There has been significant excitement in recent years about the potential of certain glycolipids as immunomodulating agents. The discovery of KRN7000 (1) can be considered a milestone in this regard (Figure 1). KRN7000 is an exogenous (α-linked) galactosylceramide, and it was shown that its action as an antigen arises by binding to a CD1d protein, followed by recognition of the complex by the semi-invariant T cell receptor (TCR) of natural killer T cells (iNKT cells). The resulting activation of NKT cells leads to a rapid release of both pro-inflammatory Th1 (e.g. IFN-γ and IL-2) and anti-inflammatory Th2 (e.g. IL-4 and IL-10) cytokines.

Unfortunately, the release of both types of cytokines limits the therapeutic potential of 1, as they antagonise each other’s action. Disruption of the carefully controlled Th1/Th2 cytokine balance can cause disease, and therapeutic strategies aimed at a restoration of this balance by in vivo modulation of iNKT cells either in vitro or in vivo are very promising. Many analogues of KRN7000 modified at the various sub-structures have been synthesised and evaluated in murine (m) and human (h) systems. For example, it has been demonstrated that a shorter acyl chain (m, h) as well as introduction of unsaturations (m, h) results in Th2 polarisation. A shorter phytosphingosine chain gives a preferential Th2 response, even when a terminal ring is introduced in a truncated sphingosine chain (h). Introduction of aromatic groups in the acyl chain generally results in a Th1 bias (m, h). Modifications in the galactose unit proved the essential role of the α-anomeric configuration (m), and the 2-OH for activity (m, h), but revealed that modifications on the 3-, 4-, or 6-OH groups are allowed (m). Interestingly, nonglycosidic (polyhydroxyl) substituted ceramides have been shown to bind to CD1d and activate iNKT cells. The carbocyclic analogue of KRN7000 induces Th1-biased cytokine production (m), as is the case for the corresponding C-glycoside (α-C-GalCer), and the phytosphingosine C1-nor-C-glycoside (m, h). Surprisingly, the corresponding α-S-GalCer analogue does not stimulate iNKT cells either in vitro or in vivo. Analogue modifications in the phytosphingosine polar region have also been investigated. The 3-OH group proved to be more important than the 4-OH group for antigenic activity (m), and the stereochemistry of the alcohols and amide substituents influence the activity (m). Other modifications in this region include conformationally restricted analogues featuring an azetidine ring (in which the amide nitrogen and C4 are linked), which show potent induction of cytokines with a slight Th2 bias (m), and a fluorinated analogue containing a 4-deoxy-4,4-difluoro modification, which shows preferential Th1 induction (m). Finally, Kim and co-workers reported a set of analogues in which the amide group is replaced by a triazole moiety, with variable lengths of the attached lipid chain (m). It was found that selected analogues elicit a Th2-polarised cytokine response, with the long-chained analogues such as 2 having a stronger response.

However, at present the relationship between glycolipid structure and cytokine polarisation is not completely understood. It has been postulated that the origin of cytokine polarisation could relate to the stability of the glycolipid complex with CD1d, or to a difference in glycolipid-presenting motifs.

The matter is complicated given the two binding events involved. X-ray crystallographic studies have given some insight into these binding events. The crystal structure of human CD1d in complex with KRN7000 shows how the long aliphatic chains fit in ‘binding grooves’ present on CD1d. Importantly, it was also discovered that a series of hydrogen bonds between the galactosylceramide and CD1d serve to position the...
teristics. We therefore introduced two fluorine atoms in the a interaction by altering the amide N/C0 was decided to investigate the functional role of this possible tween these groups short enough to suggest an interaction. It (Thr156 for mCD1d, Thr154 for hCD1d), with the distance be- drogen bond with the OH group of the adjacent Thr residue
bonds occur between hCD1d Asp151 (equivalent to mCD1d Asp153) and the galactose 2-OH group. A hydrogen bond between Thr154 and the a and the phytosphingosine 3-OH group. A hydrogen bond be-
between Thr154 and the galactose 2-OH and 3-OH groups. [26a] Interestingly, a hydrogen bond between the OH group of Thr156 and the KRN7000 actose 2-OH and 3-OH groups. [26a] Interestingly, a hydrogen
bond between the OH group of Thr156 and the KRN7000

We were intrigued by the Thr156–amide NH hydrogen bond. Inspection of the various X-ray crystallographic studies indeed revealed that the amide N–H is perfectly lined up to form a hydro-

3-phosphingosine derivative 7 with the galactosyl trichloroacetimi-de donor 6. The synthesis of the fluorinated fatty acid 5 was envisioned by a fluorinated building block approach in-
volving addition of 8 to alkene 9. Alkene 9 was synthesised in two steps from commercially available tetracosanol 10 by bromination followed by elimin-
ination (Scheme 2). The elimination was achieved using a com-

Scheme 1. Retrosynthetic analysis for compound 3.

Scheme 2. Synthesis of the fluorinated fatty acid 5: a) DDQ, TBAB, PPh3, CH2Cl2, room temperature, 30 min, 96%; b) KOH, TIPSOH, DMF/Et2O (85:15), room temperature, 16 h, 73%; c) 8, AIBN, DCE, 70 °C, 16 h, 87%; d) Bu3SnH, Et3B, air, toluene, room temperature, 1 h, 70%; e) NaOH (1 m), THF, room temperature, 16 h, 95%.

Scheme 3. The a-galacturonosylceramide (GalA-GSL) complexed to mCD1d (though GalA-GSL lacks a sphingo-
sine derivative 7 with the galactosyl trichloroacetimide donor 6. The synthesis of the fluorinated fatty acid 5 was envisioned by a fluorinated building block approach in-
volving addition of 8 to alkene 9. Alkene 9 was synthesised in two steps from commercially available tetracosanol 10 by bromination followed by elimination (Scheme 2). The elimination was achieved using a com-

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Fluorination of KRN7000 elicits CD1d-dependent TCR-activation of NKT cells

In conclusion, we have successfully synthesised a novel KRN7000 analogue that has the potential to interrogate molecular interactions at the atomic level. Our biological data show that compound 3 is able to modulate NKT cell responses and that the presence of two fluorine atoms in the amide group induces a Th2-biased immune response by murine NKT cells.

Experimental Section

Column chromatography was performed on 230–400 mesh Matrex silica gel. Preparative HPLC was carried out using a Bio-Rad Biosil D 90–10, 250 × 22 mm column elut...
ing at 20 mL min⁻¹. Melting point values are uncorrected. Reaction solvents were dried before use as follows: THF and Et₂O were distilled from sodium/benzophenone; CH₂Cl₂ and Et₂N were distilled from CaH₂; toluene was distilled from sodium; pyridine was double distilled from CaH₂ and stored in a Schlenk flask. Ethyl iododifluoracetate (light pink) was purchased from Fluorochem Ltd., and in most cases was used without further purification. If the purchased ethyl iododifluoracetate was deep purple in colour, the reagent was dissolved in Et₂O, washed with a saturated solution of Na₂SO₄, and concentrated in vacuo to give a light-yellow oil. All reaction vessels were flame dried under vacuum prior to use, and all experiments were carried out under N₂ atmosphere. All other reagents were purchased from commercial sources and used without further purification.

1-Bromotetrasacane (11): 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ; 833 mg, 3.01 mmol) was added to a mixture of alcohol tetracosanol (1.0 g, 2.73 mmol), PPh₃ (789 mg, 3.01 mmol), and tri-n-butylammonium bromide (TBAB; 970 mg, 3.01 mmol) in CH₂Cl₂ (13.6 mL), and the mixture was stirred at room temperature for 30 min. The deep-red mixture was poured into H₂O/MeOH (1:1, 100 mL), extracted with petroleum ether (3×80 mL), dried over MgSO₄, filtered, and concentrated in vacuo to give the crude amine. The crude was purified by column chromatography (petroleum ether) to afford alkene 11 as a colourless oil (1.10 g, 96%). ¹H NMR (400 MHz, CDCl₃): δ = 3.41 (2 H, t, J = 7.0 Hz), 1.87 (2 H, quintet, J = 7.0 Hz), 1.49–1.21 (42 H, m), 0.90 ppm (3 H, t, J = 6.5 Hz); ¹³C NMR (100 MHz, CDCl₃): δ = 33.9, 33.1, 32.1, 29.90–29.82 (m), 29.75, 29.64, 29.56, 29.2, 29.1, 28.4, 22.9, 14.3 ppm. ¹H and ¹³C NMR correspond to previously reported values.[19]

Tetracos-1-ene (9): KOH (352 mg, 6.27 mmol) was added to a mixture of aldehyde tetracosanol (1.13 g, 3.13 mmol) and TIPSOH (0.62 mL, 3.13 mmol) in DMF (31 mL) and Et₂O (5 mL), and the mixture was stirred at room temperature for 16 h. The mixture was poured into H₂O (60 mL), extracted with petroleum ether (3×80 mL), dried over MgSO₄, filtered, and concentrated in vacuo. The crude was purified by column chromatography (petroleum ether) to afford alkene 9 as a colourless oil (1.0 g, 27.6 mg, 0.060 mmol) in THF (1 mL). The mixture was stirred at room temperature for 16 h, followed by the addition of HCl (2 mL, 1.5 mL). The aqueous layer was extracted with Et₂O (3×10 mL), and the combined organic layer was washed with brine, dried over MgSO₄, filtered, and concentrated in vacuo. The crude was used immediately in the subsequent transformation.

Protected 2,2'-difluoroalkanoylbenzene 10: 1PMe₃ (1.0 mmol solution in THF; 1.0 mL, 1.0 mmol) was added to azide 14 (200 mg, 0.210 mmol) in THF at 0 °C, and the mixture was stirred at 0 °C for 45 min, followed by a further 3 h at room temperature. NaOH (1 mL, 2.0 mmol) was added, and the mixture was stirred for 80 min at room temperature. EtOAc (10 mL) was then added, and the organic layer was washed with H₂O (2×10 mL) and brine (10 mL), dried over Na₂SO₄, filtered, and concentrated in vacuo to give crude amine 4 (197 mg). The crude (61 mg) was used immediately in the subsequent transformation. 2) EDC (22 mg, 0.141 mmol) was added to a mixture of crude amine 4 (61 mg, 0.0658 mmol) and difluoroalkanoylbenzene 5 (20 mg, 0.0462 mmol) in CH₂Cl₂ (2 mL), and the mixture was stirred at room temperature for 16 h. The mixture was dried over Na₂SO₄, filtered, and concentrated in vacuo. The crude was purified by column chromatography (EtOAc/hexane 1:9) to afford the protected α-GalCer 15 as a solid (25 mg, 40%); mp: 56–58 °C; [α]D₂⁰ = +46.0 (c = 0.125, CHCl₃); ¹H NMR (400 MHz, CDCl₃): δ = 7.53–7.50 (2 H, m), 7.42–7.22 (23 H, m), 6.83 (1 H, d, J = 8.5 Hz), 5.45 (1 H, s), 4.91 (1 H, d, J = 3.5 Hz), 4.85 (1 H, d, J = 11.8 Hz), 4.78 (1 H, d, J = 12.0 Hz), 4.72 (1 H, d, J = 12.0 Hz), 4.71 (1 H, d, J = 11.5 Hz), 4.64 (1 H, d, J = 11.8 Hz), 4.56 (1 H, d, J = 11.6 Hz), 4.51 (1 H, d, J = 11.3 Hz), 4.49 (1 H, d, J = 11.6 Hz), 4.33 (1 H, m), 4.16–4.05 (3 H, m), 3.95–3.88 (3 H, m), 3.83–3.76 (2 H, m), 3.53–3.50 (2 H, m), 2.07–1.95 (2 H, m), 1.71–1.56 (2 H, m), 1.45–1.37 (2 H, m), 1.26 (m, br), 0.89 ppm (6 H, appt, δ = 6.85 Hz); ¹³C NMR (100 MHz, CDCl₃): δ = 138.64, 138.59, 138.3, 138.0, 137.8, 128.8, 128.5, 128.4, 128.1, 127.8 (m), 127.7, 127.60, 127.57, 126.3, 100.0, 99.6, 79.5, 79.2, 76.2, 75.5, 74.4, 73.8, 73.5, 72.0, 71.9, 69.3, 67.6, 63.1, 50.6, 33.8 (t, J = 164.6 Hz), 32.9, 32.7, 29.7 (m), 29.6, 29.4, 29.2, 25.8, 22.7, 21.6 (m), 14.1 ppm (amide and CF₂, not observed); ¹⁹F NMR (282 MHz, CDCl₃): δ = −105.4 (d, J = 252.5 Hz), −107.2 ppm (d, J = 252.5 Hz); IR: δ = −2918 (vs), 2850 (s), 1761 (m), 1465 (m), 1415 (m), 735 cm⁻¹ (m); ES MS m/z (%): 483 (M+Na⁺, 100); HRMS (ES⁺) for C₂₈H₅₄F₂O₃Na[M+Na⁺]⁺: calc. 483.3984, found 483.3984.
glycolipids

Keywords: α-galactosylceramide • cytokines • fluorination • glycolipids • NK cells

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temperature under H2 atmosphere (balloon) for 1.5 h. Pyridine (-0.2 mL) was added, and the mixture was stirred for 15 min at room temperature, filtered through celite, washed with a copious amount of CH2Cl2/MEOH (1:1) and co-evaporated with toluene under reduced pressure. The crude product was purified by column chromatography (MeOHHCH2Cl2, 10:20, then 50% v/v) to afford GalCer and compound 3 (7.2 mg, 46%).1H NMR (400 MHz, CDCl3/CD2OD); δ = 4.85 (1H, d, J = 4.0 Hz), 4.18 (1H, m), 3.89 (1H, d, J = 3.5 Hz), 3.86 (1H, dd, J = 10.5, 4.5 Hz), 3.73 (1H, m), 3.70–3.67 (2H, m), 3.66–3.61 (2H, m), 3.53–3.48 (2H, m), 3.33–3.27 (2H, m), 2.07–1.87 (2H, m), 1.57–1.06 (m), 0.86–0.72 ppm (6H, app t, J = 3.86 (1H, dd, J = 7.1 Hz).19F NMR (282 MHz, CDCl3/CD2OD); δ = −105.7 (d, J = 250.4 Hz), −108.0 ppm (d, J = 251.5 Hz); IR: ν~max = 3292 (m, br), 2916 (s), 2849 (w), 1600, 1472 (NH), 1360 (~C0) +, 1000, ~1 – 60, 2320 (~C23) 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim www.chemmedchem.org

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The synthesis of 2',2'-difluoro KRN7000 is described. In vivo evaluation demonstrates that this fluorinated glycolipid induces CD1d-dependent TCR activation of NKT cells, with a bias towards Th2 cytokine production.