

Supplementary material

Methods

Quantification of endogenous murine VWF and N8-GP binding to murine VWF in vivo

FVIII-deficient mice were dosed 280 U/kg N8-GP and blood samples isolated before, 24 h and 30 h after dosing as described under Pharmacokinetics in mice. Endogenous murine VWF was measured by Imubind[®] VWF ELISA (American Diagnostica). N8-GP complexed with endogenous murine VWF was quantified by ELISA using anti PEG-coated microtiter plates (Abcam, Epitomics) for catching and polyclonal anti VWF antibody for detection. Pools of five-fold diluted plasma from six mice were added and incubated 1 h. The plates were washed with PBS (Gibco) containing 0.05% Tween, and bound VWF detected with horse radish peroxidase-conjugated polyclonal anti-VWF antibody (DAKO) and TMB substrate (Kem-En-Tec). A calibration curve was made by spiking of defined concentrations (0, 0.03; 0.06; 0.012 and 0.25 nmol/L) of N8-GP to plasma from FVIII-deficient mice containing excess murine VWF.

SDS-PAGE

Samples for electrophoresis were prepared in lithium dodecyl sulphate (LDS) sample buffer (Invitrogen) containing 50 mM DTT and incubated at 56°C for 10 min. Electrophoresis were performed using 7% tris-acetate Pre-Cast Novex polyacrylamide gel with tris-acetate buffer (Invitrogen). Electrophoresis was carried out for 60 min at a limiting voltage of 150 V. The gels were either stained with coomassie using GelCode (Pierce) according to the manufacturer's description or by PEG staining as described by Kurfurst et al.

Trypsin digest and RP-HPLC peptide map.

Samples were buffer exchanged into 2 M urea, 50 mM Tris, 2 mM CaCl₂ and 8 mM methylamine, pH 7.8, using a NAP5 column (GE Healthcare) and incubated overnight at 37°C with porcine trypsin (Promega) at a ratio of 1:20 (w/w). The digests were terminated by acidification with trifluoroacetic acid (TFA). The peptides were separated by RP-HPLC using a Jupiter Proteo 90A column (2 x 250 mm, Phenomenex) using 0.06% TFA in water (solvent A) and 0.055% TFA in 90% acetonitrile (solvent B) and a linear gradient from 2.3-51.5% solvent B at 0-100 min, and 51.5-100% solvent B from 100-120 min. The column temperature was 40°C, flow rate 0.2 mL/min, and absorbance was detected at 215 nm.

Glycan analysis

Samples were buffer exchanged into 2M urea, 10 mM EDTA, 25 mM NH_4HCO_3 using a 50K Micron[®] centrifugal filter (Millipore). The buffer-exchanged sample was adjusted to 0.1% RapiGest[™] and digested by 1U PNGase F (Roche). Released glycans were purified on HyperSep Hypercarb SPE Plates with 10 mg bed weight; 1 mL column volume (Thermo Fisher Scientific). The wells were successively primed with 0.5 mL methanol, 0.5 mL 1M NaOH, 1 mL water, 1 mL 30% acetic acid, and 1 mL water before equilibration with 5% acetonitrile in 0.1 % (TFA). Subsequently, the PNGase F digests were loaded on the plate and rinsed with equilibration buffer. Glycans were eluted with 0.1% TFA in 50% acetonitrile and dried under a stream of nitrogen. Labelling reagent was prepared by dissolving 2-aminobenzamid (2-AB, 50 mg/mL) in 30% acetic acid in DMSO. Sodium cyanoborohydride (1.0 M) in Tetrahydrofuran (THF, Sigma) was added to the 2-AB solution at a ratio of 1:9 THF to 2-AB solution (vol:vol) before adding 5 μL to the samples and incubating 3 hours at 65°C. Excess labelling reagent was removed with a 96 well MassPREP[™] Hydrophilic interaction chromatography (HILIC) plate (Waters) operated as described by the manufacturer. Briefly, the HILIC wells were rinsed with water and equilibrated with 90% acetonitrile. Labelled samples were adjusted to 80% acetonitrile prior to loading. The wells were rinsed with equilibration buffer and labelled glycans were eluted with 25 μL 10 mM ammonium citrate in 25% acetonitrile.

Glycan profiles were obtained by anion-exchange HPLC analysis on a CarboPac PA-100 column (Dionex Corporation) using 150 mM NaOH (eluent A) and 300 mM sodium acetate in 150 mM NaOH (eluent B). The chromatogram was developed using linear gradient from 0% to 35% eluent B over 52 minutes follow by 13 minutes regeneration of the column at 100% eluent B. The flow was 0.5 mL/min and 2-AB fluorescence was measured by excitation at 330 nm and emission at 420 nm.

N8-GP uptake by U87MG cells

U87MG cells were obtained from American Type Culture Collection (ATCC no. HTB-14) and cultured in MEM with non-essential amino acids and sodium pyroval (Gibco, 12492) supplemented with 10% (v/v) fetal calf serum, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco) in tissue culture flasks and plates (Gibco) coated with bovine fibronectin (Sigma, 40 $\mu\text{g}/\text{mL}$ in PBS). The medium was changed every 2nd or 3rd day and the cells passed once weekly by trypsination (0.25% trypsin, 1 mM EDTA in PBS). Cells were seeded in 48 well plates, and experiments

performed after 2 days when the cells had reached 95% confluence. Cells were washed with buffer A (100 mM HEPES, 150 mM NaCl, 4 KCl, 11 mM Glucose, pH 7.4) and incubated for 15 min at 37°C with buffer B (buffer A supplemented with 5 mM CaCl₂ and 1 mg/mL BSA). Some wells were pre-incubated for 5-10 min with receptor associated protein (RAP, 0.5 μM) prior to adding radioligand. Then, ¹²⁵I-N8-GP or ¹²⁵I-FVIII (turoctocog alfa) were added at a concentration of 2 nM alone or together with unlabelled N8-GP (225 nM), FVIII (225 nM or 1 μM) or VWF (25 or 50 nM), and incubated for 1 hour at 37°C. Cells were subsequently placed on ice and washed three times with cold buffer B to remove non-bound material. To separate surface-bound ¹²⁵I-FVIII from internalized, cells were incubated with 50 ug/mL trypsin (Sigma), 50 ug/mL proteinase K (Sigma), and 5 mM EDTA (Merck) in PBS for 60 min on ice. The cell suspension was centrifuged 5 min at 294×g at 4°C. The radioactivity in supernatants (surface bound material) and cell pellets (internalized material) were counted in a γ-counter (Cobra). Each experiment was performed in four replicates and the average values of each experiment used for calculating the mean and standard deviation.

Table S1. Endogenous murine VWF level and N8-GP – murine VWF complex after administration of 280 U/kg FVIII or N8-GP to FVIII-deficient mice.

Time (h)	Endogenous mVWF (%)*		N8-GP in complex with murine VWF (nmol/L) [#]	N8-GP concentration (nmol/L) [§]
	Mice receiving FVIII	Mice receiving N8-GP		
0	100	100	0	0
24	68±12	71±23	0.75	0.96±0.11
30	115±45	117±35	0.84	0.80±0.10

*) Values are expressed as % of the murine VWF level in each individual mouse before dosing. Data are mean and standard deviation from six mice

[#]) N8-GP - VWF complex was measured on a pool of plasma from six mice.

[§]) FVIII activity was measured in a chromogenic assay and values converted to molar concentration. Data are mean and standard deviation from six mice.

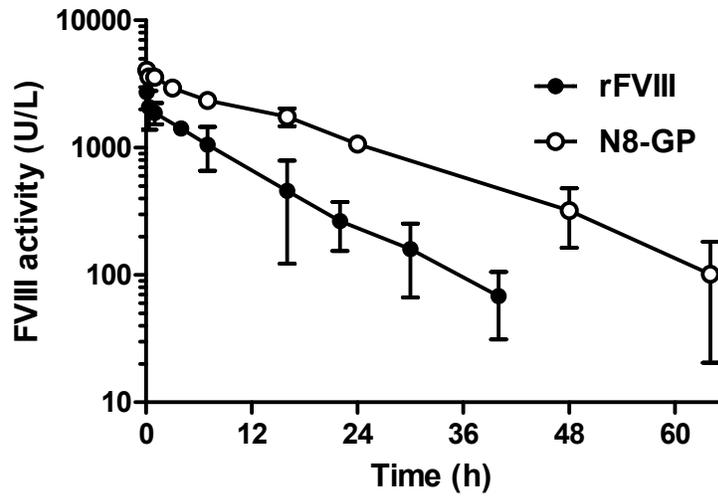


Figure S1. Increased exposure of N8-GP in FVIII-deficient mice. Mice were dosed i.v. with 280 U/kg FVIII (turoctocog alfa) or N8-GP. Blood was sampled at the indicated time points and FVIII concentration analyzed in a chromogenic assay. Data are mean and SD (n=3).

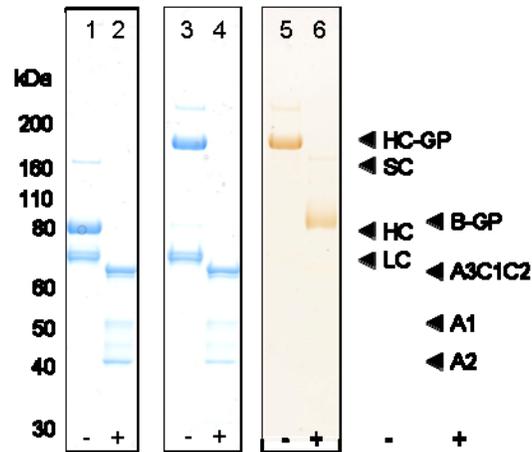


Figure S2. SDS-PAGE of N8-GP and FVIII. FVIII (turoctocog alfa, lane 1, 2) and N8-GP (lane 3-6) were analyzed by SDS-PAGE with (+) or without (-) prior cleavage with thrombin as indicated at the bottom of each lane. The gels were stained with coomassie (lane 1-4) or barium iodide that stain the PEG moiety (lane 5-6). Note that the 40K-PEGylated B-domain peptide (L-GP) released by thrombin cleavage migrates approximately as the heavy chain. The following fragments are indicated next to the gel. HC-GP: GlycoPEGylated heavy chain; SC: single chain FVIII; HC: Heavy chain; LC: Light chain; L-GP: GlycoPEGylated B-domain linker peptide; A3C1C2: Light chain without a3; A1: Heavy chain A1 domain and A2: Heavy chain A2 domain.

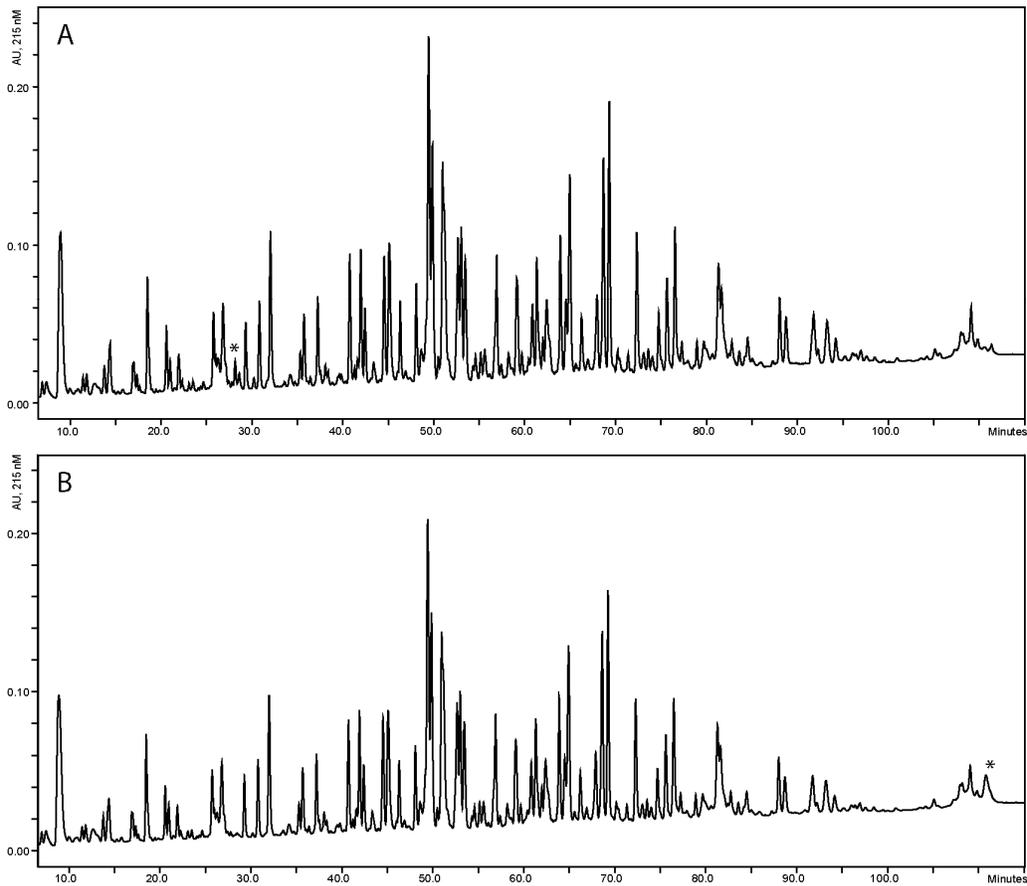


Figure S3. Tryptic peptide map of FVIII and N8-GP.

RP-HPLC peptide maps of FVIII (turoctocog alpha, A) and N8-GP (B) were comparable. The O-glycosylated peptide (His₇₄₈-Lys₇₅₇) is indicated by the asterisk in the two peptide maps. For FVIII the peptide elutes at 28 min whereas the glycoPEGylated peptide from N8-GP elutes at 111 min. The peptides identity has been confirmed by N-terminal amino acid sequencing.

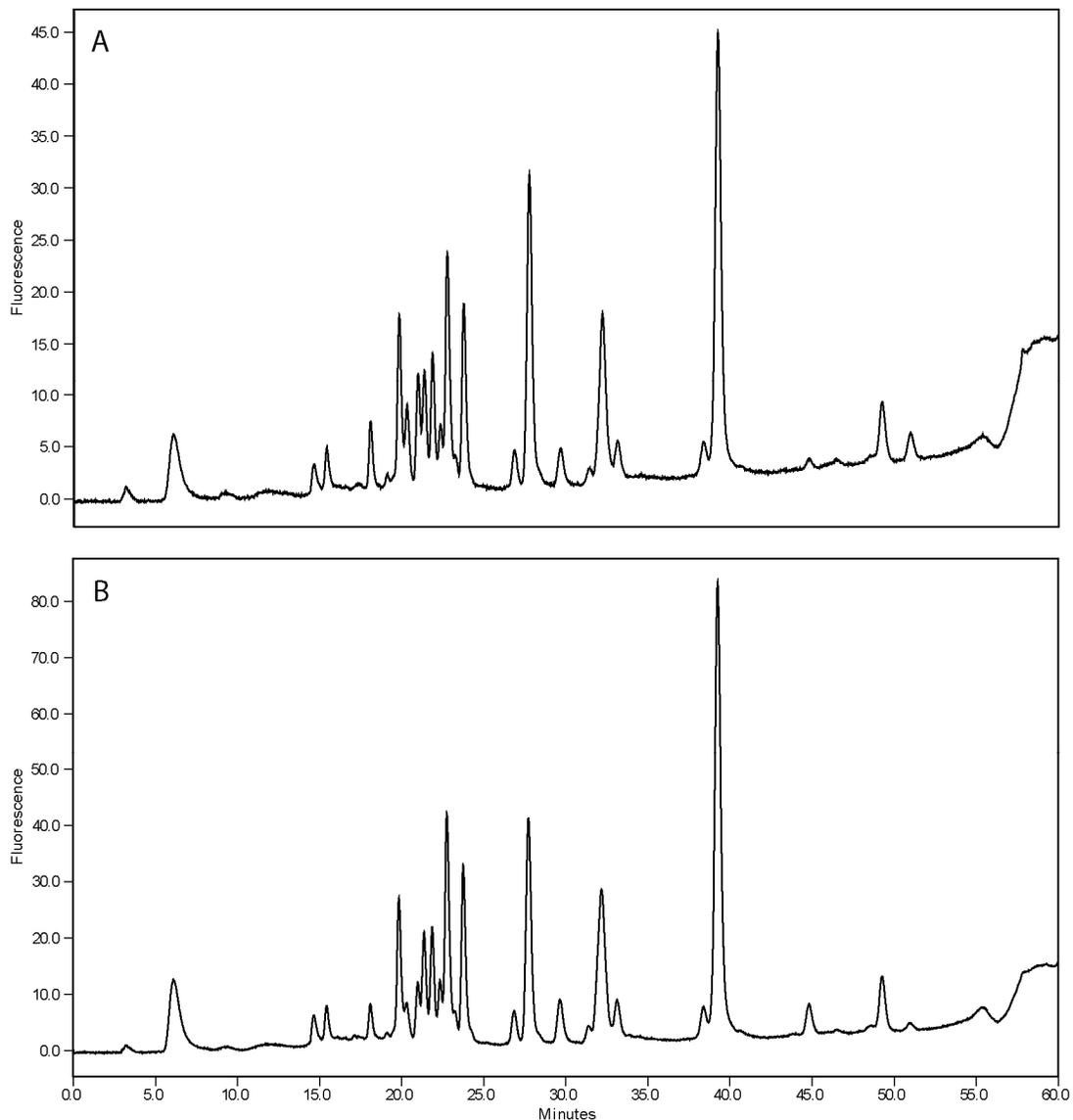


Figure S4. Glycoprofiles of FVIII and N8-GP. N-glycans from FVIII (turoctocog alfa, A) and N8-GP (B) were released by PNGase F digestion and labelled with 2-aminobenzamide before analysis by anion exchange HPLC. Neutral glycans eluted from 5 to 25 min and charged glycans with one or more sialic acid residues eluted later than 23 min. Minor variations were observed, i.e. a minor increase in the number of charged complex type glycans and a more than four-fold reduction of N-glycolylneuraminic acid eluting at 51 min. The ratio among high mannose type glycans and other neutral glycans (retention time 20-25 min) was consistent between FVIII and N8-GP.

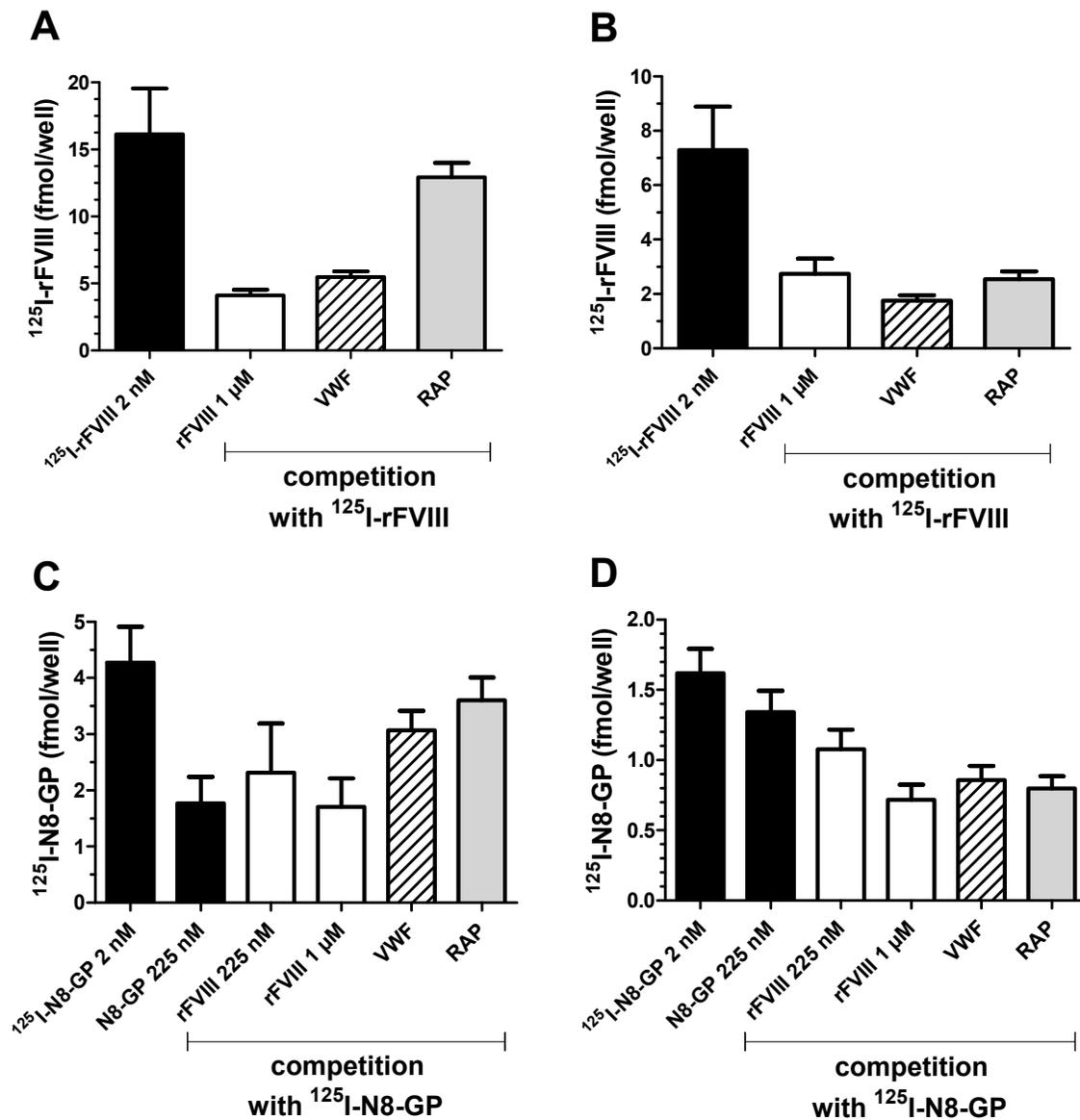


Figure S5. Decreased cell binding of N8-GP compared to FVIII. Data are mean and standard deviation ($n=5$) of $^{125}\text{I-FVIII}$ (A, B) or $^{125}\text{I-N8-GP}$ (C,D) binding (A,C) and internalization (B, D) in U87MG cells alone or in the presence of the noted competitors/binding proteins. Note that scales on y-axes are different. Five-fold less N8-GP compared to FVIII was bound to the cells. The LRP antagonist RAP inhibited internalization of both FVIII and N8-GP but had limited effect on binding.

Reference

Kurfurst MM. Detection and molecular weight determination of polyethylene glycol- modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem* 1992; 200(2): 244-248.