Development of a Novel Therapy for Lipo-oligosaccharide-induced Experimental Neuritis: Use of Peptide Glycomimics

S. Usuki[1], K. Taguchi[3], Y.-H. Gu[1**], S.A. Thompson[2], and R. K. Yu[1*]

Appendix S1 (Supplemental Information)

S1.1. Preparation of mAb R24 specific to GD3

The hybridoma cell line mAb R24 (anti-GD3, IgG3 class) was cultivated in a serum–free cell culture medium (BD Cell MAb Medium Serum Free, Becton Dickinson and Co., Sparks, MD). The mAb R24-IgG was purified from the conditioned medium by the procedure as previously described(Usuki et al., 2010). Briefly, the conditioned medium was concentrated by approximately 20-fold using an Amicon concentrator (model 8200) with an ultrafiltration membrane YM10 (Millipore Corp., Bedford, MA), followed by IgG precipitation with a saturated solution of ammonium sulfate (80 g dissolved in 100 mL of hot water), dialyzed against phosphate buffered saline (PBS) in water for 4 days at 4°C. The dialyzed IgG proteins were further purified by affinity column chromatography (HiTrap Protein G HP, 1mL) (Amersham Bioscience, Uppsala, Sweden). The IgG fraction was recovered from the column by eluting with glycine buffer (0.1 M glycine-HCl, pH 2.7) and then dialysis against PBS for 3 days at 4°C. The dialyzed mAb R24 IgG protein was used for ELISA and for constructing the mAb R24 affinity column.

S1.2. Biotinylation of P_{GD3}-4

One of the GD3-like peptides (P_{GD3}-4) was biotinylated using an EZ-Link®Sulfo-

NHS-LC-Biotinylation Kit (Pierce, Rockford, IL). Ten mg of P_{GD3}-4 was dissolved in 2 mL of PBS according to the instruction kit. After addition of the Sulfo-NHS-LC-Biotin solution, the reaction mixture was incubated for 1 h at room temperature. The biotinylated product (bP_{GD3}-4) was purified by a Zeba^{TM} Desalt Spin Column. The biotin-peptide ratio of bP_{GD3}-4 was determined using a HABA (4'-hydroxyazobenzene-2-carboxylic acid) Biotin Quantitation Kit (Pierce, Rockford, IL) and a LavaPep^{TM} Peptide Quantification Kit (Fluorotechnics, Sydney, Australia), and was expressed as nmol biotin/nmol peptide.

S1.3. Pharmacokinetic analysis

A Micro-Renathane polyurethane cannula, 0.84 mm o.d. x 0.36 mm i.d., (Braintree Scientific Inc., Braintree, MA) was inserted before dosing into the femoral vein of a female Lewis rat (n = 9; body weight = 200 ± 20 g). A single dose of bP_{GD3}-4 (100, 500, or 1000 nmol kg^{-1}; n = 3 per group) was administered i.p. Serial blood samples (~ 200 µL) were taken from the femoral vein for bP_{GD3}-4 testing at predose (0 h) and serial postdose-time points (0.5, 1, 2, 3, 6, 12, and 24 h). Blood samples were centrifuged at room temperature and the plasma harvested. The plasma samples were stored at -60°C to -80°C until analyzed by a streptavidin-coated ELISA plate for determining the total concentration of bP_{GD3}-4.

The plasma concentration-time course data of bP_{GD3}-4 were analyzed by a noncompartmental method of i.p. administration with first-order output as previously reported (Barrett *et al*., 1997; Wahl *et al*., 1988). The following parameters were calculated: maximum-, minimum-, and average-observed plasma concentration (C_{max}, C_{min}, and C_{av}), areas under the plasma concentration–time curve from zero to infinite time (AUC) plasma clearance (CL), distribution half-life ($t_{1/2}\alpha$), and elimination half-life

($t_{1/2}\beta$). Calculations of rate constants $t_{1/2}\alpha$ and $t_{1/2}\beta$ were obtained by the curve-peeling method (Gibaldi and Perrier, 1982). AUC was estimated according to the method of the trapezoidal rule by including the remaining AUC after the last measurable time point (24 h), and extrapolating the curve from the last time point to infinity.

S1. 4. Pharmacokinetic study

The dosage schedule of the 4 active peptides (P_{GD3}-3, P_{GD3}-4, P_{GD3}-5, and P_{GD3}-6) was designed by pharmacokinetic analysis based on a single dose administration into a rat. To assist in quantitation of the peptide mimics, P_{GD3}-4 was biotinylated and the specific activity for biotin labeling was determined as 0.4 for bP_{GD3}-4 (mole of biotin per mole of peptide).

Purified bP_{GD3}-4 was administered i.p. to rats as described above. Plasma concentration versus time after a single dose of 100, 500, and 1,000 nmol kg^{-1} for bP_{GD3}-4 are in Fig. S1A. None of the experimental rats died or exhibited toxicity as a result of the peptide administration.

Table S1. lists the parameter values obtained from a noncompartmental analysis of bP_{GD3}-4. Concentration over time data showed two-phase attenuation of distribution and elimination expressed as $t_{1/2}\alpha$, and $t_{1/2}\beta$. There was no change of parameter values for clearance, $t_{1/2}\alpha$ and $t_{1/2}\beta$, in the single-dose range of 100–1000 nmol kg^{-1} for bP_{GD3}-4. From these data, bP_{GD3}-4 exhibits linear pharmacokinetics after multiple doses. Elimination was rapid necessitating multiple dosing in order to determine the kinetics. In light of the $t_{1/2}\beta$ and minimum effective plasma concentrations determined from a single dose of $bP_{GD3\text{-}4}$, we adopted a chronic i.p. dose administration of P_{GD3}-4 (1,000 nmol kg^{-1} at 24-hourly intervals for 7 days). And, in order to avoid the effects of multiple i.p. dosing,

the animals were rested at two-week intervals. As shown in the chronic simulation (Fig. S1B), the resulting C_{max}, C_{min}, and C_{av} were 0.67 x 10^3, 0.33, and 0.34 x10^3 nmol/mL, respectively. At a minimum, an effective plasma concentration of peptides was achieved by multiple dosing, and this is shown by the IC_{50} values for each of the active peptides (0.3 nmol/mL for P_{GD3}-3, 0.15 nmol/mL for P_{GD3}-4, 0.1 nmol/mL for P_{GD3}-5, and 0.25 nmol/mL for P_{GD3}-6) (Fig. 2 in Text).

Supplemental Table S1. Pharmacokinetic parameters after i.p. administration of bP_{GD3}-4 to normal Lewis rats

Parameter		bP_{GD3}–4 dose group		
		n=3	n=3	n=3
Dose	(nmol kg^{-1})	100.0	500.0	1000.0
t_{max}	(h)	0.5	0.5	0.5
C_{max}	(nmol mL^{-1})	70.8 (10.0)	267.1(52.1)	667.2 (121.1)
$t_{1/2}\alpha$	(h)	0.48 (0.03)	0.42 (0.03)	0.46 (0.03)
$t_{1/2}\beta$	(h)	2.45 (0.27)	2.45 (0.33)	2.76 (0.42)
CL	(mL h^{-1} kg^{-1})	0.82 (0.24)	0.86 (0.11)	0.71 (0.04)
Vdss	(mL)	ND	ND	ND
AUC	(nmol mL h^{-1} kg^{-1})	130.0 (43.6)	589.2 (72.9)	1425.7 (78.8)

The values are the mean (SD).
ND, not determined.
Vdss, steady-state of distribution.

Supplemental Figure Legends

Figure S1. Plasma concentrations of peptide bP_{GD3}-4.

(A) Mean plasma concentration-time profiles following i.p. single dosing with 100 nmol kg^{-1}(•), 500 nmol kg^{-1}(o), and 1000 nmol kg^{-1}(▲) of bP_{GD3}-4. n = 3 rats; values are means ± SD.

(B) Expected plasma concentration-time profile simulated from multiple administration of bP_{GD3}-4 in (A) 1000 nmol kg^{-1} by time intervals of 24-hourly for 7 days. The simulation-plot was drawn according to the method of the trapezoidal rule of AUC and the parameters including $t_{1/2}\beta$ values for 1000 nmol kg^{-1} of bP_{GD3}-4 as shown in Table 2. The plasma concentrations of bP_{GD3}-4 in A and B are shown on a logarithmic scale on the vertical axis.

Figure S2. Molecular docking of GD3 and peptides into the binding pocket on the B chain of mAb R24.

The complementary determination region (CDR) 1, 2, and 3 of mAb R24 are marked by white letters, which form the binding pockets on the B chain of mAb R24.

(A) Molecular docking of the carbohydrate part of ganglioside GD3 into the binding pocket on the B chain of mAb R24, which is colored with electrostatic potentials in a range from -50.636 to 50.636 kT/e using pyMol. Red color denotes negatively charged group and blue color denotes positively charged groups. The carbohydrate head group of GD3 is shown in stick model. The blue section (positively charged) of the binding pocket interacts with terminal NeuAc-NeuAc (negatively charged).

(B-E) Molecular docking of P_{GD3}-3, P_{GD3}-4, P_{GD3}-5, and P_{GD3}-6 into the binding pocket on the B chain of mAb R24 are illustrated in each of the pictures. Using MGLTools, the

surface of the B chain of mAb R24 is marked in green and the surfaces of peptides are marked in blue.

Supplemental References

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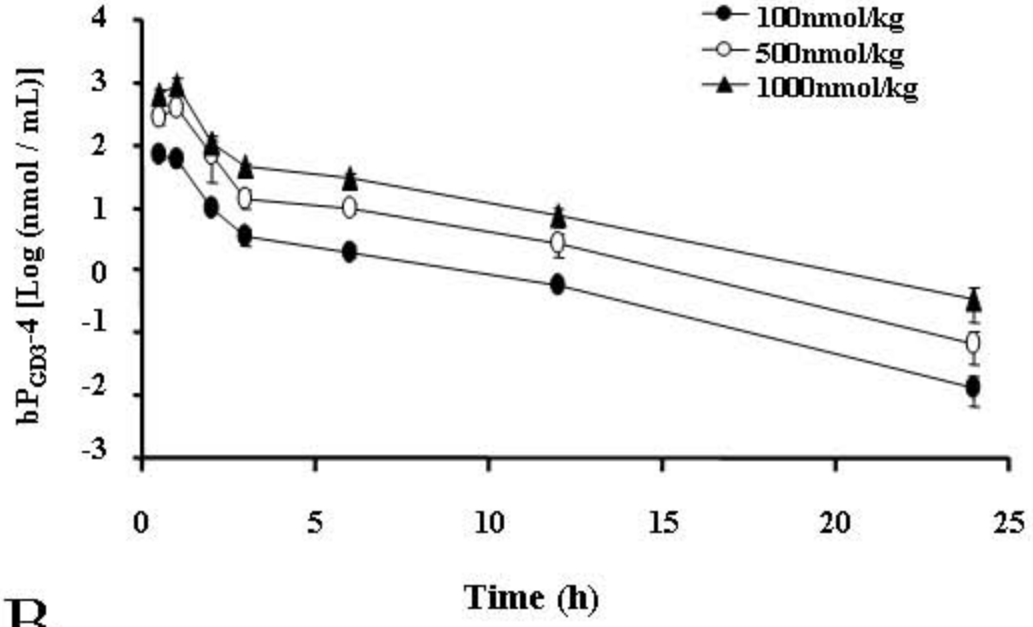
Fig. S1.

A


100nmol/kg
500nmol/kg
1000nmol/kg
bP$_{CD3}$-4 [Log (nmol / mL)]
4
3
2
1
0
-1
-2
-3
0
5
10
15
20
25
Time (h)

B

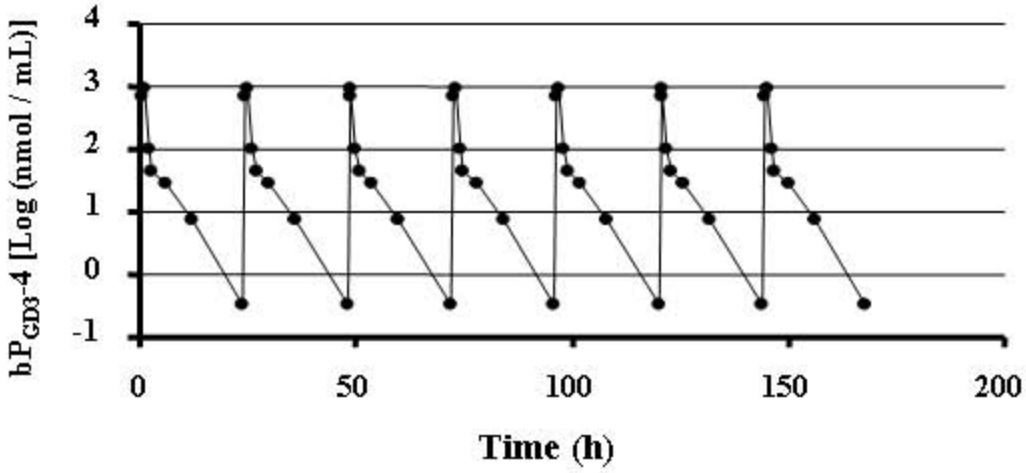
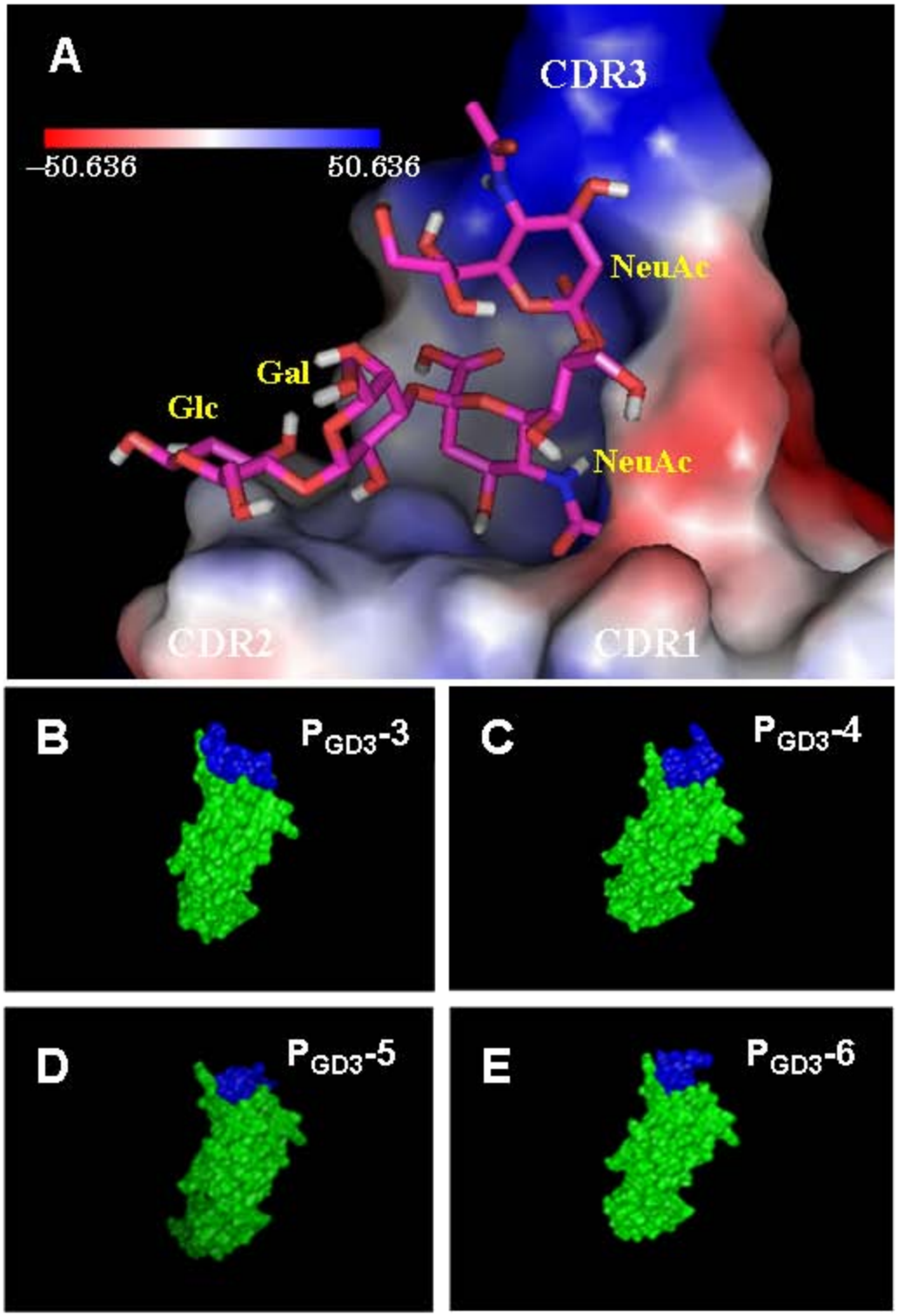

bP$_{CD3}$-4 [Log (nmol / mL)]
4
3
2
1
0
-1
0
50
100
150
200
Time (h)

Fig. S2


A
CDR3
–50.636
50.636
NeuAc
Gal
Glc
NeuAc
CDR2
CDR1
B
P_{GD3}-3
C
P_{GD3}-4
D
P_{GD3}-5
E
P_{GD3}-6