ORGANIC LETTERS

1999
Vol. 1, No. 3
359−361

Synthesis of NBD-α-galactosylceramide and Its Immunologic Properties

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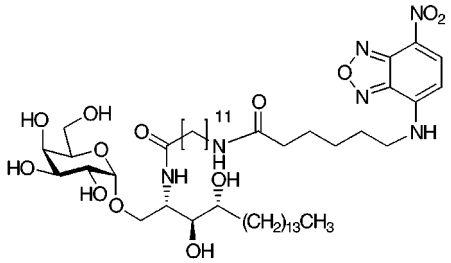
Received March 29, 1999

ABSTRACT



AGL-597 (8)

A representative α-galactosylceramide (α-GalCer), KRN7000, can activate NKT cells through CD1d molecules, which play an essential role in the generation of the strong antitumor activity of KRN7000. Our previous study has demonstrated that α-GalCer binds directly to CD1d molecules. However, it is controversial whether CD1d binds α-GalCer in endosomal compartments. To address this question, we synthesized NBD-α-GalCer, which has strong fluorescent properties. We found that the NBD-α-GalCer has immunostimulatory activity that is stronger than that of KRN7000.

KRN7000 (**1**), an α-galactosylceramide (α-GalCer, galactose bound to ceramide in an α-configuration, Figure 1), has shown significant immunostimulatory[1] and antitumor activity in mice.[2] Our studies concerning the mechanism of immunostimulation by **KRN7000** have indicated that **KRN7000** is presented to mouse Vα14/Vβ8 natural killer T (NKT) cells and human Vα24/Vβ11 NKT cells, a counterpart of mouse Vα14/Vβ8 NKT cells, by mouse CD1 and human CD1d molecules on antigen-presenting cells, leading to activation of these NKT cells.[3−8] Because it is well-known that NKT cells activated by **KRN7000** and interleukin-12 can suppress tumor metastases[9,10] and that NKT cells may play an important role in regulating the progress of several autoimmune diseases such as systemic sclerosis and type 1 diabetes,[11,12] α-GalCer has drawn significant attention as an interesting immunomodulator which will be useful for treatment of these and related diseases.

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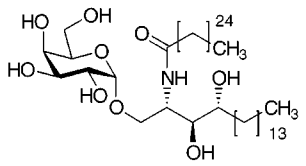
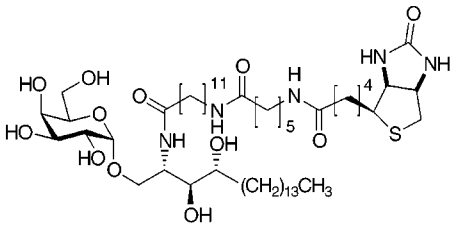
10.1021/ol9900111 CCC: $18.00
Published on Web 06/23/1999



KRN7000 (1)



AGL-592 (2)

Figure 1. Chemical structures of **KRN7000** (α-GalCer) and **AGL-592** (biotinylated α-GalCer).

The human CD1 family contains five members, designated CD1a, CD1b, CD1c, CD1d, and CD1e. CD1 can be classified into two groups based upon amino acid sequence homologies: CD1a−c (group 1) and the CD1d-like group of mouse, rat, and humans (group 2).[13] Among the group 1 CD1 molecules, CD1b has been well characterized by several research groups. These studies have demonstrated that glycolipids such as glucomonomycolate (GMM) and lipoarabinomannan (LAM) from *Mycobacteria* spp. can function as antigens for subsets of human T cells expressing $\alpha\beta$ T cell receptors.[14] In addition, it has been shown that LAM, fragments derived from LAM and GMM, can bind to CD1b molecules.[15] Furthermore, CD1b molecules broadly distribute in endosomal and lysosomal compartments of antigen-presenting cells, where the binding of glycolipid and CD1b molecules likely occurs.[16−18] However, while CD1d-like molecules also distribute in similar endosomal compartments,[19,20] it remains controversial whether CD1d binds α-GalCer in these intracellular locations.

We have recently demonstrated that α-GalCer directly binds to CD1d molecules by the method of surface plasmon resonance using immobilized biotinylated α-GalCers such as **AGL-592** (**2**, Figure 1) and soluble CD1d molecules.[21] These results suggest that biotinylated α-GalCer will be a useful tool to study the distribution of CD1d molecules in antigen-presenting cells. We therefore examined the distribution of CD1d on CD1d-transfected cells and parental untransfected cells using biotinylated α-GalCers and fluorescein isothiocyanate (FITC)-conjugated avidin. However, we could not clearly detect differences in CD1d distribution between these two types of cells, which exhibit distinct CD1d expression patterns in experiments using a biotin-conjugated anti-CD1d monoclonal antibody and FITC-conjugated avidin. This result suggested that an α-GalCer bearing a fluorescent group might be necessary to study the distribution of CD1d in antigen-presenting cells. We therefore synthesized an α-GalCer bearing a fluorescent group.

It has been reported that paclitaxel (Taxol) and docetaxel (Taxotere), potential anticancer agents, bearing a 7-nitrobenz-2-oxa-1,3-diazol (NBD) group are useful tools for the investigation of the binding site for these compounds.[22] On the basis of this finding, we synthesized an α-GalCer bearing a NBD group (NBD-α-GalCer) as shown in Scheme 1. Briefly, the lyso sphingosine (**3**)[21] was amidated with 12-*N*-(trifluoroacetoxyamino)dodecanoic acid (**4**)[23] in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (WSC·HCl) to afford galactosylceramide (**5**). Deprotection of the trifluoroacetyl group under basic conditions provided the galactosylceramide containing an ω-amino fatty acid (**6**). The NBD group was coupled with **6** through its *N*-hydroxysuccinimide ester (**7**), which was purchased from Molecular Probes, to generate the NBD-α-GalCer (**AGL-597, 8**).[24]

We previously reported that **KRN7000** stimulates the proliferation of murine spleen cells, which indicates the immunostimulatory properties of the glycolipids, in a concentration-dependent manner and has one of the strongest immunostimulatory activities among the α-GalCers which were synthesized in our laboratory.[1,25] On the basis of this finding, we examined the effect of **AGL-597** on murine pleen cells and used **KRN7000** as a positive control. At the same time, we evaluated the spleen cell proliferation stimulatory activity of the biotinylated α-GalCer, **AGL-592**, to estimate the role of fatty acid side chains in the ceramide moiety in the immune response induced by α-GalCers.

As shown in Table 1, **KRN7000** significantly stimulated the proliferation of spleen cells beginning at concentrations as low as 0.1 ng/mL. **AGL-592** showed siginificant spleen cell proliferation stimulatory activity at concentrations of 10

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(24) **AGL-597** (**8**): $[\alpha]^{23}_{D}$ +9.6° (*c* 0.1, methanol); mp 126−127 °C; FDMS *m/z* 953 $(M + H)^+$, 975 $(M + Na)^+$; ^{1}H NMR (500 MHz, CD_3OD) δ 8.52 (1H, d, J = 9.0 Hz), 6.35 (1H, d, J = 9.0 Hz), 4.80 (1H, d, J = 3.4 Hz), 4.19 (1H, m), 3.87 (2H, m), 3.83 (1H, m), 3.75 (1H, m), 3.66−3.72 (3H, m), 3.60 (1H, t, J = 6.1 Hz), 3.54 (2H, m), 3.30 (2H, m), 3.13 (2H, t, J = 7.1 Hz), 2.21 (2H, t, J = 7.6 Hz), 2.20 (2H, t, J = 7.3 Hz), 1.80 (2H, m), 1.68 (2H, m), 1.60 (2H, m), 1.47 (4H, m), 1.28 (14H, m) 0.89 (3H, t, J = 7.1 Hz). Anal. ($C_{48}H_{84}N_6O_{13}$) C, H, N.

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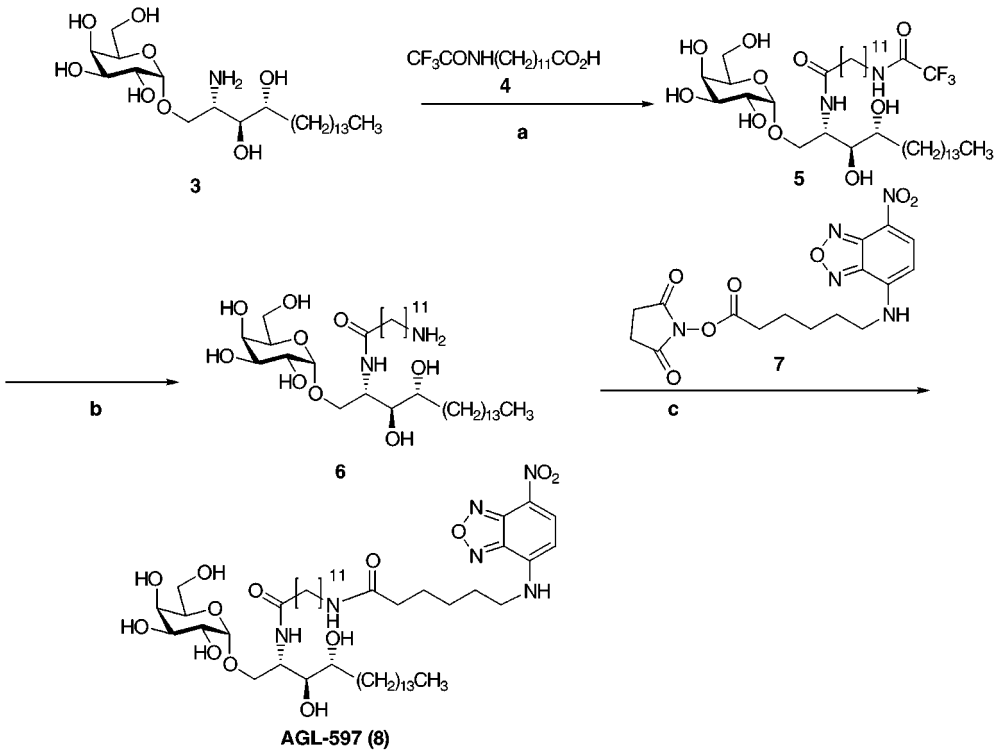
Scheme 1



(a) **4**, WSC.HCl, HOBt.H_2O, DMF, 120°C, 1h. ; (b) **5**, NaOH, THF, MeOH, H_2O, 40°C, 1h. ; (c) DMF, 25°C, 14h.

and 100 ng/mL with a potency that was approximately 10-fold weaker than that of **KRN7000**. By contrast, when **AGL-597** was similarly evaluated, it was observed that **AGL-597** indicated a strong proliferative response of spleen cells even at concentrations as low as 0.1 ng/mL, predicting a potency that is 10-fold stronger than that of **KRN7000**.

Table 1. Effects of **KRN7000**, **Agl-597**, and **Agl-592** on the Proliferation of Murine Spleen Cells[a]

	[^{3}H]-TdR incorporation (cpm)			
sample	0.1 ng/mL	1 ng/mL	10 ng/mL	100 ng/mL
vehicle	5226 ± 311	4628 ± 302	5054 ± 662	4375 ± 302
KRN7000	7270 ± 809[b]	13499 ± 246[b]	17438 ± 967[b]	17636 ± 515[b]
AGL-597	12564 ± 390[b]	17263 ± 262[b]	16973 ± 909[b]	16243 ± 722[b]
AGL-592	5074 ± 201	5273 ± 306	10951 ± 244[b]	15292 ± 262[b]

[a] 2.5 × 10^5 cells/100 μL/well of spleen cells from C57BL/6 mice suspended in RPMI 1640 medium containing 10% FCS were plated on a 96-well plate. At the same time, various concentrations of samples (10 μL/well) were added into each well and the cell suspension was cultured at 37 °C, 5% CO_2 for 18 h. Subsequently, 0.5 μCi/well of [^{3}H]-TdR was added to each well and, 8 h later, the [^{3}H]-TdR uptake into the cells was assayed with a liquid scintillation counter. Each value shows the mean ±S.D. [b] $p < 0.05$ (vs vehicle treated control group).

It has been reported that the docetaxel analogues bearing biotinyl or NBD probes are almost as active as docetaxel in an in vitro microtubular protein assay, although their activities in living cells, followed by the appearance of microtubule bundles, are diminished significantly in comparison to that of the parental docetaxel.[22] It is likely that the relationship between **KRN7000** and the biotinylated compound, **AGL-592**, is similar to that of the docetaxel analogues. It is therefore interesting that the α-GalCer modified with a NBD probe, **AGL-597**, exhibits a stronger immunostimulatory activity than the parental α-GalCer, **KRN7000**. These results clearly demonstrate that the immunostimulatory activity of α-GalCer depends on the chemical structure of the fatty acid side chain in the ceramide portion of the molecule. Furthermore, these studies suggest that the inability of **AGL-592** to detect CD1d in transfected cells may be due to its limited capacity to bind CD1d and that **AGL-597** may have a high affinity interaction with CD1d. It is likely, therefore, that NBD-α-GalCer will be a useful tool for the study of the antigen-presenting pathways related to CD1d-like molecules.

Acknowledgment. We thank Dr. Richard S. Blumberg (Brigham and Women's Hospital, Harvard Medical School) for his careful reading of the manuscript.

Supporting Information Available: Full experimental procedures for the preparation of **AGL-597** (**8**) and the method of spleen cell proliferation assay. This material is available free of charge via the Internet at http://pubs.acs.org.

OL9900111