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Amide Analogues of CD1d Agonists Modulate *i*NKT-Cell-Mediated Cytokine Production

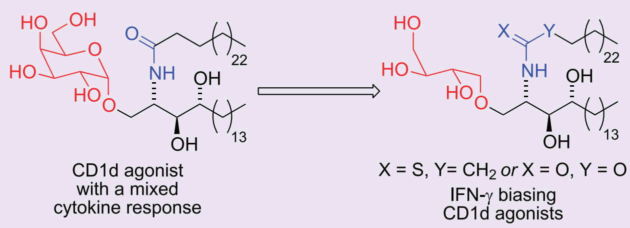
Justyna Wojno,[†,‡,∥,⊥] John-Paul Jukes,[§,⊥] Hemza Ghadbane,[§,⊥] Dawn Shepherd,[§] Gurdyal S. Besra,[*,‡] Vincenzo Cerundolo,[*,§] and Liam R. Cox[*,†]

[†]School of Chemistry and [‡]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

[§]Medical Research Council Human Immunology Unit, Nuffield Department of Medicine, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, U.K.

Supporting Information

ABSTRACT: Invariant natural killer T (*i*NKT) cells are restricted by the non-polymorphic MHC class I-like protein, CD1d, and activated following presentation of lipid antigens bound to CD1d molecules. The prototypical *i*NKT cell agonist is α-galactosyl ceramide (α-GalCer). CD1d-mediated activation of *i*NKT cells by this molecule results in the rapid secretion of a range of pro-inflammatory (Th1) and regulatory (Th2) cytokines. Polarization of the cytokine response can be achieved by modifying the structure of the glycolipid, which opens up the possibility of using CD1d agonists as therapeutic agents for a range of diseases. Analysis of crystal structures of the T-cell receptor−α-GalCer−CD1d complex led us to postulate that amide isosteres of known CD1d agonists should modulate the cytokine response profile upon *i*NKT-cell activation. To this end, we describe the synthesis and biological activity of amide analogues of α-GalCer and its non-glycosidic analogue threitol ceramide (ThrCer). All of the analogues were found to stimulate murine and human *i*NKT cells by CD1d-mediated presentation to varying degrees; however, the thioamide and carbamate analogues of ThrCer were of particular interest in that they elicited a strongly polarized cytokine response (more interferon-gamma (IFN-γ), no interleukin-4 (IL-4)) in mice. While the ThrCer-carbamate analogue was shown to transactivate natural killer (NK) cells, a mechanism that has been used to account for the preferential production of IFN-γ by other CD1d agonists, this pathway does not account for the polarized cytokine response observed for the thioamide analogue.



Natural killer T (NKT) cells have been implicated in a range of important immune surveillance mechanisms, such as host defense against external pathogens, immune tolerance, and malignancy.[1] NKT cells can be divided into two subsets, so-named Type I and Type II. Type I NKT cells have received the most attention. These cells are also known as invariant NKT (*i*NKT) cells owing to their expression of an invariant α chain T cell receptor (TCR; Vα14−Jα18 chain in mice and Vα24−Jα18 chain in humans), which is paired with a more variable β chain.[1] The *i*NKT cell TCR recognizes lipid antigens presented in the context of the non-polymorphic MHC class I-like protein, CD1d, which has been shown to bind a range of dialkyl lipids and glycolipids.[2] *i*NKT cell TCR recognition of the CD1d−lipid complex leads to the rapid proliferation and release of a range of cytokines. The activation of *i*NKT cells is an important step in "boosting" adaptive immune responses through the activation and maturation of dendritic cells (DC) and B cells through CD40−CD40L interactions and the activation of natural killer (NK) cells following interferon gamma (IFN-γ) release.[3] Since the structure of CD1d ligands has been shown to govern the released cytokine profile, the development of lipid molecules that promote the specific activation of *i*NKT cells could find application in the treatment of a wide range of disorders.[4,5]

Of the range of lipids that bind to CD1d, the glycolipid α-GalCer (**1**) is one of the most potent (Figure 1).[6] Recognition of the α-GalCer−CD1d complex by the *i*NKT cell TCR initiates a powerful immune response. However, while α-GalCer remains one of the most potent *i*NKT cell agonists and has shown potential in the treatment of various conditions,[7] it may prove difficult to use this molecule widely as a useful therapeutic agent, at least as a direct activator of *i*NKT cells. Not only does α-GalCer-mediated *i*NKT cell activation lead to the secretion of both T helper Type 1 (Th1) (*e.g.*, IFN-γ) and T helper Type 2 (Th2) (*e.g.*, interleukin-4 (IL-4)) cytokines, and therefore a mixed immune response, but more importantly, overstimulation of *i*NKT cells, which can result in their entering a long-term anergic state, *i.e.*, unresponsiveness to subsequent α-GalCer stimulation and preferential IL-4 production, which would be deleterious for long-term therapy.[8,9]

It was recently demonstrated that the non-glycosidic α-GalCer analogue threitol ceramide (ThrCer) **2** (Figure 1) overcomes the problematic *i*NKT cell activation-induced anergy associated with α-GalCer **1**.[10] While preventing α-GalCer-dependent *i*NKT cell overstimulation, ThrCer still ensures effective DC maturation,

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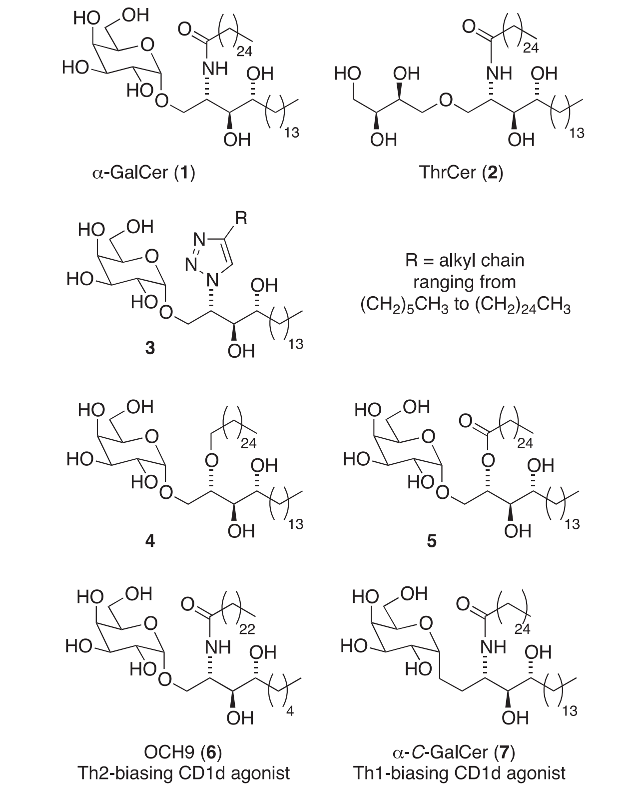


Figure 1. KRN7000 (or α-GalCer) **1** and analogues as examples of *i*NKT cell ligands.

minimizes *i*NKT cell-dependent DC lysis, and ensures optimal expansion of antigen-specific T cell responses. Thus by minimizing *i*NKT cell overstimulation and *i*NKT cell-dependent DC lysis, ThrCer rectifies some of the deficiencies of α-GalCer.

There has been much interest in studying how other glycolipids, structurally related to α-GalCer, can be used to regulate the immune response through their presentation on CD1d molecules to *i*NKT cell TCRs.[2,3,10,11] CD1d agonists, which lead to biased Th1/Th2 responses, have received particular attention,[11] while α-GalCer analogues have also helped to elucidate the structural requirements for CD1d binding and subsequent presentation to *i*NKT cell TCRs.[11] Glycolipids with different carbohydrate head groups, glycosidic linkages, different fatty acid acyl chains, and different ceramide bases have all been reported;[11] however, surprisingly little attention has been devoted to the amide bond that links the fatty acid acyl chain to the phytosphingosine base. Kim and co-workers studied a series of α-GalCer analogues **3**, in which a 1,2,3-triazole unit bearing a lipid substituent of varying length replaced the amide functionality found in α-GalCer (Figure 1).[12] Their most promising results were obtained with the analogue possessing a C_{24} alkyl chain, which exhibited a Th2 bias (*i.e.*, more IL-4 and less IFN-γ compared with that elicited by α-GalCer) in the cytokine response when administered at low concentrations. Shiozaki *et al.* recently studied α-GalCer analogues **4** and **5**, in which ether and ester functionalities, respectively, replace the amide found in α-GalCer (Figure 1).[13] The ether analogue **4** was unable to stimulate any cytokine response when administered in mice. The ester analogue **5** elicited a weaker cytokine response than α-GalCer, with IFN-γ production being very low and IL-4 production approximately two-thirds of that displayed by α-GalCer.

X-ray crystal structures of α-GalCer **1** bound to human CD1d (hCD1d),[14] an hCD1d–α-GalCer–TCR ternary complex,[15,16] and mouse CD1d (mCD1d)–α-GalCer–TCR ternary complexes[15,17] reveal a similar bound conformation of α-GalCer in both mouse and human CD1d molecules, as well as comparable *i*NKT cell TCR binding to the CD1d–α-GalCer complex. Analysis of the best-resolved (2.8 Å) crystal structure of the ternary complex containing mCD1d[15] reveals the amide NH in α-GalCer acts as a hydrogen-bond donor to the side-chain hydroxyl functionality of Thr156 in mCD1d (Figure 2).

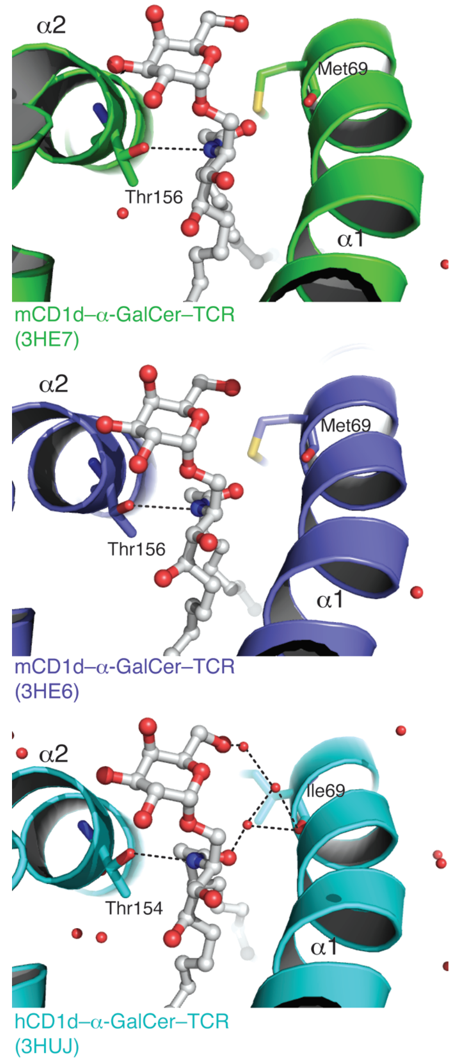
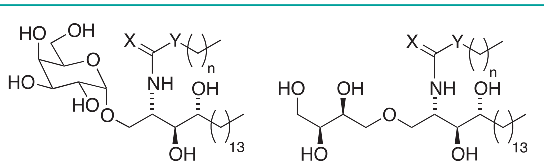


Figure 2. Key hydrogen bonds (dotted black lines) involving the amide functionality of α-GalCer in the mCD1d–α-GalCer–TCR complex (green and blue) (taken from structures 3HE7 and 3HE6 in the PDB database, respectively, ref 15) and the hCD1d–α-GalCer–TCR complex (cyan) (taken from structure 3HUJ in the PDB database, ref 15).

The OH residue of Thr156 plays a second role as a donor in a (weaker) hydrogen bond to the glycosidic oxygen in α-GalCer. We postulate that this bifunctional binding mode is important for ensuring the glycolipid adopts an appropriate bound conformation for its recognition by *i*NKT cell TCRs. A similar inspection of the best resolved (2.5 Å) crystal structure of the ternary complex containing hCD1d[15] reveals a similar hydrogen-bonding network, with the amide NH of α-GalCer forming a hydrogen bond with the equivalent hCD1d amino acid residue, Thr154. In all structures, the amide carbonyl oxygen of α-GalCer is not involved in direct hydrogen-bonding interactions with either the CD1d molecule or the TCR, although the better-resolved ternary complex crystal structure containing hCD1d reveals a hydrogen bond to a bridging water molecule, which is further hydrogen-bonded to the backbone carbonyl of Ile69 located in the α1 helix of the hCD1d molecule (Figure 2).[15] A similar interaction is not observed in the structures of the ternary complexes containing mCD1d; however, Met69 in the α1 helix of mCD1d is ideally positioned to play such a role,[15] and indeed such an interaction is observed in the mCD1d complex containing the α-GalCer analogue, OCH9[18] and other α-GalCer analogues.[19] We currently lack crystallographic structural information on the corresponding CD1d−ThrCer complexes; however, we postulate that this non-glycosidic agonist binds in a similar fashion to α-GalCer since it preserves all the key functionality that is required in α-GalCer for binding to the CD1d molecule, and its presentation by CD1d results in an IL-4/IFN-γ cytokine profile similar to that displayed by α-GalCer.[10]

RESULTS AND DISCUSSION

CD1d Agonist Design. On the basis of an analysis of the available crystal structures of the CD1d−α-GalCer−TCR complex and in particular the role of the amide functionality in ligand binding, we postulated that other carboxylic acid derivatives, which retain a hydrogen-bonding capability and in particular the capacity to function as a hydrogen-bond donor to Thr156 in mCD1d and Thr154 in hCD1d, may also be useful CD1d agonists. To this end, we proposed α-GalCer analogues **8**, **9**, and **10** and their ThrCer analogues, **11**, **12** and **13**, to test this notion (Figure 3).



8, X = S, Y = CH_2, n = 23
9, X = O, Y = NH, n = 22
10, X = O, Y = O, n = 23

11, X = S, Y = CH_2, n = 23
12, X = O, Y = NH, n = 22
13, X = O, Y = O, n = 23

Figure 3. Target α-GalCer and ThrCer analogues.

Owing to their increased polarity and N−H acidity,[20] thioamides are better hydrogen-bond donors than amides,[21] while the sulfur atom functions as a weaker hydrogen-bond acceptor.[22] Thioamides also differ from amides in their longer C═S bond (1.65 Å, *cf.* 1.20 Å for a C═O bond in amides) and the larger van der Waals radius of the sulfur atom (1.85 Å, *cf.* 1.40 Å for oxygen). We therefore postulated that thioamide analogues of α-GalCer and ThrCer should partake in a strong hydrogen bond with the side-chain hydroxyl of Thr156 in mCD1d (and Thr154 in hCD1d); however, any hydrogen bonding with a bridging water molecule would be weaker, assuming it were present at all, given the increased size of the sulfur atom that might displace a water molecule altogether.[23]

In the case of the urea[24] and carbamate analogues,[25] we expected incorporating a second heteroatom into the acyl chain would not only modulate the hydrogen-bonding capacity of both the NH involved in hydrogen bonding to Thr156 in mCD1d (and Thr154 in hCD1d)[26] and the carbonyl oxygen in a water-bridged hydrogen bond to a backbone carbonyl in the α1 helix of CD1d (Ile69 in hCD1d or Met69 in mCD1d) but also open up the possibility of additional hydrogen-bonding interactions, which might serve to stabilize the glycolipid−CD1d complex or, of course, affect the binding conformation deleteriously. At the same time, we were cognisant that the second heteroatom substituent would extend the planarity of the acyl chain to two atoms beyond the carbonyl group and therefore affect the conformation in this part of the molecule and potentially its binding to CD1d and subsequent presentation to *i*NKT cells.

Biology. Synthesis of the target molecules proceeded uneventfully and is detailed in the Supporting Information. With these new CD1d ligands in hand, their biological activity was investigated alongside α-GalCer **1** and ThrCer **2**. In a preliminary screen, all eight compounds were tested for their ability to stimulate the *i*NKT cell hybridoma DN32, following pulsing of C1R-mCD1d cells with various concentrations of ligands. The concentration of IL-2 in the supernatant released after *i*NKT cell activation was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (Figure 4).[27] Encouragingly, these experiments demonstrated

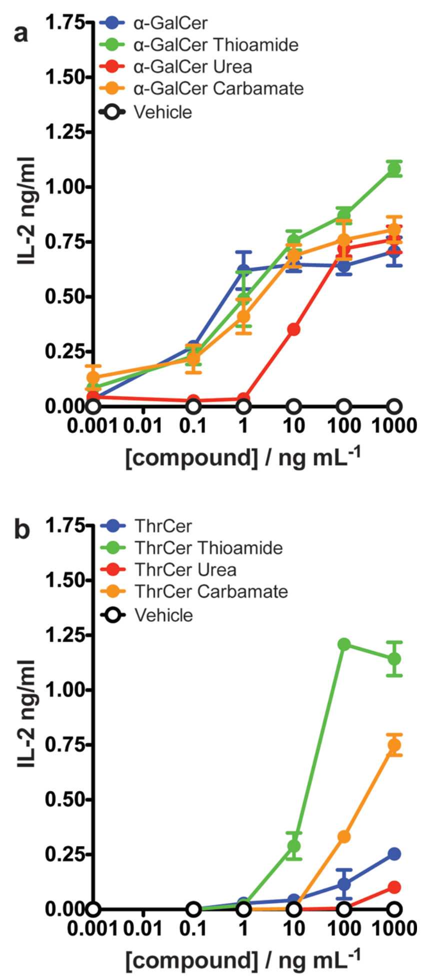


Figure 4. Activation of murine *i*NKT cells using thioamide, urea, and carbamate analogues of α-GalCer (panel a) and ThrCer (panel b).

that both ThrCer-thioamide **11** and ThrCer-carbamate **13** induced increased activation compared with ThrCer **2**, whereas the ThrCer-urea analogue **12** led to weak stimulation and only at high concentrations (Figure 4, panel b). A similar hierarchy was observed for the α-GalCer analogues, although the differences, particularly at high concentration, were less pronounced (Figure 4, panel a).

A second *in vitro* experiment was used to test functional activity, this time using a human model; thus human *i*NKT cells were co-cultured for 40 h with C1R-hCD1d cells that had been pulsed with 100 ng mL^{-1} concentrations of vehicle, α-GalCer **1**, α-GalCer-thioamide **8**, α-GalCer-urea **9**, and α-GalCer-carbamate **10** (Figure 5, panel a) and ThrCer **2**, ThrCer-thioamide **11**, ThrCer-urea **12**, and ThrCer-carbamate **13** (Figure 5, panel b). In this assay, the ability of the various ligands to activate *i*NKT cells was assessed by determining the levels of IFN-γ production after 40 h by ELISA.[10] Once again, all three ThrCer analogues stimulated human *i*NKT cells, albeit at lower levels than the α-GalCer analogues, which is in accord with the behavior of the two parent compounds. In agreement with the murine *i*NKT cell data (Figure 4), the weakest ligand at 100 ng mL^{-1} was again ThrCer-urea **12**; however in this assay, ThrCer-thioamide **11** and ThrCer-carbamate **13** were now more comparable to ThrCer in their behavior (Figure 5, panel b). All of the α-GalCer analogues stimulated human *i*NKT cells, with the urea analogue **9** proving to be the weakest activator at low concentrations (data not shown) (Figure 5, panel a).

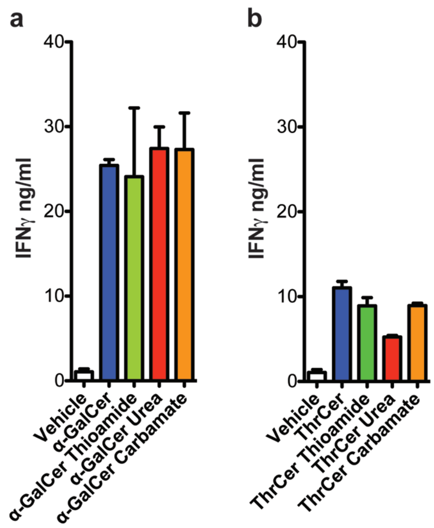


Figure 5. Activation of human *i*NKT cells using thioamide, urea, and carbamate analogues of α-GalCer (panel a) and ThrCer (panel b).

Since the two urea derivatives **9** and **12** displayed the weakest activity in our *in vitro* experiments and could not be refolded for surface plasmon resonance (SPR) experiments (*vide infra*), further studies focused solely on the thioamide and carbamate derivatives. These analogues were investigated *in vivo*, alongside the parent compounds and Th2 cytokine-biasing molecule OCH9 (**6**),[28,29] specifically to assess their ability to cause DC maturation and cytokine response profile. To this end, 1 μg of lipid was injected intravenously (i.v.) into wildtype C57 BL/6 or C57 BL/6 CD1d$^{-/-}$ (NKT-cell-deficient) mice. After 2 h, the mice were tail-bled, and IL-4 levels in the serum were measured by ELISA (Figure 6).[10] At 18 h, blood serum levels of IFN-γ were measured by ELISA (Figure 6), and cells harvested from the spleen were used to determine the extent of DC maturation by measuring the expression of the co-stimulatory molecule, CD86, using fluoresence-activated cell sorting (FACS) analysis (Figure 7).

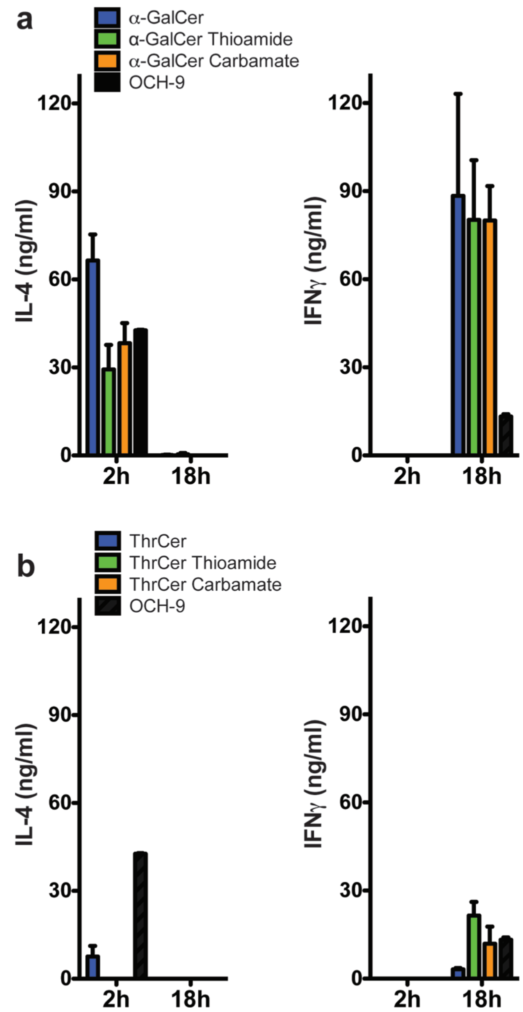


Figure 6. Cytokine production of wildtype C57 BL/6 mice after stimulation with α-GalCer analogues (panel a) and ThrCer analogues (panel b) or the Th2-biasing analogue OCH9 (**6**).

The *in vivo* activation of *i*NKT cells with the α-GalCer and ThrCer analogues was determined by analyzing the cytokine profile in blood serum at 2 and 18 h. Thus, α-GalCer analogues **8** and **10** showed a marked decrease in the ability to stimulate *i*NKT cells to produce IL-4 at 2 h post injection compared with α-GalCer, but both compounds were able to maintain IFN-γ production at 18 h, consistent with that of α-GalCer (Figure 6, panel a). Differences in cytokine production were even more pronounced with the weaker ThrCer agonists **11** and **13**, both of which did not stimulate *i*NKT cells to produce IL-4 at all when assayed at 2 h but were still able to produce IFN-γ at 18 h (Figure 6, panel b). Although there was not a statistical difference between the IFN-γ produced by ThrCer and its carbamate analogue (**13**) at 18 h ($p > 0.05$), the thioamide analogue **11** produced significantly more IFN-γ compared to ThrCer ($p = 0.01$). No cytokine production was detected in CD1d$^{-/-}$ mice injected with the α-GalCer and ThrCer analogues (data not shown). Since the presentation of CD1d–lipid complex by DC to *i*NKT cells results in activation and the subsequent maturation of DC, we also determined whether there was any difference in the ability of DC to upregulate the co-stimulatory molecule, CD86, following i.v.

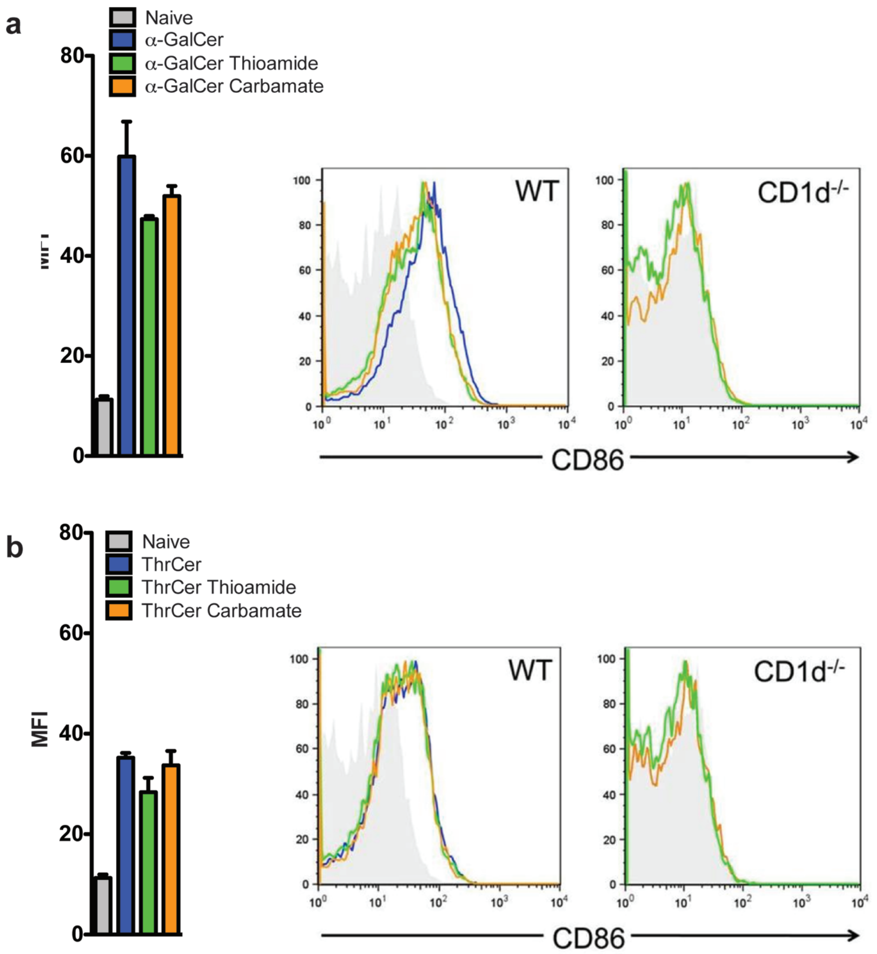


Figure 7. DC maturation, as determined by the upregulation of the co-stimulatory molecule CD86 by FACS analysis, after injection of wildtype C57 BL/6 or CD1d$^{-/-}$ (NKT-cell-deficient) mice with α-GalCer analogues (panel a) and ThrCer analogues (panel b). MFI = median fluorescent intensity.

delivery of α-GalCer and ThrCer analogues. Pleasingly, both sets of analogues induced DC maturation to a similar degree as the parent α-GalCer and ThrCer compounds in wildtype mice but not in CD1d$^{-/-}$ mice (Figure 7).

Finally, we examined the binding kinetics of our new CD1d agonists. To this end, bacterially expressed hCD1d and β-2-microglobulin (β_2M) molecules were refolded with the thioamide, and carbamate analogues of both α-GalCer and ThrCer by oxidative refolding chromatography, and then biotinylated as described previously.[30,31] The urea analogues of α-GalCer and ThrCer could not be refolded, and therefore no SPR data are available for these molecules. Soluble human *i*NKT TCR was prepared as described by McCarthy *et al.*[30] SPR experiments were used to measure the affinity and kinetics of human *i*NKT cell TCRs for hCD1d loaded with α-GalCer, ThrCer, and their thioamide and carbamate analogues (Figure 8). To this end, increasing concentrations of TCR were injected for 10 s over the indicated complex immobilized on the BIAcore chip until the specific binding reached its plateau. K_d and B_{max} were calculated by fitting the data using a non-linear regression binding kinetics model (GraphPad Prism) (Figure 8). Kinetic measurements for the k_{off} were calculated using BIAevaluation software kit; k_{on} values were calculated from the experimental k_{off} and K_d (Table 1).

Analysis. The *in vivo* experiments for the α-GalCer analogues show that the thioamide (**8**) and carbamate derivatives (**10**) both display a cytokine bias toward IFN-γ compared with α-GalCer. This bias arises from a reduction in IL-4 production relative to that of the parent α-GalCer **1**, rather than an increase in IFN-γ production, which in both cases was similar to that generated by α-GalCer **1**. Results for the ThrCer derivatives were more significant in that these molecules displayed an even more pronounced trend with stronger skewing toward IFN-γ production. Both ThrCer-thioamide **11** and ThrCer-carbamate **13** displayed *no* IL-4 production when assayed at 2 h but showed levels of IFN-γ production at 18 h, which were similar (for **13**) or higher (for **11**) than those shown for ThrCer **2** and in the case of **11** only four times lower than that displayed by the most potent CD1d agonist, α-GalCer **1**.

CD1d agonists that exhibit a cytokine response that is more Th1-biasing (more IFN-γ and less IL-4) than α-GalCer are relatively unusual[32−35] but in demand owing to their potential application as adjuvants for cancer immunotherapy and in combating infectious diseases. The *C*-glycosyl analogue of KRN7000, α-*C*-GalCer (**7**, Figure 1), is one such molecule that induces a useful Th1-biased cytokine response.[34] The thioamide and carbamate analogues and those of ThrCer in particular appear to fall into the same category.

Rationalizing the observed results is not straightforward since the mechanisms by which glycolipid CD1d agonists modulate the cytokine response on *i*NKT cell activation are multifactorial and remain poorly understood.[2,18] The stability of the

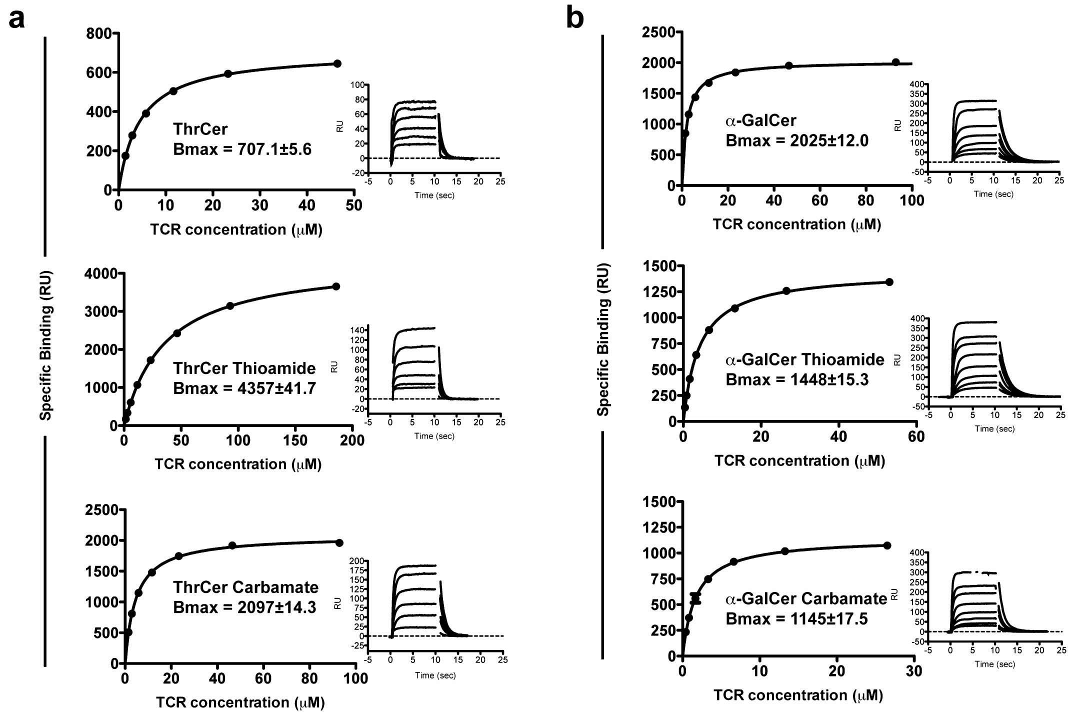


Figure 8. Binding affinities (left) and kinetics (right) of the *i*NKT cell TCR for hCD1d molecules loaded with ThrCer (panel a) and α-GalCer (panel b) analogues measured using SPR.

Table 1. K_d Values and On- and Off-Rates for α-GalCer and ThrCer and Their Thioamide and Carbamate Analogues

lipid on CD1d	exptl K_d (μM)	exptl k_{off} (s^{-1})	calcd k_{on} ($\times 10^5$ M^{-1} s^{-1})
ThrCer **2**	4.57 ± 0.12	1.18 ± 0.034	2.58 ± 0.14
ThrCer-thioamide **11**	36.06 ± 0.96	1.88 ± 0.045	0.52 ± 0.03
ThrCer-carbamate **13**	4.60 ± 0.13	1.19 ± 0.072	2.59 ± 0.23
α-GalCer **1**	2.19 ± 0.07	0.565 ± 0.008	2.58 ± 0.12
α-GalCer-thioamide **8**	4.20 ± 0.15	0.537 ± 0.011	1.28 ± 0.07
α-GalCer-carbamate **10**	1.72 ± 0.10	0.919 ± 0.035	5.34 ± 0.51

glycolipid–CD1d complex[36] and its TCR affinity[17,37] have both been invoked to be important; however, the proposal that low CD1d binding affinity and TCR affinity leads to Th2 cytokine-biasing agonists has recently been challenged by Sullivan *et al.*, who made a direct comparison between the Th2-biasing OCH9 glycolipid **6** and the Th1-biasing *C*-glycosyl analogue of α-GalCer **7**.[18] Both OCH9 **6** and the *C*-glycosyl analogue of α-GalCer **7** displayed weaker interactions than α-GalCer with the *i*NKT cell TCR, which led the authors to attribute the observed differences in cytokine response profiles to other factors including their differing pharmacokinetics properties.[18] Our own SPR experiments, which measured the binding kinetics of the TCR to glycolipid-loaded hCD1d for the thioamide and carbamate analogues of α-GalCer and ThrCer, show that these molecules also display similar or poorer TCR binding kinetics compared to the parent compounds. Thus equilibrium binding constants (K_d) for the TCR–carbamate–hCD1d complexes (compounds **13** and **10**) were similar to those of their parent compounds (**2** and **1**, respectively), with comparable association and dissociation rates (Table 1) indicating a similar TCR engagement and dissociation. On the other hand, weaker TCR binding affinity was observed toward both ThrCer and α-GalCer thioamide analogues (compounds **11** and **8**, respectively) than to their parent analogues, with the most pronounced (8-fold) reduction in K_d between the ThrCer thioamide **11**, the most Th1 cytokine-biasing analogue in our series. In this sense, the thioamide analogue of ThrCer (**11**) is behaving similarly to the Th1 cytokine-biasing *C*-glycosyl analogue of α-GalCer (**7**), which shows even lower TCR affinity than OCH9.[18] In both thioamide analogues, the weaker binding was mainly attributed to the slower association rate. We hypothesize that this slow TCR engagement may be a result of the disturbance of bridging water molecules in the thioamide–CD1d complex, arising from the replacement of the carbonyl oxygen with a larger sulfur atom. In both α-GalCer and ThrCer series, the urea analogues displayed poor activity, and we were unable to obtain TCR binding and kinetics data for these two substrates, which may suggest that the additional NH functionality incorporated into the acyl chain disrupts glycolipid binding and subsequent presentation.

With little correlation between TCR binding affinity for a CD1d–glycolipid complex and the measured cytokine profile, researchers have attributed differences in cytokine response profiles to other factors, including their differing pharmacokinetics properties and ability to transactivate NK cells downstream of *i*NKT cell activation. For example, the Th2 cytokine-biasing response of OCH9 has been attributed to its reduced ability to transactivate NK cells, which are responsible for a significant proportion of the IFN-γ produced after glycolipid stimulation.[38] The Th1 cytokine-biasing *C*-glycosyl analogue of α-GalCer (**7**), conversely, is capable of transactivating NK cells *via* a CD40-dependent mechanism.[18] Since the thioamide and carbamate analogues of ThrCer showed similar (**13**) or higher (**11**) levels of IFN-γ compared to ThrCer at 18 h (Figure 6), we investigated the contribution of NK cell transactivation to IFN-γ production following i.v. injection of these two ThrCer analogues. Interestingly, ThrCer **2** and ThrCer-carbamate **13**, but not ThrCer-thioamide **11**, showed evidence of NK cell transactivation at 24 h post i.v., as determined by intracellular IFN-γ staining (see the Supporting Information). The observed levels of NK cell transactivation in the case of ThrCer **2** and its carbamate analogue **13** were similar to those previously reported using α-GalCer **1**.[34] Furthermore, these findings correlated with prolonged IFN-γ levels in blood serum following the administration of ThrCer-carbamate and ThrCer, but not ThrCer-thioamide (see the Supporting Information). Thus while the enhanced production of IFN-γ at 18 h post i.v. using the carbamate analogue of ThrCer can (at least in part) be rationalized by this CD1d agonist transactivating NK cells, in analogy to the behavior of the well-known Th1 cytokine-biasing analogue, **7**, the cytokine profile observed for the thioamide analogue **11** cannot be attributed to this mechanism of IFN-γ production. We hypothesize that the decreased "on rate" of ThrCer-thioamide, as shown by SPR (Figure 8), may be detrimental for sustained IFN-γ production through this mechanism.

Conclusions. Ever since it was demonstrated that α-GalCer **1** functions as a potent CD1d agonist, numerous structural modifications have probed structure−activity relationships and led to the discovery of CD1d agonists that are capable of polarizing cytokine production. Structural variation around the amide bond in **1** has to-date received scant attention. To this end, we prepared thioamide, carbamate, and urea analogues of α-GalCer and its non-glycosidic analogue, ThrCer, and carried out an investigation of their biological activity. While the carbamate and thioamide analogues of α-GalCer are similar in behavior to the parent molecule, the same changes in ThrCer led to two substrates that display a markedly different cytokine response profile upon *i*NKT cell activation. This study shows for the first time that amide isosteres of CD1d agonists can be used to elicit significant changes in cytokine response. We propose that the carbamate analogue **13** behaves similarly to the known Th1 cytokine-biasing analogue **7**, with transactivation of NK cells, at least in part accounting for the observed increase in IFN-γ production. This mechanism cannot account for the observations with the thioamide analogue **11**, which does not transactivate NK cells, and we tentatively propose that other factors such as the location of glycolipid loading and processing are important in this case. Further studies will seek to shed further insight into what may be a novel mode of action of this attractive CD1d agonist.

METHODS

Mice and Reagents. C57BL/6 and CD1d$^{-/-}$ (NKT cell-deficient) mice were used. Animal experiments were carried out under the authority of a U.K. Home Office Project License. Compounds were solubilized in 150 mM $NaCl_{(aq)}$ and 0.5% Tween 20 (vehicle).

***In Vitro* and *in Vivo* Activation of *i*NKT Cells.** For *in vitro* activation of murine *i*NKT cells, 1×10^5 C1R-mCD1d cells were pulsed with α-GalCer, ThrCer, and analogues or vehicle overnight. Following washes, 2×10^4 murine *i*NKT (DN32) hybridoma cells were added to the cultures for 24 h, and the presence of IL-2 was determined by ELISA.[27] For *in vitro* activation of human *i*NKT cells, 1×10^5 C1R-hCD1d cells were pulsed with α-GalCer, ThrCer, and analogues or vehicle overnight. Following washes, 2×10^4 *i*NKT cells were added to the cultures for 40 h, and the presence of IFN-γ was determined by ELISA.[27]

For *in vivo* activation of *i*NKT cells, C57 BL/6 WT or CD1d$^{-/-}$ mice were injected intravenously (i.v.) with 1 μg of lipids, blood serum was taken at 2 or 18 h, and the presence of IL-4 and IFN-γ was determined by ELISA.[10]

Phenotype of Murine APCs. Expression of CD86 on CD11c$^+$ splenocytes was assessed by flow cytometry following i.v. delivery of 1 μg of lipids to C57 BL/6 or CD1d$^{-/-}$ mice at 18 h post injection. Abs for flow cytometry were from eBioscience, and flow cytometry was performed on a FACSCalibur device with CellQuest software.

Protein Expression and Purification. hCD1d and β2 m were refolded with GalCer and ThrCer analogues by oxidative chromatography, following the method described by Karadimitris *et al.*[31] In summary, CD1d and β2 m were overexpressed in *E. coli* BL21 using a prokaryotic expression system. The individual proteins were purified from inclusion bodies as described in Dunbar *et al.*,[39] then refolded with the corresponding lipid, and biotinylated, and the complex was purified as described by Karadimitris *et al.*[31]

Preparation of Human *i*NKT TCR. Soluble TCR was prepared according to the protocol described by McCarthy *et al.*,[30] where both Vα24 and Vβ11 chains were individually overexpressed in *E. coli*, purified from the inclusion bodies, then refolded, and purified to generate the TCR heterodimers according to the method previously published by Boulter *et al.*[40]

Surface Plasmon Resonance. SPR experiments were performed with a model 3000 Biacore to measure the affinity and kinetics of NKT TCR binding to hCD1d−ligand complexes. In brief, approximately 1000 RU of the biotinylated hCD1d-lipid complexes were immobilized onto streptavidin-coated CM5 sensor chips (Biacore). Aliquots of purified TCR with increasing concentrations were passed on the immobilized hCD1d−lipid at a flow rate of 10 μL min^{-1} for the equilibrium binding experiments or 50 μL min^{-1} for the kinetics experiments. The K_d values were calculated by fitting the data from the equilibrium binding experiment to a non-linear regression saturation binding model (GraphPad Prism 5.0), whereas the k_{off} data were estimated from the kinetics experiments by fitting the data with the built-in models of the BIAeval 3.1 software (BIAcore).

ASSOCIATED CONTENT

Supporting Information

Transactivation of NK cells, statistical analysis, experimental procedures and full characterization data for all new compounds, and scanned copies of ^{1}H NMR and ^{13}C NMR spectra for all new compounds. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: g.besra@bham.ac.uk; vincenzo.cerundolo@imm.ox.ac.uk; l.r.cox@bham.ac.uk.

Present Address

‖WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow G1 1XL, U.K.

Author Contributions

⊥These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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