Supplementary material Saito et al

Preparation of adaGb$_3$ and analogues

a) Reagents.

Air sensitive reagents were handled under an inert atmosphere. Molecular sieves (3Å) were activated at 250 ˚C for 24 hours and were allowed to cool to room temperature under vacuum. Dry solvent mixture (DDT) : DMF: CH_2Cl_2: Et_3N; 5: 5: 1, was dried over activated molecular sieves (3 Å) for 16 hours. AdaAcOH: 0.5 M solution in DDT. Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP): 0.2 M solution in DDT. Unless specified, Teflon-lined screw capped Kimax® glass tubes were used as reaction tubes and for the storage of products. GSL precursors were dried in a P_2O_5 desiccators.

b) Mass Spectroscopic analysis.

Electro spray mass spectroscopic (ESMS) analyses were performed on a QSTAR$_{XL}$ MS/MS spectrometer with a nano-spray source (ABI / MDS Sciex, Concord, ON). The sample was loaded in a PicoTip^{TM} EMITTER and was attached to the nano-spray source. Compounds were dissolved in MeOH-NaCl solution to a final concentration of 0.5 mg/mL. MeOH-NaCl was prepared by adding a saturated aqueous NaCl solution (3 mL) to MeOH-MS (10 mL). Theoretical mass was calculated with ChemDraw®.

c) Syntheses.

1. (2S, 3R, 4E)-2-(1-Adamantane)-acetamido-3-hydroxyl-4-octadecenyl-(α-D-galactotopyranosyl)-(1-4)-(β-galactopyranosyl)-(1-4)-β-D-glucopyranoside, AdaGb$_3$.

To a cooled (-78 ˚C) solution of 1-adamantaneaceticacid (26 μmols) in DDT (0.5 mLs) was added BOP (26 μmols) and the mixture was stirred for 10 minutes. A solution of LysoGb$_3$ (7 mgs, 9 μmols) in DDT (0.5 mLs) added drop wise and the resulting mixture was stirred for 1 hour at -78 ˚C. Water (200 μL) was added and the mixture was allowed to warm up to room temperature and dried. Crude sample was dissolved in a minimum volume of 90: 15: 1; $CHCl_3$: MeOH: H_2O; and loaded on to a silica column (bed volume; height, 2 cm; diameter, 1 cm) equilibrated with the same solvent mixture and, eluted with the following solvents (number of fractions, volume of each fraction): 90: 15: 1; $CHCl_3$: MeOH: H_2O (8, 4 mLs); 80: 20: 2; $CHCl_3$: MeOH: H_2O (6, 4 mLs); 65: 25: 4; $CHCl_3$: MeOH: H_2O (6, 4 mLs). The product eluted in the 65: 25: 4; $CHCl_3$: MeOH: H_2O fractions. ESMS, (ion) (found, expected): ($M+H^+$) (962.53, 962.57); ($M+ Na^+$) (984.53, 984.55).

2. (2S, 3R, 4E)-2-[1-(3-Carboxymethyl)-adamantanacetamido]-3-hydroxyl-4-octadecenyl-(α-D-galactotopyranosyl)-(1-4)-(β-galactopyranosyl)-(1-4)-β-D-glucopyranoside, carboxyadaGb$_3$

A solution of 1,3-adamantanediaceticacid (26 mmols) in DDT (0.5 mLs) was cooled (-78 ˚C) and BOP (26 mmols) was added and the mixture was stirred for 10 minutes. A solution of LysoGb$_3$ (7 mgs, 9 mmols) in DDT (0.5 mLs) added drop wise and the resulting mixture was stirred for 1 hour at -78 ˚C. Water (200 mL) was added and the mixture was allowed to warm up to room temperature and dried. Crude sample was dissolved in a minimum volume of 90: 15: 1; $CHCl_3$: MeOH: H_2O; and loaded on to a

silica column (bed volume; height, 2 cm; diameter, 1 cm) equilibrated with the same solvent mixture and, eluted with the following solvents (number of fractions, volume of each fraction): 90: 15: 1; $CHCl_3$: MeOH: H_2O (8, 4 mLs); 80: 20: 2; $CHCl_3$: MeOH: H_2O (6, 4 mLs); 65: 25: 4; $CHCl_3$: MeOH: H_2O (6, 4 mLs). The product eluted in the 65: 25: 4; $CHCl_3$: MeOH: H_2O. ESMS, (ion) (found, expected): ($M—H^+$) (1018.553, 1018.559); ($M+ H^+$) (1042.590, 1043.556).

3. (2S, 3R, 4E)-2-[1-(3-(1,3-Diisopropyl)-ureido)-adamantanacetamido]-3-hydroxyl-4-octadecenyl-(α-D-galactotopyranosyl)-(1-4)-(β-galactopyranosyl)-(1-4)-β-D-glucopyranoside. urea adaGb$_3$.

To a solution of carboxyadaGb$_3$ (2 mgs, 1.8 mmols) in dimethylformamide (DMF) (0.5 mLs) was added a 0.2 M solution of N,N'-diisopropylcarbodiimide (65 mL, 13 mmols) and the mixture was stirred for 1 h at 70 ˚C. The mixture was then dried under a flow of nitrogen and the resulting crude product was dissolved in a minimum volume of 90: 15: 1; $CHCl_3$: MeOH: H_2O; and loaded on to a silica column (bed volume; height, 2.5 cm; diameter, 0.5 cm) equilibrated with the same solvent mixture and, eluted with the following solvents (number of fractions, volume of each fraction): 90: 15: 1; $CHCl_3$: MeOH: H_2O (6, 4 mLs); 80: 20: 2; $CHCl_3$: MeOH: H_2O (8, 4 mLs); 65: 25: 4; $CHCl_3$: MeOH: H_2O (6, 4 mLs). The product eluted in the 65: 25: 4; $CHCl_3$: MeOH: H_2O fractions. MALDI-TOF, (ion) (found, expected): ($M+Na^+$) (1168.57, 1168.67).

4**. (2S, 3R, 4E)-2-[1-(3-(*N,* 2-hydroxyethyl)-carbamoyl)-adamantanacetamido]-3-hydroxyl-4-octadecenyl-(α-D-galactotopyranosyl)-(1-4)-(β-galactopyranosyl)-(1-4)-β-D-glucopyranoside. Hydroxyethyl adaGb$_3$.**

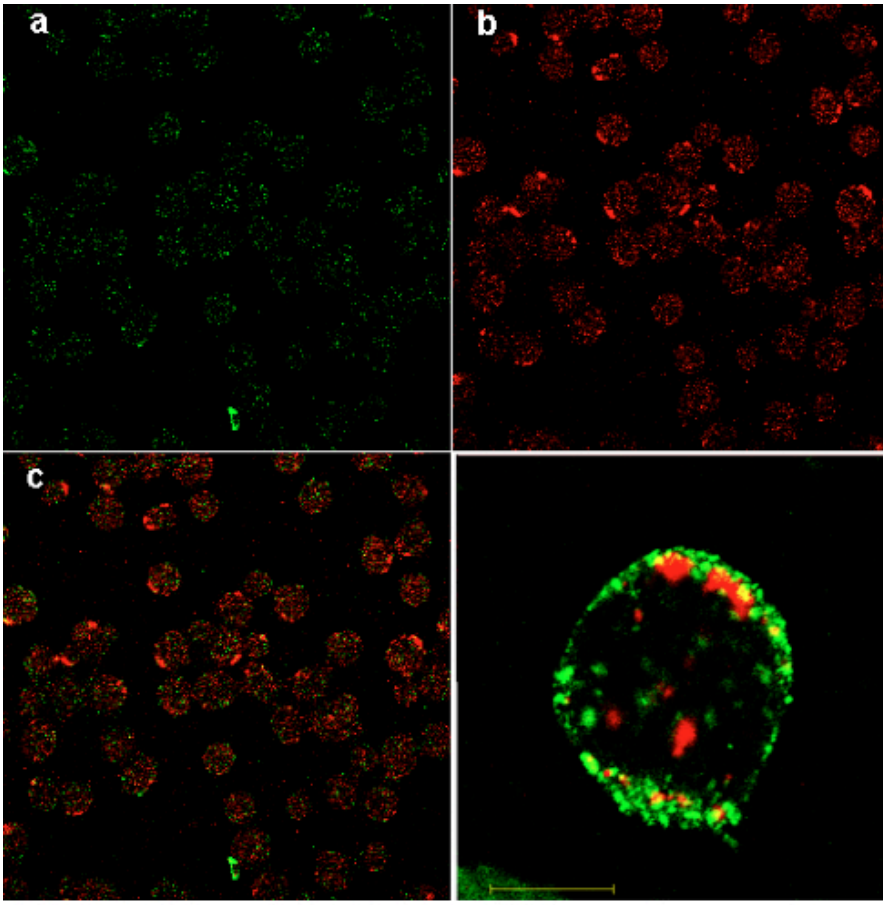
To a cooled (-78˚C) solution of carboxyadaGb$_3$ (2 mgs, 2.5 μmols) in DDT (0.5 mLs) was added BOP (5 μmols) and the mixture was stirred for 10 minutes. A solution of ethanolamine (12.5 μLs of a 1M solution in DMF, 12.5 μmols) was added drop wise and the resulting mixture was stirred for 2 hour at -78 ˚C. Water (200 mL) was added and the mixture was allowed to warm to room temperature and dried. Crude product was dissolved in a minimum volume of 90: 15: 1; $CHCl_3$: MeOH: H_2O; and loaded on to a silica column (bed volume; height, 2 cm; diameter, 0.75 cm) equilibrated with the same solvent mixture and, eluted with the following solvents (number of fractions, volume of each fraction): 90: 15: 1; $CHCl_3$: MeOH: H_2O (4, 4 mLs); 80: 20: 2; $CHCl_3$: MeOH: H_2O (6, 4 mLs); 65: 25: 4; $CHCl_3$: MeOH: H_2O (6, 4 mLs). The product eluted in the 65: 25: 4; $CHCl_3$: MeOH: H_2O fractions. MALDI-TOF, (ion) (found, expected): ($M+Na^+$) (1085.48, 1085.60).

5. **Adamantyl bis-globotriaoside.**

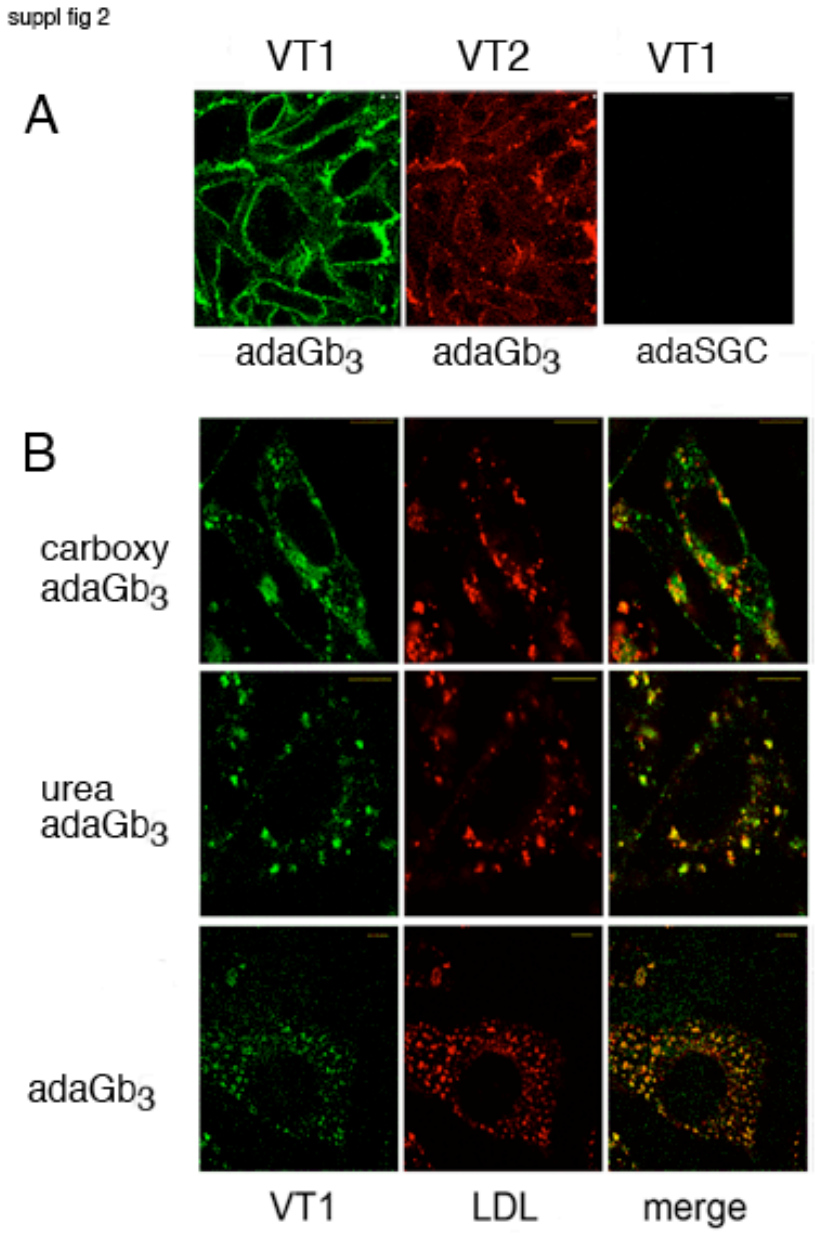
A solution of carboxyadaGb$_3$ (5.4 mmols) in DDT (1 mLs) was cooled (-78 ˚C) and BOP (13 mmols) was added the mixture was stirred for 10 minutes. A solution of LysoGb$_3$ (6.5 mmols) in DDT (1 mLs) added drop wise and the resulting mixture was stirred for 2 hour at -78 ˚C. Water (200 mL) was added and the mixture was allowed to warm to room temperature and dried. Crude product was dissolved in 4: 1; $CHCl_3$: MeOH; and loaded on to a DEAE column (acetate form, bed volume; height, 12 cm; diameter, 0.75 cm) equilibrated with the same solvent mixture and, eluted with the following solvents (number of fractions, volume of each fraction): 4:1; $CHCl_3$: MeOH (12, 3 mLs); 30:60:8; $CHCl_3$: MeOH: H_2O (8, 4 mLs). The product eluted in the 30: 60: 8; $CHCl_3$: MeOH: H_2O fractions. Then the product was further purified on a silica column similar to as

described for carboxyadaGb$_3$. ESMS, (ion) (found, expected): (M+ Na^+) (1810.0314, 1809.9861).

The structures of adaGb$_3$, hydroxyethyladaGb$_3$ carboxyadaGb$_3$, urea adaGb$_3$ and adabisGb$_3$ are illustrated in manuscript Figure 1. Adamantyl sulfogalactosyl ceramide (adaSGC) was synthesized as previously (Whetstone, D., and Lingwood, C. (2003) *Biochem* **42**, 1611-1617).



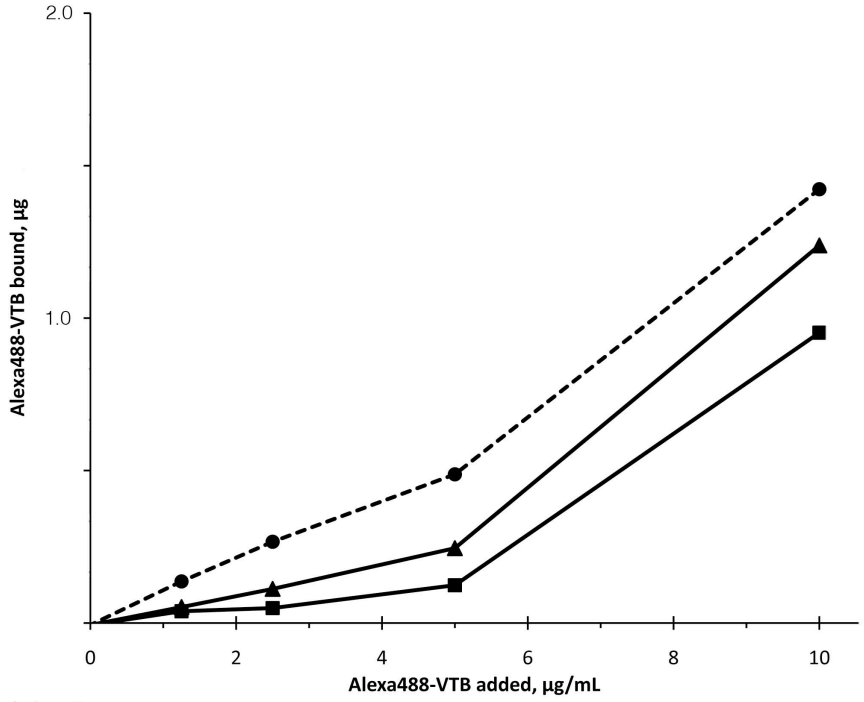
Suppl Figure1 **No colocalization of lamp 2 and VT1 in adaGb$_3$ treated Jurkat cells** AdaGb$_3$-treated Jurkat cells were incubated with Alexa488-VT1 for 1hr at 37ºC. Cells were permeabilized and stained with Alexa594 labeled anti Lamp2 antibody. Low power image (a,b,c) shows cell labeling for VT1(green), lamp 2(red) and merged image respectively. High power image (d) shows distinct labeling of lysosomal lamp 2(red) and VT1 (green), bar=10μm.



Suppl Figure 2. **Only adamantylGb_3 analogues bind VT to target endosomes of CHO cells.** Panel A AdaGb$_3$ or adaSGC reconstituted CHO cells were double labeled with Alexa488-VT1 and Texas Red-VT2 for 30 mins at 4^{o}C. Cell surface labeling occurred for adaGb$_3$ treated cells but no VT1 (or VT2, not shown) labeling was observed for adaSGC treated cells. Panel B VT1 binding and internalization into CHO cells reconstituted with different adaGb$_3$ analogues were compared to Dil-LDL (red) binding its endosomal receptor. Reconstituted cells were incubated with Alexa488-VT1 (green)

for 1hr at 37°C. Internalized VT1 localized in LDL defined endosomes of cells reconstituted with either adaGb$_3$, urea adaGb$_3$ or carboxyadaGb$_3$. Bar=10µm.

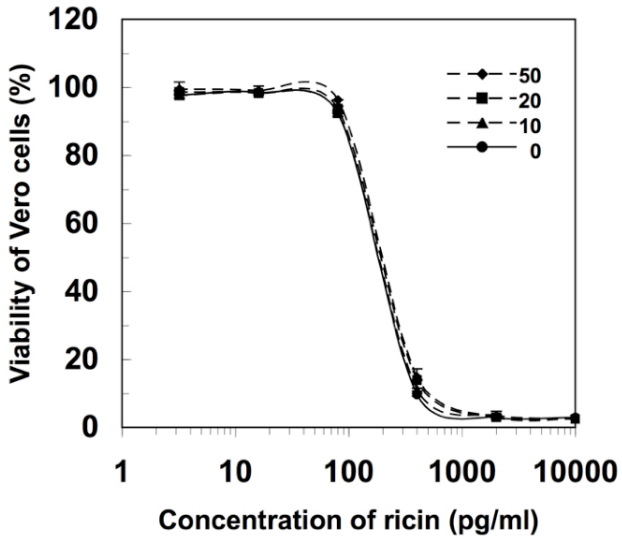
The effect of adaGb$_3$ insertion on VT1B binding to Vero cells was determined as follows: Vero cells in 96 well plates were incubated on ice with adaGb$_3$ (0, 10 or 50µM) according to the standard insertion protocol. Alexa488-VT1B was titrated and bound to the cells for 1 hour on ice. The cells were washed with cold PBS, solublized with 50µL 1% Triton X-100, and the fluorescence measured using a Molecular Devices SpectraMax Gemini EM spectrofluorometer (Ex= 485nm, Em= 538 nm). VT1B bound was quantitated from an Alexa488-VT1B standard curve.



suppl. Figure 3

***Suppl. Figure 3*. Effect of adaGb$_3$ incorporation on vero cell VT1B binding**. Vero cells monolayers were treated with 10 or 50µM adaGb$_3$ for 1hr at 4°C. Cells were then incubated with Alexa-VT1B for 30mins at 4°C, washed and bound VT1B determined using a…fluorimeter (--●--) control, (-▲-) 10µM adaGb$_3$, (-■-) 50µM adaGb$_3$ treated vero cells. Cell surface VT1B binding was reduced after in corporation of adaGb$_3$, particularly at low VT1B concentrations.

Suppl. Figure 4



Suppl. Figure 4 **Ricin toxicity for vero cells treated with adaGb$_3$.** Vero cells were incubated with 10 μM(▲), 20 μM(■), 50 μM(◆) of adaGb$_3$ or without analogues(●) at 4°C for 1 hour according to the standard insertion protocol. Cells were treated with tenfold serial diluted ricin and incubated at 37°C. Cell viability was monitored after 72 hours and expressed as a percentage of control cells, treated with neither ricin nor adaGb$_3$.

Quantitation of internalized VT1/VT2: Staining density was quantified from randomized fields and expressed as average using **Image J** software .

Table 1 VT1/VT2 colocalization with organelle markers

Fig 4D		
10min (EEA1)	%VT1 colocalized with EEA1	%EEA1 colocalized with VT1
vero cells	53.1	80.5
adaGb$_3$-treated CHO cells	65.2	48.8
adaGb$_3$-treated JKT cells	67.2	44.3
OHEtadaGb$_3$-treated CHO cells	74.5	62.7
OHEtadaGb$_3$-treated JKT cells	65.4	41.3
1h (EEA1)	%VT1 colocalized with EEA1	%EEA1 colocalized with VT1
vero cells	64.0	68.4
adaGb$_3$-treated CHO cells	75.1	12.3
adaGb$_3$-treated JKT cells	61.0	15.5
OHEtadaGb$_3$-treated CHO cells	83.0	28.2
OHEtadaGb$_3$-treated JKT cells	47.7	12.9
Fig4E		
1h (Rab6)	%VT1 colocalized with Rab6	%Rab6 colocalized with VT1
vero cells	18.9	51.4
adaGb$_3$-treated CHO cells	0.5	1.3
adaGb$_3$-treated JKT cells	11.0*	0.4
OHEtadaGb$_3$-treated CHO cells	14.2*	0.1
OHEtadaGb$_3$-treated JKT cells	2.6*	0.9
6h (calnexin)	%VT1 colocalized with calnexin	%calnexin colocalized with VT1
vero cells	55.7	42.9
adaGb$_3$-treated CHO cells	10.3*	0.0
adaGb$_3$-treated JKT cells	13.3*	0.6
OHEtadaGb$_3$-treated CHO cells	16.9*	0.4
OHEtadaGb$_3$-treated JKT cells	17.7*	0.1
Fig 5C		
10min (EEA1)	%VT1 colocalized with EEA1	%EEA1 colocalized with VT1
adaGb$_3$-treated vero cells	92.4	56.2
OHEtadaGb$_3$-treated vero cells	75.1	54.1
1h (EEA1)	%VT1 colocalized with EEA1	%EEA1 colocalized with VT1
adaGb$_3$-treated vero cells	13.0	7.6
OHEtadaGb$_3$-treated vero cells	90.7	58.1
1h (Rab6)	%VT1 colocalized with Rab6	%Rab6 colocalized with VT1
adaGb$_3$-treated vero cells	1.9	10.7
OHEtadaGb$_3$-treated vero cells	8.8	25.5
6h (calnexin)	%VT1 colocalized with calnexin	%calnexin colocalized with VT1
adaGb$_3$-treated vero cells	27.2	11.3

OHEtadaGb$_3$-treated vero cells	21.0	4.6
Fig 5D		
1h (Rab6)	%VT2 colocalized with Rab6	%Rab6 colocalized with VT2
non-treated vero cells	14.5	34.7
adaGb$_3$-treated vero cells	2.7	6.2
OHEtadaGb$_3$-treated vero cells	14.2	21.5
6h (calnexin)	%VT2 colocalized with calnexin	%calnexin colocalized with VT2
non-treated vero cells	25.9	11.6
adaGb$_3$-treated vero cells	10.0	1.8
OHEtadaGb$_3$-treated vero cells	36.4	27.8

* calculated values overestimates due to toxin loss (table 2),

Table 2 Time dependent loss of intracellular VT1 at 37°C

	10min	1h	6h
non-treated vero cells	100	55.5	77.6
adaGb$_3$-treated vero cells	100	91.3	103.6
adaGb$_3$-treated CHO cells	100	32.9	0
adaGb$_3$-treated JKT cells	100	37.5	0
OHEtadaGb$_3$-treated vero cells	100	164.6	43.6
OHEtadaGb$_3$-treated CHO cells	100	19.3	0