ng/ml concentration. IL-4 estimation (figure 3a) from cell culture supernatants was analyzed, and the T1204B and

T1206B analogues induced a substantial increase in IL-4 at 2 h with a gradual decrease up to 24 h compared to α-GalCer. Study of kinetic release of IFN-γ (figure 3b) revealed no significant changes in the cytokine expression up to 12 h. However, a remarkable increase in the IFN-γ levels was observed at 24 h compared to that of α-GalCer. Therefore, compounds T1204B and T1206B exhibited a good stimulatory effect expressing high levels of both the IL-4 and IFN-γ cytokines.

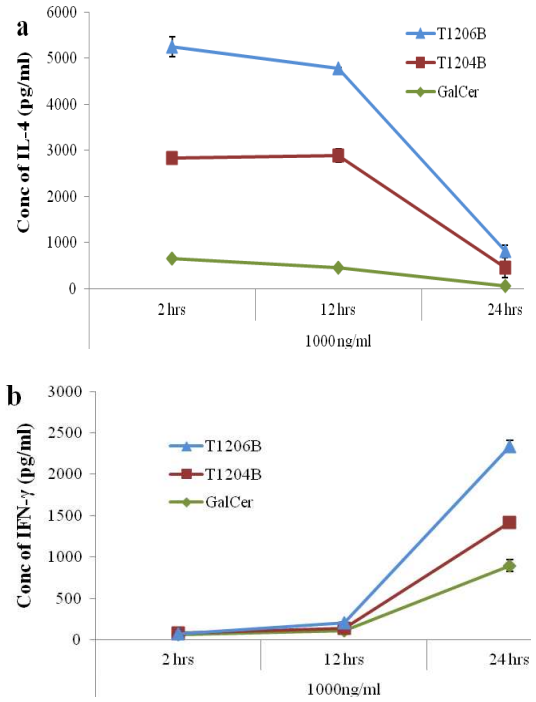


Figure 3. Kinetic release of cell-secreted cytokines (IL-4 and IFN-γ) when stimulated with α-GalCer and its analogues.

The test compounds (i.e., T1204B and T1206B) exhibited cytokine expression polarized towards Th1 after 48 h (figures 2b and 2c). However, the cytokine levels in kinetic release studies at 2 h, 12 h and 24 h confirmed a bias towards Th2 behavior. Further studies on *i*NKT stimulation and *in vivo* kinetic release of test compounds were carried out with direct comparison of Kim's triazolyl derivative (i.e., α-GalCer-analogue-8, which is referred to as GC-8) under similar conditions.

To further confirm that the triazole analogues are activators of *i*NKT cells, *i*NKT hybridoma cells were treated with T1204B, T1206B, GC-8 and α-GalCer, incubated with CD1d protein for 16 h and assayed for IL-2 production (figure 4). The level of cytokine IL-2 that was expressed in the *in vitro* study revealed that all three compounds were efficient stimulators of *i*NKT cells at a concentration of 100 ng/ml even though slightly less compared to that α-GalCer. The T1206B analogue slightly enhanced IL-2 stimulation compared to the GC-8 compound (figure 4).

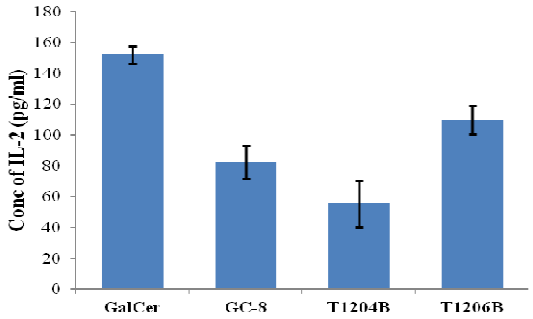


Figure 4. IL-2 expression on compound treatment with CD1d protein with Vα14 Vβ8.2 DN3A4-1.2 (1.2) *i*NKT hybridoma cells

In addition, *in vivo* experiments were performed with all four compounds individually administered to BALB/c mice intravenously at a concentration of 1 μg, and both analogues (i.e., T1204B and T1206B) stimulated low levels of IFN-γ (figure 5a) and IL-4 (figure 5b) expression compared to that of α-GalCer or GC-8. Interestingly, these derivatives also exhibit signature trends of expression of both representative cytokines, as observed for α-GalCer.

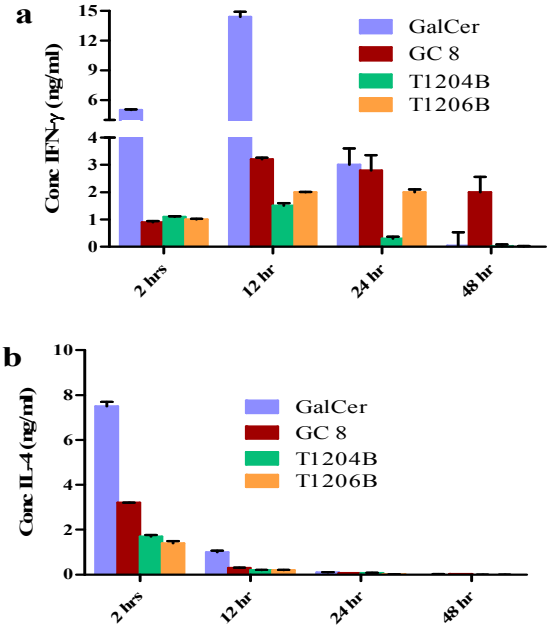


Figure 5. IFN-γ and IL-4 production by BALB/c mice on *in vivo* treatment of compounds at a concentration of 1 μg.

In conclusion, the syntheses and immunological results of a focused library of novel benzyloxyalkyl-substituted triazolyl α-GalCer analogues that are useful immunomodulators have been reported. The results from the bioassays indicated that T1204B and T1206B exhibited significantly improved Th1 as well as Th2 cytokine expression, which may be due to the presence of directing moie-

ties in their chemical structure. However, similar levels of expression were not observed when these tests were performed *in vitro* on isolated *i*NKT cells or *in vivo* in an animal model. The observed immune response may be due to the dose-dependent stimulation of other heterogeneous innate cell populations present in spleen cells by the test compounds in the *in vivo* environment. Overall, it is important to note that the additive effect observed in the current study will aid in designing analogues that induce higher levels of either Th1 or Th2 immunity and direct the immune response of interest. Modulation of Th1/Th2 activation by these α-GalCer analogues makes them promising candidates for their possible application as vaccine adjuvants and ligands to treat autoimmune disorders.

ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures including biology and chemistry, analytical data of all compounds, ^{1}H NMR, ^{13}C NMR and HRMS spectra for selected compounds. This material is available free of charge via the internet at http://pubs.acs.org.

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Notes

Data analysis All of the data in the graphs are expressed as the mean ± SD of triplicates of each sample. The statistical significance of the secretion levels was determined by Student's t test. a$p < 0.05$, b$p < 0.01$ and c$p < 0.001$.

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ABBREVIATIONS

IFN-γ, interferon gamma; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme linked immunosorbent assay; HIV, human immunodeficiency virus; OD, optical density; TBAF, tetrabutylammonium fluoride; MS, molecular sieves; THF, tetrahydrofuran.

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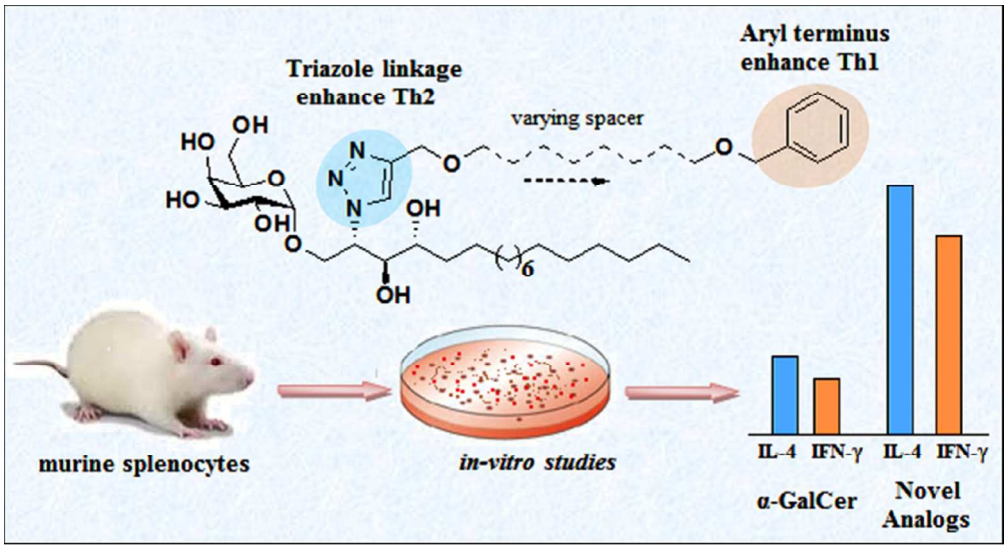

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