Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 697–702

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

EFFECTS OF 3α- and 3β-GALACTOSYLATED α-GALACTOSYLCERAMIDES ON THE IMMUNE SYSTEM

Teruyuki Sakai, Masahiro Morita, Naoki Matsunaga, Kohji Akimoto, Takashi Yokoyama, Hiroshi Iijima and Yasuhiko Koezuka*

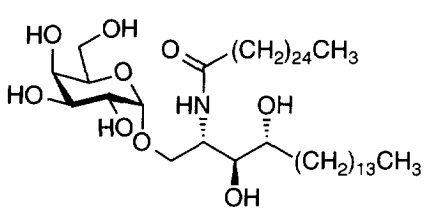
Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd.
3 Miyahara-cho, Takasaki-shi, Gunma 370-1295, Japan

Received 9 December 1998; accepted 21 January 1999

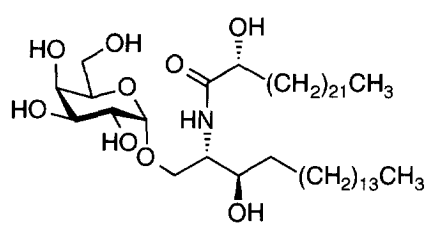
Abstract: We compared effects of α-galactosylceramide (α-GalCer) and its 3α- or 3β-galactosylated derivatives on the proliferation of murine spleen cells. The 3α-galactosylated α-GalCer showed stronger proliferative response than the parental α-GalCer, but the 3β-galactosylated α-GalCer possessed weaker activity than the α-GalCer. In addition, α-Gal-3-Cer did not show immunostimulatory activity.

Introduction

We previously reported that representative α-galactosylceramide (α-GalCer, galactose bound to ceramide in an α-configuration), named **KRN7000** (**1**) (Fig. 1), has strong immunostimulatory and antitumor activity.[1,2] **KRN7000** has also drawn researchers' attention as a ligand for mouse and human natural killer T (NKT) cells.[3-8] Our structure-activity relationship study using α-GalCers with different ceramide moieties indicated that the 3 hydroxyl group (3-OH) in the ceramide plays an essential role in the manifestation of immunostimulatory activity induced by α-GalCers, demonstrating that α-GalCer with only the 3-OH is the minimal structure to stimulate the immune system through the activation of NKT cells.[1,3]



1



2

Figure 1 Structures of **KRN7000** (**1**) and **AGL-506** (**2**)

Other researchers also attempted to isolate α-glycosylceramides from marine sponges, and they succeeded in the isolation of various types of α-GalCers, monoglycosylated α-GalCers, and diglycosylated α-

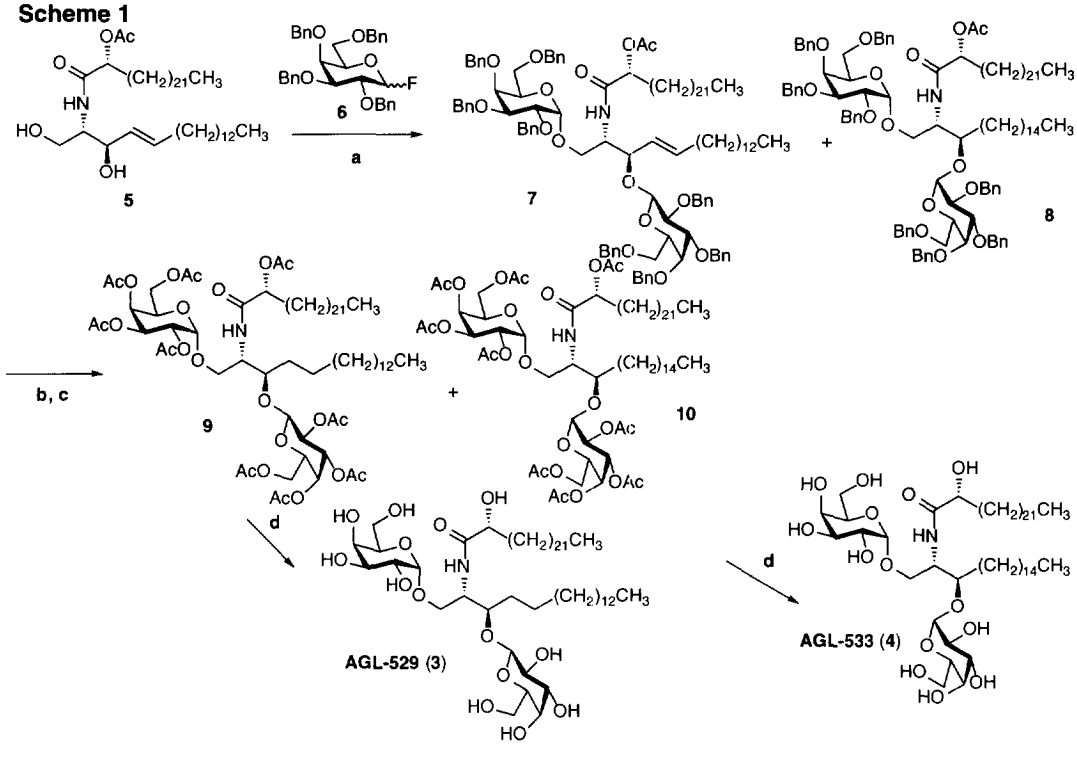
0960-894X/99/$ - see front matter
PII: S0960-894X(99)00054-2

GalCers,[7-9] and it has been reported that several mono- or diglycosylated α-GalCers as well as α-GalCers possess immunostimulatory effects.[12-14] In these studies using mono- or diglycosylated α-GalCers, it was demonstrated that the 3"-glycosylated α-GalCer have much less immunostimulatory activity than the parental α-GalCer.[13,14] These findings arose a question as to whether the 3-glycosylated α-GalCer also has weaker immunostimulatory activity than the parental α-GalCer. In order to address this question, we synthesized two kinds of 3-galactosylated α-GalCers, and compared effects among these two compounds and **AGL-506** (**2**)[1] (Fig. 1), the parental α-GalCer, on the proliferation of murine spleen cells.

Chemistry

The synthetic procedures of **AGL-529** (3β-galactosylated α-GalCer, **3**) and **AGL-533** (3α-galactosylated α-GalCer, **4**) are shown in Scheme 1. The synthesis of ceramide (**5**) was reported previously.[1] The ceramide (**5**) was glycosylated with 3 eq. galactosyl donor (**6**) to give **7** and **8**. The mixture of **7** and **8** were directly hydrogenated and subsequent acetylated to give **9** and **10**. Deacetylation of **9** and **10** gave **AGL-529** (**3**)[15] and **AGL-533** (**4**),[16] respectively.

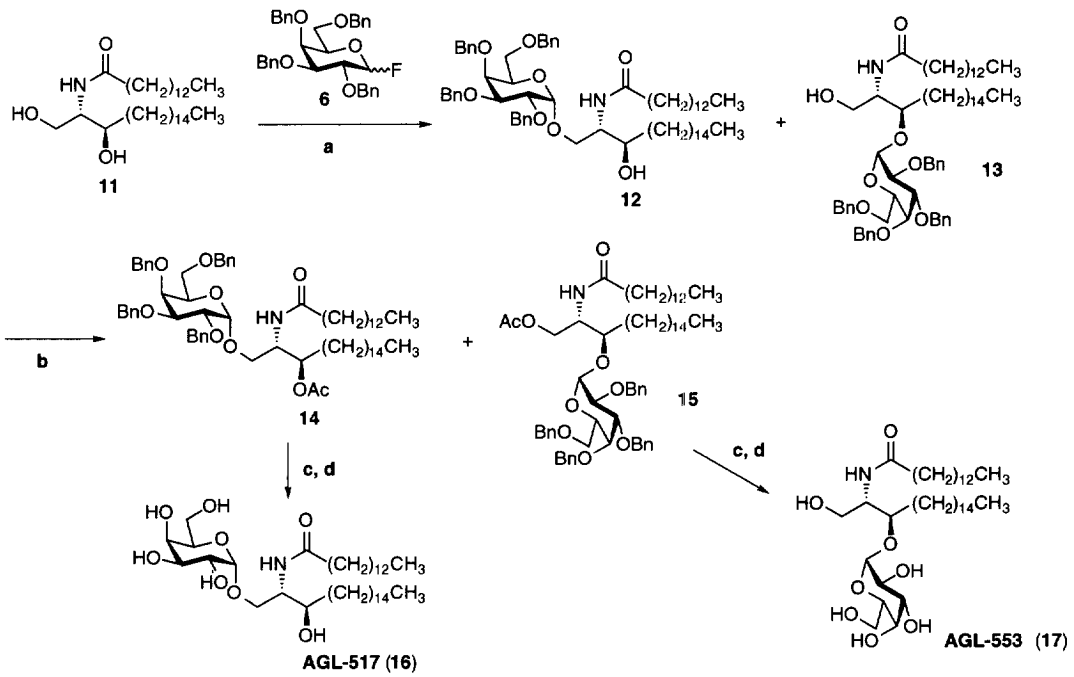
Scheme 1



Scheme 1; Reagents; (a) **6**(3eq), $SnCl_2$, $AgClO_4$, MS4A / THF, -10 ℃- r.t., 2hr, 65%; (b) H_2, Pd-black / THF, r.t., 24hr, 95%; (c) Ac_2O, Et_3N / CH_2Cl_2, r.t., 5hr, **9** (15%), **10** (33%); (d) NaOMe, MeOH, 1hr, 90%.

The synthetic procedures of α-Gal-1-Cer (**AGL-517**, **16**) and α-Gal-3-Cer (**AGL-553**, **17**) are shown in Scheme 2. Briefly, the synthesis of ceramide **11** was reported previously.[13] The ceramide **11** was directly glycosylated with 1.2 eq (**6**) to give **12** and **13**. The mixture of **12** and **13** were directly acetylated to give **14** and **15**. Debenzylation and subsequent deacetylation of **14** and **15** gave **AGL-517** (**16**)[1] and **AGL-553** (**17**),[17] respectively.

Scheme 2



Scheme 2; Reagents; (a) **6**(1.2eq), $SnCl_2$, $AgClO_4$, MS4A / THF, -10 ℃- r.t., 2hr, 65%; (b) Ac_2O, Et_3N / CH_2Cl_2, r.t., 5hr, **14** (48%), **15** (26%); (c) H_2, Pd-black / THF, r.t., 24hr, 95%; (d) NaOMe, MeOH, 1hr, 90%.

Results and Discussion

We compared the effects of **AGL-506** (α-GalCer, **2**), **AGL-529** (3β-galactosylated α-GalCer, **3**), and **AGL-533** (3α-galactosylated α-GalCer, **4**) on the proliferation of mouse spleen cells. As shown in Table 1, **AGL-506** stimulated the proliferation of spleen cells in a concentration-dependent fashion, and significant stimulatory activity was observed from the concentration of 1 ng/ml. Although **AGL-529** showed significant enhancement of the proliferative effect from the concentration of 10 ng/ml, its potency was weaker than that of **AGL-506**, the parental α-GalCer. By contrast, **AGL-533** significantly stimulated the proliferation of spleen cells from the concentration of 1 ng/ml, and it appeared that **AGL-533** has stronger immunostimulatory activity than **AGL-506**. It is quite interesting that the configuration of the glycosidic linkage between galactose

and ceramide in the 3-position greatly affects the α-GalCer induced immunostimulatory effects, i.e., the α-linkage enhances the immunostimulatory effect of α-GalCer, but the β-linkage reduces the activity.

Table 1. Effects of **AGL-506** (**2**), **AGL-529** (**3**) and **AGL-533** (**4**) on the proliferation of murine spleen cells.

	^{3}H-TdR incorpolation (cpm)		
Sample	1 ng/ml	10 ng/ml	100 ng/ml
Vehicle	6962 ± 758	7858 ± 1065	6280 ± 823
AGL-506 (**2**)	9454 ± 844*	20283 ± 3420*	45966 ± 2196*
AGL-529 (**3**)	7892 ± 1320	13195 ± 1818*	45036 ± 231*
AGL-533 (**4**)	16138 ± 944*	34892 ± 1259*	44867 ± 4529*

2.5 x 10^5 cells/100μl/well of spleen cells from BALB/c mice suspended in 10 % FCS RPMI 1640 medium 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 2 days. Then 0.5 μCi/well of tritium-thymidine (^{3}H-TdR) was added into each well, and 16 hours later, the ^{3}H-TdR uptake into the cells was measured by a liquid scintillation counter. Each value shows the mean ± S.D. *; $p < 0.05$ (vs. vehicle treated group).

Because 3α-galactosylated α-GalCer (**AGL-533**) showed stronger immunostimulatory activity than the parental α-GalCer (**AGL-506**) (Table 1), the result arose a question as to whether α-galactosyl-3-ceramide (α-Gal-3-Cer, galactose bound to ceramide in the 3-position) can stimulate the proliferation of spleen cells as α-Gal-1-Cers such as **KRN7000** and **AGL-506** can do. To address this question, we synthesized two kinds of α-GalCers with only the 3-OH, α-Gal-1-Cer (**AGL-517**, **16**) and α-Gal-3-Cer (**AGL-553**, **17**), by the methods shown in Scheme 2.

Table 2. Effects of **AGL-517** (**16**) and **AGL-553** (**17**) on murine spleen cells.

	^{3}H-TdR incorpolation (cpm)		
Sample	1 ng/ml	10 ng/ml	100 ng/ml
Vehicle	6962 ± 758	7858 ± 1065	6280 ± 823
AGL-517 (**16**)	9018 ± 541*	44395 ± 2157*	44395 ± 2157*
AGL-553 (**17**)	7722 ± 230	7412 ± 1783	6141 ± 1040

The speen cell proliferation assay was done by the same method shown in Table 1. Each value shows the mean ± S.D. *; $p < 0.05$ (vs. vehicle treated group).

We compared the effects of α-Gal-1-Cer, **AGL-517** (**16**), and α-Gal-3-Cer, **AGL-553** (**17**), on the proliferation of mouse spleen cells. As shown in Table 2, **AGL-517** stimulated the proliferation of spleen cells in a concentration-dependent fashion, and significant stimulatory activity was observed from the concentration of 1 ng/ml. By contrast, **AGL-553** did not stimulate the proliferation of spleen cells even at the highest concentration of 100 ng/ml. Although several research groups have published the reports on various types of

α-GalCers and their derivatives which were isolated from marine sponges[9-13,18,19] or were totally synthesized,[1,14,20] all compounds are α-Gal-1-Cers and their derivatives. Since there is no report on α-Gal-3-Cer as far as we know, this is the first finding that α-Gal-3-Cer (at least 100 ng/ml) has no immunostimulatory effect. The results shown in Table 1 and 2 demonstrate that α-binding of galactose not in the 3-position but in the 1-position is essential for the manifestation of immunostimulatory activity by α-GalCers, and that the 3-position is a preferable site to enhance the activity by the glycosylation.

It was demonstrated that CD1d-transfectant cells pretreated with **KRN7000** activate murine Vα14 positive NKT cell clones and human Vα24 positive NKT cell clones, suggesting that CD1d molecule plays an essential role in the activation of these NKT cells.[4-8] In addition, it was recently proven that radiolabeled **KRN7000**[21] and biotinylated α-GalCers[22] actualy bind to mouse CD1 and human CD1d molecules. These results suggest that the complex of α-GalCer and CD1d molecule is recognized by Vα14 or Vα24 T cell receptor on NKT cells. Our computer-aided molecular modeling demonstrated that active compounds such as α-GalCer and α-glucosylceramide (α-GlcCer) share active conformations which are not accessible to β-GalCer and β-GlcCer.[23] Furthermore, the crystallographic structure of mouse CD1 was recently solved by Zeng et al.[24] Taken these findings together, it seems to be possible to construct the binding model of CD1d/α-GalCer complex using these data. It is considered that the present study using **AGL-506** (**2**), **AGL-529** (**3**), **AGL-533** (**4**), **AGL-517** (**16**) and **AGL-553** (**17**) will contribute to the modeling study of CD1d/α-GalCer complex and will be useful to investigate the interaction mechanism among CD1d molecules, α-GalCer and T cell receptor.

References and Notes

1. Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sakai, T.; Sawa, E.; Yamaji, K.; Kobayashi, E.; Fukushima, H.; Koezuka, Y. *J. Med. Chem.*, **1995**, *38*, 2176-2187.

2. Nakagawa, R.; Motoki, K.; Ueno, H.; Iijima, R.; Nakamura, H.; Kobayashi, E.; Shimosaka, A.; Koezuka, Y. *Cancer Res.*, **1998**, *58*, 1202-1207.

3. Kawano, T.; Cui, J.; Koezuka, Y.; Toura, I.; Kaneko, Y.; Motoki, K.; Ueno, H.; Nakagawa, R.; Sato, H.; Kondo, E.; Koseki, H.; Taniguchi, M. *Science*, **1997**, *278*, 1626-1629.

4. Burdin, N.; Brossay, L.; Koezuka, Y; Smiley, S.; Grusby, M.; Gui, M; Taniguchi, M.; Hayakawa, K; Kronenberg, M. *J. Immunol.*, **1998**, *161*, 3271-3281.

5. Spada, F.; Koezuka, Y.; Porcelli, S. A. *J. Exp. Med.*, **1998**, *188*, 1529-1534.

6. Brossay, L.; Chioda, M. C.; Burdin, N.; Koezuka, Y.; Casorati, G.; Dellabona, P.; Kronenberg, M. *J. Exp. Med.*, **1998**, *188*, 1521-1528.

7. Couedel, C.; Reyrat, M. -A.; Brossay, L.; Koezuka, Y.; Porcelli, S.; Davodeau, F.; Bonneville, M., *Eur. J. Immunol.*, **1998**, *28*, 4391-4397.

8. Nieda, M.; Nicol, A.; Koezuka, Y.; Kikuchi, A.; Nakamura, H.; Takahashi, T.; Furukawa, H.; Yabe, T.; Ishikawa, Y.; Tadokoro, K.; Juji, T., *Hum. Immunol.*, in press.

9. Cafieri, F.; Fattorusso, E.; Mahajnah, Y.; Mangoni, A. *Liebigs Ann. Chem.*, **1994**, 1187.

10. Costantino, V; Fattorusso, E.; Mangoni, A.; Akinin, M.; Gaydou, E. *Liebigs Ann. Chem.*, **1994**, 1181.

11. Cafieri, F.; Fattorusso, E.; Mangoni, A; Taglialatela-Scafati, O. *Liebigs Ann. Chem.*, **1995**, 1477.

12. Costantino, V.; Fattorusso, E.; Mangoni, A.; Rosa, M. D.; Ianaro, A.; Maffia, P. *Tetrahedron*, **1996**, 52, 1573.

13. Uchimura, A.; Shimizu, T.; Nakajima, M.; Ueno, H.; Motoki, K.; Fukushima, H.; Natori, T.; Koezuka,Y. *Bioorg. Med. Chem.*, **1997**, *5*, 1447-1452.

14. Uchimura, A.; Shimizu, T.; Morita, M.; Ueno, H.; Motoki, K.; Fukushima, H.; Natori, T.; Koezuka,Y. *Bioorg. Med. Chem.*, **1997**, *5*, 2245-2249.

15. **AGL-529** (**3**): $[\alpha]_D^{25}$+44.3° (*c* 0.42, pyridine); FDMS m/z 993 (MH^+); IR (KBr) 3340, 2890, 2810, 1630, 1515, 1460, 1140, 1060 cm^{-1}; ^1H NMR(500MHz, C_5D_5N) δ 8.64 (1H, d, J=9.2Hz), 5.24 (1H, d, J=3.7Hz), 4.82 (1H, d, J=7.9Hz), 4.75-4.80 (1H, m), 4.72 (1H, dd, J=7.9Hz, 12.2Hz), 4.67 (1H, dd, J=3.7, 7.9Hz), 4.47-4.60 (4H, m), 4.37-4.46 (3H,m), 4.26-4.36 (3H, m), 4.12-4.21 (3H, m), 3.94 (1H, dd, J=6.7Hz, 10.4Hz), 2.24-2.32 (1H, m), 2.05-2.12 (1H, m), 1.96-2.04 (1H, m), 1.70-1.80 (1H, m), 1.48-1.60 (1H, m), 1.20-1.40 (66H, m), 0.88 (6H, t, J=5.5Hz).

16. **AGL-533** (**4**): $[\alpha]_D^{25}$+80.6° (*c* 1.0, pyridine); FDMS m/z 993 (MH^+); IR (KBr) 3330, 2900, 2830, 1635, 1520, 1460, 1035 cm^{-1}; ^1H NMR(500MHz, C_5D_5N) δ 8.57 (1H, d, J=9.8Hz), 5.78 (1H, d, J=3.7Hz), 5.46 (1H, d, J=3.1Hz), 4.95 (1H, m), 4.66-4.73 (2H, m), 4.60-4.65 (3H, m), 4.40-4.60 (8H, m), 4.25-4.40 (3H, m), 1.95-2.23 (2H,m), 1.65-1.90 (2H, m), 1.20-1.50 (66H, m), 0.882 (3H, t, J=6.7Hz) , 0.876 (3H, t, J=6.7Hz) .

17. **AGL-553** (**17**): $[\alpha]_D^{24}$+24.4° (*c* 0.05, pyridine); FDMS m/z 675 (MH^+); IR (KBr) 3375, 2900, 2825, 1620, 1540, 1460, 1030 cm^{-1}; 1H NMR(500MHz, C_5D_5N) δ 8.52 (1H, d, J=8.6Hz), 5.59 (1H, d, J=3.7Hz), 4.78-4.85 (1H, m), 4.65-4.75 (2H, m), 4.61 (1H, t, J=6.1Hz), 4.44-4.54 (2H, m), 4.41 (1H, d, J=4.9, 9.8Hz), 4.34 (1H, dd, J=5.5, 11.0Hz), 4.10-4.25 (2H, m), 2.45 (2H, t, J=7.3Hz), 1.70-2.10 (4H, m), 1.62 (1H, m), 1.05-1.45 (45H, m), 0.87 (6H, t, J=6.1Hz).

18. Natori, T.; Koezuka, Y.; Higa, T. *Tetrahedron lett.*, **1993**, *34*, 5591-5592.

19. Natori, T.; Morita, M.; Akimoto, K.; Koezuka, Y. *Tetrahedron*, **1994**, 50, 2771-2784.

20. Motoki, K.; Morita, M.; Kobayashi, E.; Uchida, T.; Akimoto, K.; Fukushima, H.; Koezuka, Y. *Biol. Pharm. Bull.*, **1995**, *18*, 1487-1491.

21. Maher, J. K.; Naidenko, O. V; Ernst, W. A; Sakai, T.; Modolin, R.; Kronenberg, M. submitted for publication.

22. Sakai, T.; Naidenko, O. V.; Iijima, H.; Kronenberg, M.; Koezuka, Y. submitted for publication.

23. Iijima, H.; Kimura, K.; Sakai, T.; Uchimura, A.; Shimizu, T.; Ueno, H.; Natori, T.; Koezuka, Y. *Bioorg. Med. Chem.*, **1998**, *6*, 1905-1910.

24. Zeng, Z. H.; Castano, A. R.; Segelke, B.; Stura, E. A.; Peterson, P. A.; Wilson, I. A. *Science*, **1997**, *277*, 339-345.