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ANTITUMOR ACTIVITIES OF α-, β-MONOGALACTOSYLCERAMIDES AND FOUR DIASTEREOMERS OF AN α-GALACTOSYLCERAMIDE

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Abstract: We examined antitumor activities of α-, β-monoglycosylceramides (MonoCers) and four diastereomers of an α-galactosylceramide (α-GalCer), and found that the α-GalCer among MonoCers and the 2*S*, 3*R* type among diastereomers show the strongest antitumor effects.

Agelasphines (**AGLs**) have been isolated from an extract of the marine sponge, *Agelas mauritianus*, as active substances in the course of screening of antitumor agents.[1,2] We compared the life span prolonging effects of **AGL-7a**, **AGL-9b**, **AGL-11**, **AGL-13**, and **AGL-10** against mice intraperitoneally inoculated murine melanoma B16 cells. As shown in Table 1, all **AGLs** at a dose of 1 mg/kg markedly prolonged the survival period of B16-bearing mice (T/C > 125 %), although α-galactosylceramide (α-GalCer) types were more effective than β-glucosylceramide (β-GluCer) type.

In contrast to the case of B16-bearing mice, when the survival period prolonging effects of these **AGLs** against mice intraperitoneally implanted murine leukemia P388 cells were examined, none of the **AGLs** prolonged the life span of the P388-bearing mice (T/C < 110 %, Table 2).

These findings are quite interesting because chemotherapeutic agents generally show more potent antitumor activities against P388-bearing mice than B16-bearing mice. Antitumor agents are generally classified into the following two types; chemotherapeutic agents which are highly cytotoxic against tumor cells, and biological response modifiers (BRMs)[3] which show antitumor effects via activating immune system. These findings suggested that **AGLs**, especially α-GalCer types, are BRMs. To confirm this postulation, we studied the lymphocyte proliferation (LP) stimulatory effects of five **AGLs** on the allogeneic mixed lymphocyte reaction (MLR).[4] As shown in Table 3, **AGLs** which are α-GalCers markedly stimulated the proliferation of lymphocyte on allogeneic MLR at a concentration of 1 ng/ml or higher, though **AGL-10** (β-GluCer) showed weaker LP stimulatory activities even at a concentration of 100 ng/ml than those effected by 1 ng/ml of α-GalCer type. These findings suggested that α-GalCers show marked antitumor effects via activating the immune system, that their biological activities are stronger than that of β-GluCer, and that **AGLs** having α-GalCer structures are BRMs classified nonspecific immunostimulating agents.[5]

Table 1. Life span prolonging effects of **AGL-7a**, **AGL-9b**, **AGL-11**, **AGL-13**, and **AGL-10** against mice intraperitoneally inoculated B16 cells.

AGLs	Dose mg/kg	Survival days Mean ± S.D.	T/C %
Control	–	18.5 ± 1.8	100
AGL-7a	0.1	26.8 ± 4.5	145
	1.0	31.5 ± 4.0	170
AGL-9b	0.1	27.6 ± 2.6	149
	1.0	27.0 ± 2.2	146
AGL-11	0.1	28.2 ± 2.2	152
	1.0	34.5 ± 3.9	187
AGL-13	0.1	28.0 ± 2.4	151
	1.0	32.0 ± 3.0	173
Control	–	18.6 ± 1.7	100
AGL-10	0.1	21.3 ± 2.0	115
	1.0	23.7 ± 2.0	127

Six female BDF_1 (6 weeks old) mice purchased from Nippon SLC Co., Ltd. were used per group, and inoculated intraperitoneally 1 x 10^6 cells/mouse of B16 cells on day 0. **AGLs** were administered intravenously on day 1, 5, 9. The survival or death of each mouse was observed.

Several GalCers have been isolated from organ tissues[6,7] or marine organisms,[8,9] but they have not been reported to have any marked biological activities yet. The difference in biological activities between **AGLs** of

Table 2. Survival period prolonging effects of **AGL-7a**, **AGL-9b**, **AGL-11**, **AGL-13**, and **AGL-10** against mice intraperitoneally inoculated P388 cells.

AGLs	Survival days Mean ± S.D.	T/C %
Control	10.4 ± 0.5	100
AGL-7a	11.0 ± 1.3	106
AGL-9b	11.3 ± 1.0	109
AGL-11	11.2 ± 0.4	108
AGL-13	11.2 ± 0.4	108
AGL-10	11.3 ± 0.5	109

Six female CDF_1 (6 weeks old) were used per group, and implanted intraperitoneally 1 x 10^6 cells/mouse of P388 cells on day 0. **AGLs** were given at a dose of 0.1 mg/kg intravenously on day 1, 5, 9. The survival or death of each mouse was observed.

Table 3. Lymphocyte proliferation stimulatory effects of **AGL-7a**, **AGL-9b**, **AGL-11**, **AGL-13**, and **AGL-10** on allogeneic MLR.

	^{3}H-TdR incorpolation (% of control)		
AGLs	1 ng/ml	10 ng/nl	100 ng/ml
AGL-7a	253	258	350
AGL-9b	184	220	236
AGL-11	179	209	255
AGL-13	233	281	319
AGL-10	142	134	169

1 x 10^5 cells/50μl/well of spleen cells from BALB/c mice (responder cells) and the same number of Mitomycin C-treated (50 μg/ml, 30 min) spleen cells from C57BL/6 mice (stimulator cells) suspended in 10 % FCS RPMI 1640 medium were plated on a 96-well plate. At the same time, various concentrations of **AGLs** (10 μl/well) were added into each well, and the cell suspension was cultured at 37 °C, 5% CO_2 for 3 days. Then 0.5 μCi/well of tritium-thymidine (^{3}H-TdR) was added into each well, and 6 hours later, the ^{3}H-TdR uptake into the cells was measured by a liquid scintillation counter.

α-GalCer type and the GalCers previously reported are quite interesting. Furthermore, various GluCers also have been isolated[10,11] and their marked biological activities also have not been reported yet. Recently, several types of GluCers which have cytotoxic activities against tumor cells at the concentration of 2 μg/ml were isolated from a starfish.[12] However, **AGL-9b** has no cytotoxic activities against tumor cells even at the concentration of 10 μg/ml. These findings suggested that not only the manner of combination between sugar and ceramide but also the type of sugar combining to ceramide greatly affects the biological activities of monoglycosylated ceramides (MonoCers).

Table 4. Tumor growth inhibitory effects of **AGL-517**, **AGL-564**, **AGL-563**, and **AGL-562** against mice subcutaneously inoculated B16 cells.

	TGIR (%)			
Sample	day 9	day 12	day 16	day 20
AGL-517 (α-GalCer)	59.5	62.0	64.8	54.0
AGL-564 (β-GalCer)	29.0	38.0	35.6	28.3
AGL-563 (α-GluCer)	35.3	45.2	26.8	4.2
AGL-562 (β-GluCer)	-28.0	2.7	1.4	-7.6
MMC	35.2	53.9	59.7	48.6

Six female BDF_1 mice were used per group, and inoculated subcutaneously 1 x 10^6 cells/mouse of B16 cells on day 0. **AGLs** (100 μg/kg) were intravenously administered on day 1, 5, 9 and MMC (5 mg/kg) was given intraperitoneally on day 1. Each tumor volume (length x width x height / 2) per mouse was measured. TGIR (tumor growth inhibition ratio) was calculated by the following formula: TGIR (%) = [1 – (Mean tumor volume of test group / Mean tumor volume of control group)] x 100.

To confirm this possibility, we synthesized **AGL-517**, **AGL-564**, **AGL-563**, and **AGL-562**, and examined the inhibitory effects of these compounds on the tumor growth of mice subcutaneously inoculated murine melanoma B16 cells. As shown in Table 4, **AGL-517** suppressed the tumor growth the most potently, and the suppressing effect of **AGL-517** was similar to that by the treatment with Mitomycin C (MMC) which is a chemotherapeutic agent.

Table 5. Tumor growth inhibitory effects of **AGL-517**, **AGL-564**, **AGL-563**, and **AGL-562** against mice subcutaneously inoculated Meth A cells.

	TGIR (%)			
Sample	day 10	day 13	day 16	day 20
AGL-517 (α-GalCer)	69.0	68.1	58.1	36.7
AGL-564 (β-GalCer)	26.9	33.1	22.1	3.9
AGL-563 (α-GluCer)	45.6	41.5	28.9	21.3
AGL-562 (β-GluCer)	45.2	45.0	29.2	7.0
MMC	80.1	85.1	80.0	75.1

Six female CDF_1 mice were used per group, and inoculated subcutaneously 1 x 10^6 cells/mouse of Meth A cells on day 0. **AGLs** (100 μg/kg) were intravenously administered on day 1, 5, 9 and MMC (5 mg/kg) was given intravenously on day 1. Each tumor volume (length x width x height / 2) per mouse was measured. TGIR (tumor growth inhibition ratio) was calculated by the following formula: TGIR (%) = [1 – (Mean tumor volume of test group / Mean tumor volume of control group)] x 100.

We examined the tumor growth inhibitory activities of these compounds against mice subcutaneously implanted murine fibrosarcoma Meth A cells. As shown in Table 5, **AGL-517** also showed the strongest suppressive effect of tumor growth on Meth A-bearing mice. These results demonstrated that among these

Table 6. Tumor growth inhibitory effects of **KRN7000** and **AGL-583** against mice subcutaneously inoculated B16 cells.

	TGIR (%)			
Sample	day 11	day 14	day 18	day 22
KRN7000 (α-GalCer)	41.1	50.9	51.1	40.2
AGL-583 (β-GalCer)	16.1	17.2	20.3	-3.8
MMC	-8.6	29.7	51.2	60.0

Five female BDF_1 mice were used per group, and inoculated subcutaneously 1.5 x 10^6 cells/mouse of B16 cells on day 0. 100 μg/kg of **KRN7000** or **AGL-583** was intravenously administered on day 7, 11, 15 and MMC (5 mg/kg) was given intravenously on day 7. Each tumor volume (length x width x height / 2) per mouse was measured. TGIR (tumor growth inhibition ratio) was calculated by the following formula: TGIR (%) = [1 – (Mean tumor volume of test group / Mean tumor volume of control group)] x 100.

compounds, α-GalCer shows the strongest tumor growth inhibitory activities against not only B16-bearing but also Meth A-bearing mice.

Furthermore, to examine whether GalCers which have the different ceramide portion from **AGL-517** show the similar relationship, we synthesized **KRN7000** (α-GalCer) and **AGL-583** (β-GalCer). We examined the tumor growth inhibitory effects of these compounds, and found that **KRN7000** also suppresses tumor growth more potently than **AGL-583** (Table 6).

The results demonstrated that in the case of GalCers and GluCers which have the same ceramide, not only the manner of combination between sugar and ceramide but also the type of sugar combining to ceramide plays an important role in their antitumor activities and that α-GalCer has the most potent antitumor activity.

Table 7. Tumor growth inhibitory effects of **AGL-555**, **AGL-556**, **AGL-558**, and **AGL-559** against mice subcutaneously inoculated B16 cells.

		TGIR (%)			
	Sample	day 8	day 12	day 16	day 20
A	**AGL-555**	N.M.	4.9	-8.5	13.1
	AGL-556	N.M.	-13.0	-11.0	5.1
	MMC	N.M.	38.8	52.8	68.1
B	**AGL-558**	76.5	66.6	66.4	67.9
	AGL-559	55.9	52.6	42.2	40.1
	MMC	90.0	89.7	86.0	83.2

N.M.; not measured.

1×10^6 cells/mouse of B16 cells were subcutaneously inoculated into female BDF_1 (6 weeks old) mice on day 0. **A**: Intravenous administrations of **AGLs** (100 μg/kg) or MMC (5 mg/kg) were performed on day 10, 14, 18 or day 10, respectively. **B**: Intravenous administrations of **AGLs** (100 μg/kg) or intraperitoneal treatment of MMC (5 mg/kg) were undertaken on day 1, 5, 9 or day 1, respectively. Each tumor volume (length x width x height / 2) per mouse was measured. TGIR (tumor growth inhibition ratio) was calculated by the following formula: TGIR (%) = [1 – (Mean tumor volume of test group / Mean tumor volume of control group)] x 100.

We previously reported that the structure of the ceramide portion in α-GalCer greatly affects their antitumor activities[13], although the biological activities of the diastereomers of the ceramide portion have not been reported yet. To examine the biological activities, we synthesized four kinds of diastereomers (**AGL-555**, **AGL-556**, **AGL-558**, and **AGL-559**) having α-GalCer structures, and compared the tumor growth inhibitory effects of four diastereomers against mice subcutaneously inoculated murine melanoma B16 cells. As shown in Table 7, **AGL-558** suppressed the tumor growth of B16 bearing-mice the most markedly similar to Mitomycin C (MMC) used as a positive control. These findings demonstrated that the absolute configuration of the ceramide portion plays an important role in the biological activities of α-GalCer, and that the diastereomer (2*S*, 3*R*) which is the naturally occurring type[1-12] shows the strongest antitumor effects.

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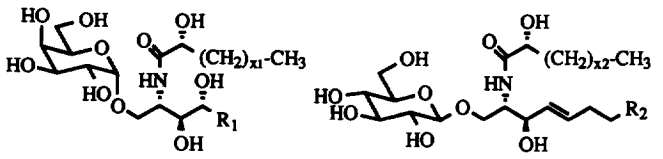
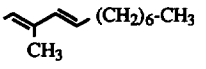
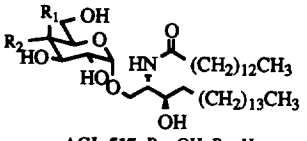
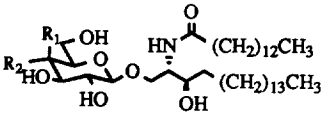


	X_1	R_1
AGL-7a	21	$-(CH_2)_{11}-CH_3$
AGL-9b	21	$-(CH_2)_{11}-CH(CH_3)_2$
AGL-11	21	$-(CH_2)_{11}-CH(CH_3)-C_2H_5$
AGL-13	22	$-(CH_2)_{11}-CH(CH_3)-C_2H_5$

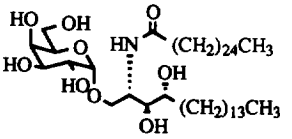
	X_2	R_2
AGL-10	21	



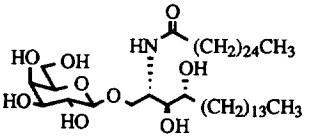
AGL-517; R_1=OH, R_2=H
AGL-563; R_1=H, R_2=OH



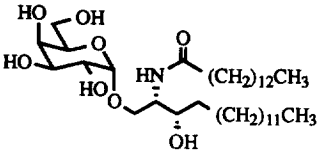
AGL-564; R_1=OH, R_2=H
AGL-562; R_1=H, R_2=OH



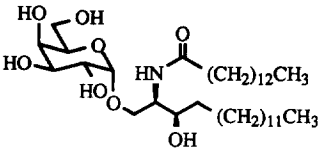
KRN7000



AGL-583



AGL-555; (2*S*, 3*S*)



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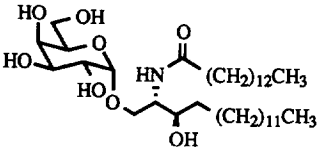
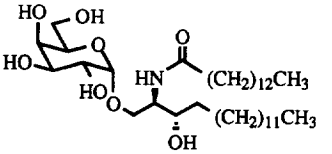


AGL-558; (2*S*, 3*R*)



AGL-559; (2*R*, 3*S*)

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