Engineering Antibody Reactivity for Efficient Derivatization to Generate $Na_V1.7$ Inhibitory GpTx-1 Peptide-Antibody Conjugates

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Supporting Information

SI MATERIALS AND METHODS

Purity methods. Peptide linker purity by LCMS: Elution from a Phenomenex Max-RP column (2.5 μm, 2.0 x 50 mm) using a gradient of 5-50% acetonitrile in H_2O (0.1%TFA) over 10 min at a flow rate of 750 μL/min on an Agilent 1290 LC-MS system. All peptide-linkers were >95% in purity. Conjugate purity by size exclusion chromatography: listed in Table S1.

Peptide-Linker 2 Preparation. The preparation of GpTx-1 peptide analogs has been described previously.[11,12] Alkyne-containing peptide was subjected to copper-catalyzed 1,3-dipolar cycloaddition with azido-PEG bromoacetamides of differing lengths to obtain the site-specifically PEGylated peptides with a triazole linkage, thus converting a propargylglycine or Pra residue in the sequence to a 3-(1,2,3-triazol-4-yl)alanine or Atz residue. Peptide **1** (reference

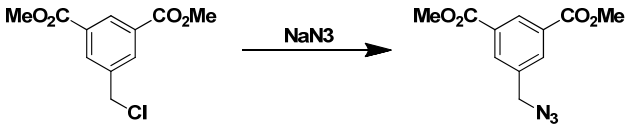
23) containing a propargylglycine (Pra) at position 13 (7.4 mM in 6.7 mL water), azido-PEG11-bromoacetamide (150 mM, 0.964 mL in water), tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA, 10 mM, 2.2 mL in DMSO), sodium ascorbate (50 mM, 10.1 mL in water), and copper (II) sulfate (35 mM, 2.9 mL in water) solutions were prepared fresh. Peptide and reagents were added in the following order to a 50 mL centrifuge tube to achieve the following final concentrations. Peptide stock solution (6.7 mL) was added for a final concentration of 3.7 mM, followed by addition of azido-PEG11-bromoacetamide (0.402 mL) for a final concentration of 4.5 mM. TBTA (0.603 mL) was added for a final concentration of 0.45 mM and mixed thoroughly. Sodium ascorbate (4.522 mL) was then added for a final concentration of 16.9 mM and mixed thoroughly. $CuSO_4$ (1.292 mL) was added for a final concentration of 3.4 mM. The solution was allowed to stand for 1 h at which time it was judged to be complete by LC-MS. To a 25mL SPE filter tube was added SP Sepharose High Performance resin as a slurry (8 mL) with a pipette. The gel was conditioned with wash buffer (50 mL 20 mM NaOAc, pH = 4.0), loaded with the peptide solution, washed (50 mL, 20 mM NaOAc, pH = 4.0 and 50 mL, 20 mM NaOAc, pH = 4.0, 0.1M NaCl), and eluted with 45 ml 1M NaCl, 20 mM NaOAc, pH = 4.0) into a 50 mL centrifuge tube. The product mixture was purified by loading onto a preparative HPLC column (Phenomenex Luna 5u C18(2) 100A AXIA, 250 x 30mm) at 30 mL/min using an Agilent prep HPLC pump. The column was flushed for 10 min with 10% B solvent. The column was attached to a prep HPLC (Agilent), and the peptide was eluted with a 10-40% B gradient over 60 min (A solvent: 0.1% TFA in water, B solvent: 0.1% TFA in acetonitrile). The fractions were analyzed by LC-MS, pooled, and lyophilized to afford pure peptide-linker construct (123 mg, 52% yield) for conjugation to IgG or

Fc domain. ESI-MS: Calc. MW for $C_{203}H_{321}BrN_{58}O_{55}S_6$ = 4726.37, Observed MW = 1576.1 $(M+3H)^{3+}$, 1182.5 $(M+4H)^{4+}$.

Capping of Peptide-linker 2 with 2-mercaptoethanol to provide peptide linker control 21 (Ala5,Phe6,Atz(PEG11-(hydroxyethyl)thio)acetamide)13,Leu26,Arg28]GpTx-1).

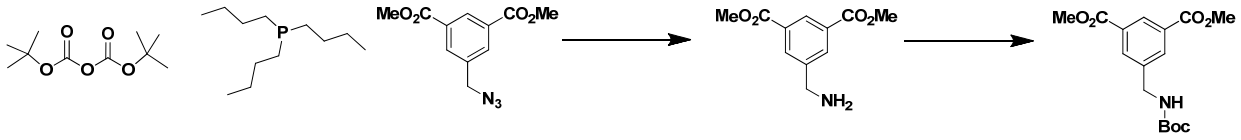
[Ala5,Phe6,Atz(PEG11-bromoacetamide)13,Leu26,Arg28]GpTx-1 (compound **2**, 2.0 mg, 0.423 μmol) was dissolved in 500 uL of water, and 50 uL of 1M Tris (pH = 7.5) was added to make 0.1 M Tris pH 7.5 (total solvent volume 550 μL) followed by 2-mercaptoethanol (63.5 μL, 0.635 μmol). The reaction was incubated overnight at room temperature. The reaction mixtue was diluted to 2 mL and purified by mass-triggered semi-prep HPLC (Agilent, using a Synergi 4u Max-RP 80 Å column, 250 x 10 mm 4 micron) with a gradient of 15-30% B over 45 min. The collected fractions were pooled and dried using a lyophilizer to afford a white powder (1 mg, 50% yield). ESI-MS: Calc. MW for $C_{205}H_{326}N_{58}O_{56}S_7$ = 4723.59, Observed MW = 1575.0 $(M+3H)^{3+}$, 1181.5 $(M+4H)^{4+}$.

Bis-(Azido-PEG$_{23}$) Linker. The following series of reactions was conducted.

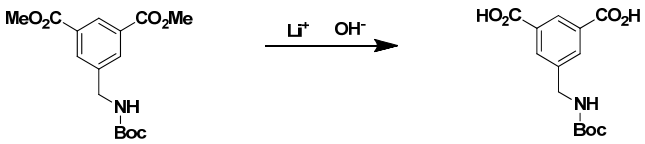


Dimethyl 5-(chloromethyl)isophthalate (CAS# 252210-01-8 (Anichem), 5.69 g, 23.45 mmol) was dissolved in acetone (90 mL) and water (30 mL). Sodium azide (9.15 g, 141 mmol) was added and the solution was refluxed for 14h. The reaction mixture was cooled to room temperature and concentrated in vacuo, the residue re-dissolved in $CHCl_3$ (250 mL) and the organic layer was washed with water (3 x 200 mL) and brine (200 mL). The organic layer was

then dried over $MgSO_4$, filtered and concentrated in vacuo to afford dimethyl 5-(azidomethyl)isophthalate (5.34 g; yield 91%).

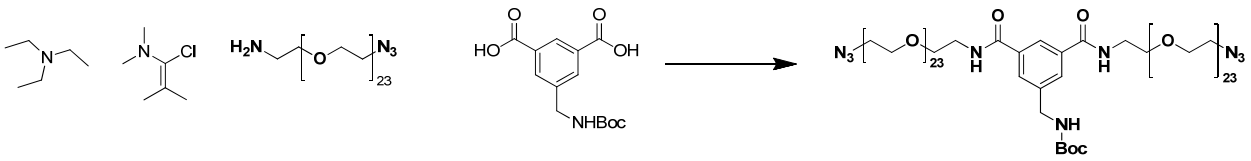


Dimethyl 5-(azidomethyl)isophthalate (1.5 g, 6.02 mmol) was dissolved in 40 mL of diethyl ether (40 mL). Tri-n-butylphosphine (1.654 mL, 6.62 mmol) was added slowly and the reaction mixture was stirred for 45 minutes at room temperature and then cooled to -50^{o}C. The solution of di-tert-butyl dicarbonate (1.417 mL, 6.62 mmol) in diethyl ether (20 mL) was slowly added (~10 min) and the reaction mixture was stirred at -50^{o}C for 1h and then quenched with saturated $NaHCO_3$ (20 mL). The reaction mixture was extracted with diethyl ether (20 mL). The organic phase was dried with $MgSO_4$, filtered and concentrated in vacuo. The crude amine was dissolved in a mixture of DCM and MeOH, sillica gel was added, concentrated and purified by silica gel chromatogrpahy using 0 - 30 % of EtOAc in hexanes affolding the Boc-protected benzyl amine which was used directly in the next step (760 mg; yield 75%).

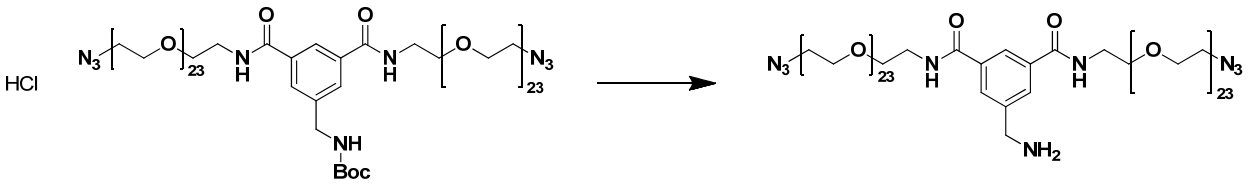


A solution of dimethyl 5-(((tert-butoxycarbonyl)amino)methyl)isophthalate (830 mg, 2.57 mmol) in MeOH (40 ml) was treated with lithium hydroxide (984 mg, 41.1 mmol) dissolved with water (20 mL). The reaction mixture was stirred at 45°C for 6h. The reaction solution was concentrated in vacuo to remove the MeOH and the aqueous layer was extracted with diethyl ether (50 mL). The aqueous layer was then acidified with HCl (2M) at 0 °C until pH 3-4 was

reached and precipitation of the desired product occurred, which was collected by filtration, washed with diethyl ether (2 x 10 mL) and dried in air to afford 5-(((tert-butoxycarbonyl)amino)methyl)isophthalic acid (570 mg; yield 75%).

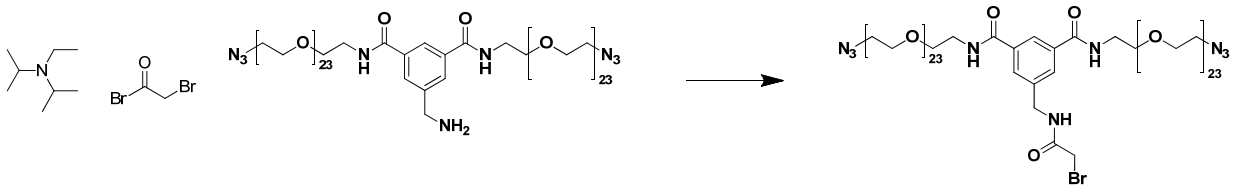


A suspension of 5-(((tert-butoxycarbonyl)amino)methyl)isophthalic acid (150 mg, 0.508 mmol) in DCM (5 mL) was treated with 1-chloro-N,N,2-trimethylprop-1-en-1-amine (Aldrich, 0.155 mL, 1.168 mmol). The reaction was stirred at 23 °C. After 1h, the reaction was concentrated and dried under vacuum. The crude acid chloride was dissolved in DCM (5 mL), and treated with azido-PEG23-amine (Quanta BioDesign,1675 mg, 1.524 mmol). Triethylamine (0.354 mL, 2.54 mmol) was then added in dropwise fashion. The reaction was stirred under nitrogen at 23 °C, and subsequently concentrated after 4h and dried under vacuum. The crude mixture was dissolved in 30% MeCN/water (3 mL) and filtered through a Whatman 0.45 µm filter, and purifed on HPLC using a Phenomenex Synergi 4 µm MAX-RP 80 Å 250 x 30 mm column and a gradient: 10-55% MeCN/water + 0.1%TFA in 35 min @ 30ml/min flow rate (5 runs of 1.5 mL each). The pooled fractions were frozen and lyophilized to afford a colorless semi-solid (675 mg; yield 54%).



A solution of the Boc-amine (0.675 g, 0.275 mmol) in hydrogen chloride, 4.0M solution in 1,4-dioxane (10.30 ml, 41.2 mmol) was stirred at 23 °C. After 19h, the reaction mixture was

concentrated and dried under vacuum affording a white solid. It was dissolved in 5 ml water and converted to a free base using VariPure IPE® carbonate resin (Agilent). The resin (~ 400 mg) was conditioned in a column with methanol (6 mL), followed by water (6 mL). The product HCl salt in water was applied and the flow through was collected. The column was washed with water (3x6 mL) and the filtrates were combined with the initial flow through and frozen. The solid was lyophilized to a white fluffy solid (580 mg; yield 90%). ESI-MS: Calc. MW for $C_{105}H_{201}N_9O_{48}$ = 2356.3, Observed MW = 1179.4 $(M+2H)^{2+}$.



A solution of the benzylic amine from the previous step (580 mg, 0.246 mmol) in DCM (5 mL) was cooled to 0 °C under nitrogen and was treated with *N*,*N*'-diisopropylethylamine (0.107 mL, 0.615 mmol), followed by bromacetyl bromide (0.027 mL, 0.307 mmol). The reaction was stirred at 0 °C. LCMS analysis at 20 min showed complete reaction to product. The solvent was removed under reduced pressure and the reaction was dried under vaccuum. The crude mixture was dissolved in 30% MeCN/water (8 mL) and filtered through a Whatman 0.45 µm filter, and purifed via HPLC using a Phenomenex Synergi 4 µm MAX-RP 80 Å 250 x 30 mm column and a gradient: 10-55% MeCN/water + 0.1%TFA in 35 min @ 30ml/min flow rate (4 runs of 2 mL each). The pooled fractions were frozen and lyophilized to afford a light brown solid characterized by LC-MS (355 mg; yield 58%). ESI-MS: Calc. MW for $C_{107}H_{202}BrN_9O_{49}$ = 2476.2, Observed MW = 1239.8 $(M+2H)^{2+}$.

General Procedure for Conjugation Reaction Screening using Partial Reduction Method.

Antibodies (0.25 mg each) and buffer (50 mM phosphate, 2 mM EDTA, pH 7.5) was added to wells in a 96 well plate (Corning 3365, polypropylene round bottom). Varying amounts of TCEP was added to the wells (1.0, 1.2, 1.5 or 2.0 eq. TCEP/engineered Cys). The final volume in each well was 95 uL. The plate was sealed with clear plastic film and shaken on an Eppendorf Thermomixer R at 300 RPM at 20 °C for 1 h. I-LC-biotin/DMF solution (5 uL of 2 mM solution, 3 eq/engineered Cys) was added to each reaction well, and the plate was re-sealed and shaken for an additional 4 h. The solutions were then desalted using Zeba Spin de-salting (40,000 MWCO) 96-well plate. The crude, intact conjugates were deglycosylated using PNGase: 15 uL buffer (pH 7.5) was added to a 96 well Corning 3365 polypropylene plate followed by PNGase F (2.0 uL, P0705L, New England Biolabs) and then each conjugate (12 uL). The plate was sealed and incubated at 37 °C for 16 h. A 12 uL aliquot of each deglycosylated sample was transferred to a second 96 well plate. 15 uL DPBS was added to the original deglycosylated sample to bring volume back up to >20 uL. 15 uL 0.5 M TCEP solution was added to each of the wells. The plate was sealed and gently shaken at 37 °C for 60 min and LC/TOF-MS data was obtained to identify ratio of the mono-alkylated peak to sum of unreacted, mono and bis-alkylated peaks in the MS spectrum.

General Procedure for Conjugation Reaction Screening using Redox Method.

Antibodies (0.25 mg each) and buffer (50 mM phosphate, 2 mM EDTA, pH 7.5) was added to wells in a 96 well plate (Corning 3365, polypropylene round bottom). Varying amounts of TCEP was added to the wells (2, 3, 6 or 10 eq. TCEP/engineered Cys). The final volume in each well was 95 uL. The plate was sealed with clear plastic film and shaken on an Eppendorf

Thermomixer R at 300 RPM at 20 °C for 1 h and desalted using a Zeba Spin de-salting (40,000 MWCO) 96-well plate. The reduced, de-salted Abs were treated with varying amounts of DHAA (2:3, 3:4.5, 6:9, 10:15 TCEP:DHAA/ engineered Cys). The plate was sealed and shaken on an Eppendorf Thermomixer R at 20 °C, for 1 h. I-LC-biotin/DMF solution (5 uL of 2 mM solution, 3 eq/engineered Cys) was added to each reaction well, and the plate was re-sealed and shaken for an additional 4 h. The solutions were then desalted using Zeba Spin de-salting (40,000 MWCO) 96-well plate. The crude, intact conjugates were deglycosylated using PNGase: 15 uL buffer (pH 7.5) was added to a 96 well Corning 3365 polypropylene plate followed by PNGase F (2.0 uL, P0705L, New England Biolabs) and then each conjugate (12 uL). The plate was sealed and incubated at 37 °C for 16 h. A 12 uL aliquot of each deglycosylated sample was transferred to a second 96 well plate. 15 uL DPBS was added to the original deglycosylated sample to bring volume back up to >20 uL. 15 uL 0.5 M TCEP solution was added to each of the wells. The plate was sealed and gently shaken at 37 °C for 60 min and LC/TOF-MS data was obtained to identify ratio of the mono-alkylated peak to sum of unreacted, mono and bis-alkylated peaks in the MS spectrum.

Antibody Conjugation via Partial Reduction Method. Anti-DNP mAb (E384C) (kappa/IgG1z) was diluted to ~5mg/mL in reaction buffer (20mM sodium phosphate pH 6.8, 2mM EDTA). Reduction of engineered cysteines was done by incubating mAb with a 1:1 molar ratio of TCEP to each cysteine at room temperature for 30 minutes. TCEP was removed using a Zeba spin desalting column (7000 MWOC). Large scale preps were concentrated to appropriate volume prior to loading using Amicon Ultra (30,000MWCO) centrifugal concentrators. Lyophilized peptide-linker bromoacetamide **2** was re-suspended in water at 20 mg/mL

immediately prior to conjugation reaction. Peptide and reduced mAb were mixed at a 2.5:1 molar ratio of peptide to cysteine (5:1 peptide to mAb) in reaction buffer at mAb concentration of 5 mg/ml and incubated for 16 h at 4^{o}C. The conjugation reaction mixture was subsequently desalted to remove excess free peptide and loaded onto a HiTrap SP-HP column (GE Healthcare). The column was rinsed in 5 column volumes of 90% buffer A (10 mM sodium phosphate pH 6.5, 5% ethanol) - 10% buffer B (10 mM sodium phosphate pH 6.5, 5% ethanol, 1M NaCl). The conjugate was eluted over a 20 column volume gradient from 10% buffer B to 70% buffer B. The unmodified mAb eluted first, followed by monvalent imunoglobulin-peptide conjugate **7** and then by bivalent imunoglobulin-peptide conjugate **5**. Following purification, conjugates were formulated into A5Su storage buffer (10 mM sodium acetate pH 5.0, 9% sucrose) and concentrated to 10 mg/mL. Conjugates were run on reducing SDS-PAGE to confirm conjugation to heavy chain (increase in size by ~5kD) and non-reducing SDS-PAGE to confirm internal disulfides remained intact. LC/MS spectrometry of peptide conjugates was recorded using an Agilent 6224 TOF LC/MS spectrometer with a non-reduced method. After calibration, a 5 μg intact sample was loaded onto a Zorbax 300SB C6 column (1 x 50 mm, 3.5 μm, Agilent Technologies) operation at 75 ºC with a flow rate of 50 μL/min. The conjugate was eluted from the column using a gradient of 90% *n*-propanol with 0.1% TFA (Buffer B) and 0.1% TFA in water (Buffer A). The gradient condition was 20% to 90 % buffer B in 14 min. From 20 mg of anti-DNP E384C mAb and 5.3 mg of peptide-linker contruct 8 mg of bivalent conjugate **5** was obtained after purification (40% yield).

Antibody Conjugation via Redox Method. TCEP (4.0 mM in water, 0.608 mL, 2.432 μmol) was added to a solution of αDNP E384C mAb (13.1 mg/mL, 60 mg, 4.58 mL, 0.405 μmol) in

17.29 mL reaction buffer (50 mM sodium phosphate, 2.0 mM EDTA, pH 7.5) in a 50 mL conical vial. The solution was shaken for 1 h at 20 ºC (300 rpm, eppendorf heater/shaker) before being desalted into reaction buffer. Six Zeba desalt coumns (MWCO 40000) were prepared for use according to the buffer exchange procedure (Thermo Scientific), using reaction buffer (50 mM sodium phosphate, 2 mM EDTA, pH 7.5, centrifuge to remove storage buffer 2 min, add 5 mL reaction buffer, centrifuge 2 min, repeat 2X, centrifuge 6 min after 3rd buffer addition, centrifuge speed 1000 g). Samples were added to the desalt columns (4 mL each column) and centrifuged for 4 minutes to collect the desalted samples. The eluent from each of the 6 columns were combined into a tube. A solution of dehydroascorbic acid (DHAA, 4 mM in DPBS, 0.912 mL, 3.65 μmol) was added to the reaction mixture and it was lightly shaken for 15 min at 20 ºC (300 rpm, eppendorf heater/shaker). To the solution was added peptide **2** (4.0 mM solution in water, 0.608 mL, 2.432 μmol). The mixture was shaken at 20 ºC for 4h, then placed in a 4 ºC refrigerator overnight. The crude reaction mixture was transferred to 2 different Amicon-Ultra 15 filters (MWCO 10,000). They were each concentrated to around 1 mL in volume (4000 g for ~30 min). Each sample was transferred to a glass autosampler vial, then the filters were washed with 1 mL A5Su buffer and transferred to the vials. The resulting samples were treated with 1 mL of 2 M $(NH_4)_2SO_4$, 20 mM NaOAc (pH 5.0). Each of the samples was injected as a single injection for purification using hydrophobic interaction chromatography (Dionex ProPac HIC-10 5 um, 300 A, 7.8x75 mm, Buffer A: 20 mM NaOAc, 1 M $(NH_4)_2SO_4$ pH=5.0, Buffer B: 20 mM NaOAc, 10% CH3CN, pH=5.0, 0-100% B over 40 min, 2.0 mL/min). The fractions corresponding to conjugate **5** were collected and combined. The conjugation reaction above was repeated twice more to give a total of 180 mg mAb starting material (3 reactions of 60 mg each).

From all purification lots, the fractions containing conjugate **5** were combined and transferred to a Thermo-Pierce dialysis flask (20,000 MWCO, 250 mL). The mixture was dialyzed against 3 L of A5Su buffer at 4 ºC. The buffer solution was changed at 2 h, and 14 h. The final dialysis proceeded for 3 h before collection of the sample. The resulting solution was added in 15 mL aliquots to 6 Amicon ultra-15 (MWCO 10,000) centrifugal filters, spun at 4000 g for 20 min, followed by the addition of more sample to each tube, then spun for 30 min at 4000 g to get volumes of between 1 and 1.5 mL. The solutions were transferred to a 15 mL conical vial and the filters were washed with A5Su buffer (1 mL each). The wash was also transferred to the vial to give about 12 mL solution. This solution was filtered through a 0.22 um PES syringe filter (Millipore), collecting into a tared 15 mL conical vial, affording 12.43 mL of conjugate **5** @ 11.2 mg/mL concentration, measured by UV-Vis absorption at 280 nm (139 mg, 72% isolated yield). Monomeric content was determined to be 97% by size-exclusion chromatography, mass was determined to be 157,085 by HPLC-TOF. A small portion of the compound was deglycosylated by adding 2 µL conjugate **5** solution, 2 µL PNGase F (New England Biolabs), and 30 µL reaction buffer (50 mM sodium phosphate, 2 mM EDTA, pH 7.5), then heating at 37 °C for 5 h. A portion of this sample (12 µL) was removed and diluted with 15 uL of 4 mM TCEP solution. The original sample was diluted with 15 µL reaction buffer (50 mM sodium phosphate, 2 mM EDTA, pH 7.5) and both samples were heated to 37 °C for an additional hour. Both solutions were analyzed by HPLC-TOF: deglycosylated **5** molar mass was 153,978, reduced sample showed unmodified light chain (MW = 23,220) and singly-modified heavy chain (MW = 53,725).

Peptide Mapping of Peptide-Ab Conjugates. The site of conjugation (engineered cysteine or native cysteine) for antibody conjugates prepared by the two different routes (partial reduction vs. redox) was investigated. Peptide mapping analysis was performed on the conjugate **5** samples from partial reduction and redox preparations. Briefly, 100 µg of each sample were reduced in 30mM DTT and incubated for 1 hr at 37 °C, followed by alkylation at room temperature and in the dark with 65mM iodoacetimide (IAM) for 45 min. Proteolytic digestion was performed overnight at 37 °C with endopeptidase Wako Lys-C (1:20 enzyme/substrate ratio). A Waters NanoAcquity UPLC coupled with a Thermo Scientific Oribtrap Velos equipped with HESI-II probe was used for LC-MS analyses. Peptide separation was achieved using formic acid buffers (mobile phase A = 0.1% formic acid in water; mobile phase B = 0.1% formic acid / 99.9% acetonitrile) with a 180 µm x 20 mm (5µm particle) trap column linked to an Agilent Zorbax 300SB-C18 0.5 mm x 250 mm (5µm particle) analytical column. The linear elution gradient performed at 15 µL/min, starting at 3% B and ending at 45% B in 85 min, was followed by a rise to 95% B in 1 min, and was held isocratic for 6 min before re-equilibration at 3% B for 20 min. The Mass Spec parameters are as follows: Full MS scan [300-2000 m/z], 30K Resolution, CID fragmentation on top 10 precursors, isolation width 2.0, and normalized collision energy = 35. Conjugation was only observed on the engineered cysteine, E384C in the anti-DNP mAb heavy chain. Various forms of alkylated mAb peptide conjugated to the $Na_V1.7$ inhibitory peptide were observed. Iodoacetimide (IAM) was added to prevent cysteines from reforming disulfides. The apparent observed mass addition due to conjugation minus IAM modifications is 1394.7 Da. No significant difference was observed between the lots prepared by redox vs. partial reduction. No off-site conjugation to native cysteines was observed in peptide maps. Analysis was screened by UV peak differences between the starting material and

conjugate lots. All MS data was analyzed by in-house MassAnalyzer software, as well as manually scrutinized for any potential masses related to conjugation on native cysteines.

Figure 3 conjugate modeling A homology model of αDNP huIgG1 antibody using 1HZH.pdb as template was created using modeling package MOE. Extended linkers were built and minimized in MOE using AMBER10:EHT force field and Generalized Born implicit solvation. The first member of the GpTx-1 NMR ensemble[11] was tethered at peptide position 13. Figures are for illustration purposes only and do not represent an atomic model of conjugates in solution.

Electrophysiology All automated and manual patch clamp electrophysiology experiments were conducted as previously described.[11,12] All cell lines were validated by RT-PCR to express the indicated Na_V isoform and mycoplasma free. Na_V current inhibition was determined after 5 min of conjugate addition at which point, inhibition reached near steady state levels for concentrations at or above IC_{50} values. For all PatchXpress experiments, cells were not reused for subsequent conjugate testing if currents did not return to >80% starting levels following conjugate washout. In addition, steady state inactivation curves were determined following washout of each concentration of conjugate and preceding subsequent concentrations tested to ensure the same level of Na_V channel inactivation prior to conjugate addition.

Pharmacokinetic studies.

Animal Welfare. All experimental procedures involving animals were conducted at an AAALAC International accredited facility in accordance to the *Guide for the Care and Use of Laboratory Animals*, 8th Edition. The research protocols were approved by the Amgen

Institutional Animal Care and Use Committee. All animals were maintained on a 12:12 h light:dark cycle, and the ambient temperature and humidity range was at 68 to 79° F and 30 to 70%, respectively. Animals in all studies had access to food and water *ad libitum.* No animals were excluded from analyses and randomization was not employed. Investigators were not blinded.

Conjugate **5**. A pharmacokinetic (PK) study was conducted with 10 week-old male CD-1 mice from Charles River Laboratories. Conjugate **5** in A5Su buffer (10 mM sodium acetate, 9% sucrose, pH 5.2) was dosed to 9 mice at 10 mg/kg subcutaneously under the skin in the mid-scapular region. Blood samples were taken at various time points using a composite sampling scheme with no more than 3 samples taken from an individual mouse. For each sample, approximately 60 μL of blood was collected into a serum separator tube via submandibular vein puncture. Samples were allowed to clot at room temperature for 20 minutes and then centrifuged under refrigerated conditions (2-8 °C) for 15 minutes at approximately 11500 x g. Serum was transferred to a 96-well plate and frozen at -80˚C until analysis.

Conjugate **5** LC-MS/MS Bioanalysis Method. Materials and Reagents.

Biotinylated murine anti-human IgG Fc monoclonal antibody (mAb, Clone no. 1.35.1), conjugate **5**, peptide **22** and WT GpTx-1 peptide were prepared and purified as described (this work, references 9,10). Stable [$^{13}C_6$,^{15}N]-leucine labeled VVSVLTVLHQDWLNGK flanking peptide was obtained from Mid-West Biotech, Inc. (Fishers, IN). Dulbecco phosphate buffered saline, 1x (DPBS), HALT Protease Inhibitor Cocktail (100x), Streptavidin Dynal M-280 magnetic beads and trypsin (TPCK treated) were obtained from Thermo Fisher Scientific (Waltham, MA). Tris(2-carboxyethyl)phosphine (TCEP), urea, iodoacetamide, acetonitrile

(ACN), water, methanol, formic acid and trifluoroacetic acid were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Oasis HLB 96-well μElution Plates, 2 mg Sorbent per Well, 30 μm particle size were obtained from Waters Corp. (Milford, MA). 1 M Tris-HCl buffer, pH 7.5 was obtained from Teknova, Inc. (Hollister, CA).

Preparation of conjugate **5** calibration standards (STDs), quality control (QC) samples, and working internal standard (WIS) solutions

Calibration standards in the range of 50 – 10,000 ng/mL and quality controls (QC) 75, 750 and 7500 ng/mL were prepared by spiking a 100 μg/mL conjugate **5** working stock solution (in A5Su buffer) into mouse serum treated with 2x HALT protease inhibitor cocktail, followed by serial dilution. WIS solutions of 1.0 μg/mL were prepared in methanol/water (50/50, v/v) for [$^{13}C_6$,^{15}N]-leucine labeled VVSVLTVLHQDWLNGK flanking peptide and WT GpTx-1 peptide.

Sample Preparation. To a 96 deep-well plate, 25 μL of serum sample, 25 μL of DPBS with 2x HALT protease inhibitor cocktail and 25 μL of 50 μg/mL biotinylated murine anti-human IgG Fc monoclonal antibody (Clone no. 1.35.1) immobilized, Streptavidin Dynal M-280 magnetic beads, were added and vortexed for 1 hour at room temperature to allow for immunocapture treatment.

The anti-human IgG Fc captured beads were then washed four times with 800 μL of 250 mM Tris-HCl buffer (pH 7.5) using a Tomtec Quadra3 instrument (Tomtec, Hamden, CT) with a magnetic nest attachment. Reduction was carried out by the addition of 30 μL of 7.5 mM TCEP in denaturation buffer (250 mM Tris-HCl buffer, pH 7.5 containing 8 M urea) with 1.0 μg/mL of WIS solutions. Alkylation was performed by adding 25 μL of 25 mM iodoacetamide in water,

vortexed and incubated for 45 minutes at 60°C, followed by digestion using 50 µL of 0.5 mg/mL trypsin with microwave set at 400W for 10 min at 30°C. The digestion was quenched with 15 µL of 20% formic acid and vortexed for 1 min. The magnetic beads were pulled down to the sample well by centrifugation at 4500 RPM (~2550 x g) for 10 min; 125 µL of the supernatant was then transferred to a fresh plate for LC-MS/MS analysis.

Instrumentation. A 10 µL volume of eluate was injected onto an Acquity UHPLC system (Waters, Corp.) and the selected peptides were separated onto a Phenomenex Aeris C18 column (2.1x 100mm, 1.7 µm) at 70°C and detected with a Sciex QTRAP® 5500 mass spectrometer (AB Sciex, Foster City, CA) operated in the electrospray positive ion MRM mode**.** The ion transitions of the selected surrogate peptides are listed in Table 1. The mobile phases were: (A) 0.1% formic acid in acetonitrile/water (5/95, v/v) and (B) 0.1% formic acid in acetonitrile /water (95/5, v/v). The LC gradient in min/% of mobile phase B was: 0.00/5, 1.00/5, 3.00/35, 3.10/95, 4.60/95, 4.70/5 and 5.00/5. The flow rate was 0.500 mL/min and the run time was 5.00 min.

Table 1. Surrogate Peptide Sequences and MRM Ion Transitions.

Analyte	**Surrogate Peptide**	**Q1/Q3 MRM**
Total aDNP E384C Ab	VVSVLTVLHQDWLNGK	603.5/805.5
Internal Standard for total Ab	VVSV***L**TVLHQDW***L**NGK	608.0/812.4
Conjugate **5**	DCLGAFR	419.8/450.2
WT GpTx-1 peptide (Internal Standard for conjugate **5**)	DCLG**FM**R	449.7/510.2

*denotes [$^{13}C_6$,^{15}N]-Leu

Peptide **22.** The PK study was conducted with 7 week-old unmodified CD-I mice from Taconic Biosciences (Oxnard, CA). [Ala5]GpTx-I (**22**) in saline was dosed at 5 mg/kg subcutaneously. At 0.5, 1, 1.25, 1.5,2,3, and 4 hours post-dose, 3 mice were euthanized and blood samples were collected in EDTA-treated microtainers and centrifuged at 13000 rpm for 5 minutes, after which plasma was extracted and frozen at -80°C in a 96-well plate.

Peptide **22** LC-MS/MS Bioanalysis Method. Preparation of peptide **22** calibration standards and working internal standard (WIS) solution.

Calibration standards in the range of 5.00 – 5,000 ng/mL were prepared by spiking a 100 µg/mL of peptide **22** working stock solution (in methanol/water (50/50, v/v) into mouse plasma, followed by serial dilution. A 100 ng/mL WIS solution containing WT GpTx-1 peptide was prepared in methanol/water (30/70, v/v).

To a 96 deep-well plate, 25 µL of plasma sample, 25 µL of WIS solution and 50 µL of 0.1 M trifluoroacetic acid were added and briefly vortexed. The sample pretreatment mixture then underwent solid phase extraction using an Oasis HLB 96-well µElution plate. The eluate was collected to a fresh plate for LC-MS/MS analysis.

Instrumentation. A 10 µL volume of eluate was injected onto an Acquity UHPLC system and the selected peptides were separated onto a Waters BEH C18 column (1.0 x 50 mm, 1.7 µm) at 70°C and detected with a Sciex QTRAP® 5500 mass spectrometer operated in the electrospray positive ion MRM mode**.** The ion transitions of the GpTx-1 peptides are listed in Table 2. The mobile phases were: (A) 0.1% formic acid in acetonitrile/water (5/95, v/v) and (B) 0.1% formic acid in acetonitrile /water (95/5, v/v). The LC gradient in min/% of mobile phase B was: 0.00/2,

0.60/2, 1.60/50, 1.70/95, 2.20/95, 2.30/2 and 2.50/2. The flow rate was 0.250 mL/min and the run time was 2.50 min.

Table 2. GpTx-1 Peptide MRM Ion Transitions.

Analyte	**MW**	**Q1/Q3 MRM**
Peptide **22**	3997.76 Da	667.1/659.7
WT GpTx-1 peptide (Internal Standard)	4073.96 Da	679.7/672.3

Quantitative and PK Analysis . A universal surrogate peptide with sequence VVSVLTVLHQDWLNGK found in the Fc region of human mAb drug candidates that contain human immunoglobulin G1 (IgG1) and (IgG4) was used for total aDNP human IgG1 antibody quantitation with stable isotope labeled surrogate peptide VVSV***L**TVLHQDW***L**NGK as the internal standard. The unique surrogate peptide DCLGAFR was used for intact conjugate **5** quantitation, with surrogate WT GpTx-1 peptide as the internal standard. The internal standard for peptide **22** quantitation was WT GpTx-1 peptide. All data was collected and processed using AB Sciex Analyst® software (version 1.5.1). Concentrations of conjugate **5**, total antibody (aDNP E384C antibody) and peptide **22** were calculated from calibration curves derived from the peak area ratios (selected peptide/internal standard) using $1/x^2$ weighted linear least-squares regression. Concentrations and PK analysis were both calculated utilizing Watson LIMS™ software.

Imaging studies.

Animals, **dosing, and tissue collection.** Experimental procedures were approved by Amgen's Institutional Animal Care and Use Committee in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and conducted at an AALAC-accredited facility. No animals were excluded from analyses and randomization was not employed. Male CD-1 mice were obtained from Charles River Laboratories and used at 3 months of age. Male and female $Na_V1.7$ wild-type and knockout mice on a mixed C57Bl6-CD1 background were bred and cared for as previously described (24) and used at 4-5 months of age. Animals were housed at 22–24°C with a 12-hour light/dark cycle, allowed to acclimate for at least 7 days before experiments and given *ad libitum* access to fresh water and food. Two separate mice for each time point were injected via the tail vein with 20 mpk conjugate **5** or parent Ab **3** respectively in A5Su buffer. Mice were anesthetized by intraperitoneal injection of 100 mg/kg Fatal Plus (Western Medical Supply, Cat# 6030) 7 days after compound injection and transcardially perfusion-fixed with phosphate buffer saline (PBS;Invitrogen, Cat# 14190) followed by 4% paraformaldehyde in PBS (Affymetrix, Cat# 19943). Sciatic nerves and L5 dorsal roots were left *in situ* for 2 hours, dissected, placed in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose (Sigma, Cat# 57930) in PBS for 24 hours at 4ºC. 12 μm tissue sections were cut on a cryostat (Leica Biosystems, CM3050S) and mounted on Superfrost Plus microscope slides (Fisher Scientific, Cat# 22-037-246).

Immunohistochemistry. Sections were washed in PBS and non-specific binding was blocked using 3% normal goat serum (Vector Lab, Cat# S-1000) in 0.3% Triton X-100/PBS (Sigma, Cat#

X-100-500mL) at room temperature (RT) for one hour. Sections were then incubated with Fluorescence Signal Enhancer (MaxVision Biosciences, MaxFluor Cat# MF01-M) for 30 min, washed three times in PBS (5 min per wash) followed by incubation of 1:500 Alexa Fluor 488 goat anti-human IgG (Invitrogen, Cat# A-11013) at RT for one hour in 1% NGS/0.1% Triton X-100/PBS. Following PBS washes as above, sections were incubated in 1:1000 rabbit polyclonal anti-PGP9.5 (AbD Serotec, Cat# 7863-0504) or 1:300 rabbit polyclonal anti-peripherin (Sigma, Cat# SAB4502419) in blocking solution at 4 ºC overnight. After washing with PBS as above, sections were incubated in 1:500 Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, Cat# A-21428) in 1% NGS/0.1 % Triton-X-100, washed with PBS as above and coversliped with Vectashield mounting medium containing DAPI (Vector Lab, Cat# H-1200). Sections were imaged using a Zeiss Axioimager M2 microscope with AxioVision software 4.8.1 using identical exposure parameters between slides. Three separate techinical replicates were conducted for each staining condition with 3-5 tissue sections per dorsal root or sciatic nerve sample. Experimentalist was blinded to sample identities until completion of all staining procedures and analyses. Control experiments included imaging dorsal root and sciatic nerve sections from mice injected with conjugate **5** without Alexa Fluor 488 goat anti-human IgG, or staining dorsal root and sciatic nerve sections from mice receiving no conjugate injection with Alexa Fluor 488 goat anti-human IgG. In both cases, no nerve labeling was observed (Figure S12). The specificity of rabbit anti-PGP9.5 (Figure S13) and rabbit anti-peripherin (Figure S14) staining were assessed by replacing these primary antibodies with rabbit IgG or no primary antibody. In both cases, no nerve labeling was observed.

SI FIGURES

S1A. The amino acid sequence of human IgG heavy chain (variable region underlined; the enigneered cysteine linkage site **E384C** in the C_H2 domain in red font):

QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVIWYDGSNKYYADSVKGRF
TISRDNSKNTLYLQMNSLRAEDTAVYYCARYNFNYGMDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSG
GTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT
KVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP**C**VKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT
LPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK

The amino acid sequence of human IgG kappa light chain (variable region underlined):

DIQMTQSPSSVSASVGDRVTITCRASQGISRRLAWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTL
TISSLQPEDFATYYCQQANSFPFTFGPGTKVDIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

S1B

Modified AHo numbering for Cys mutants	Corresponding EU numbering
D88C	D70C
V152C	V109C
A154C	A111C
E99C	E89C
A150C	A118C
S156C	S124C
E189C	E152C
D377C	V265C
E384C	E272C
Y421C	Y300C
E473C	E345C
T487C	T359C
N526C	N390C

Figure S1. (A) Sequence of E384C cysteine-engineered anti-2,4-dinitrophenol huIG1 antibody used for conjugate **5**. Residues are numbered using a modified version of the AHo numbering system, expanding the structurally-based numbering system developed by Annemarie Hoeneggar for Ig variable domains[1] across the constant domains. (B) Corresponding EU numbering of each site

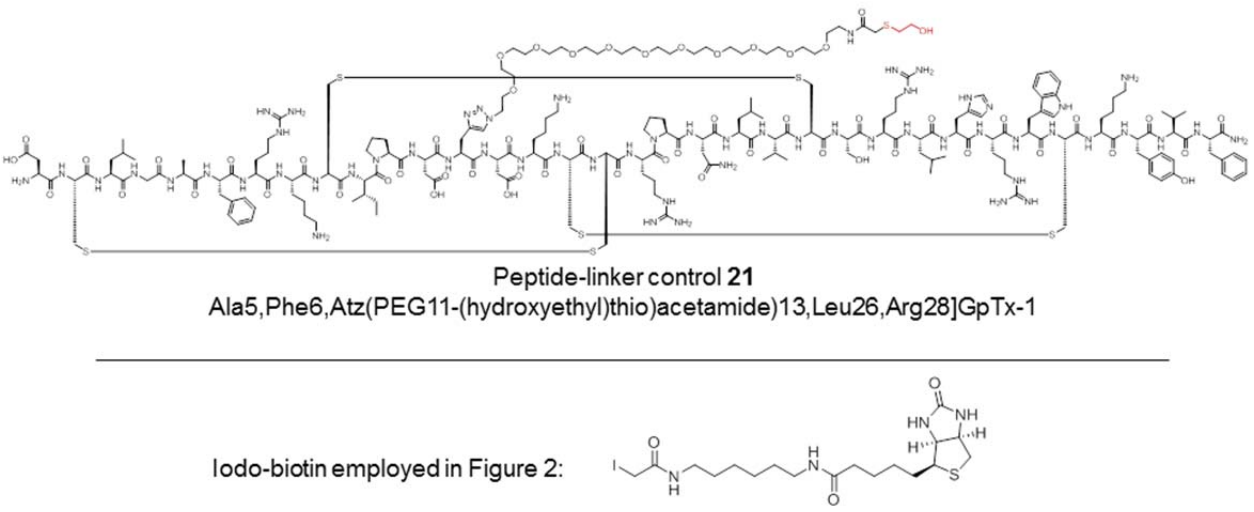


Figure S2. Peptide linker control **21** and iodo-biotin reagent.

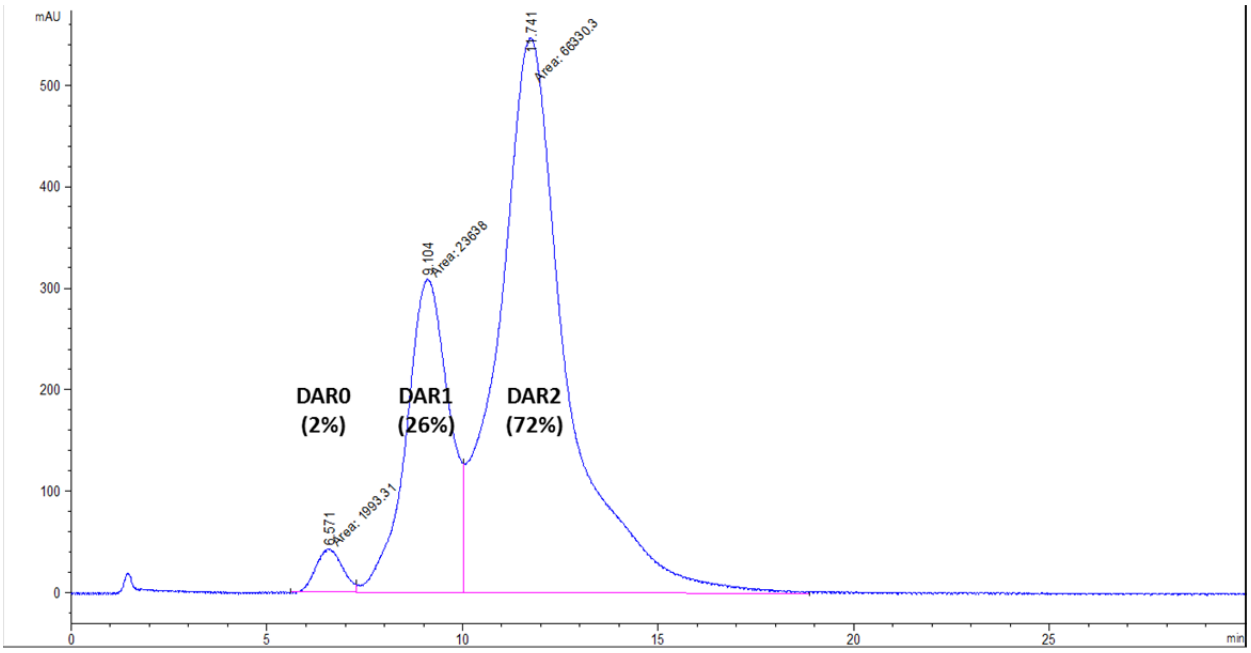


Figure S3. Conjugate reaction of peptide-linker **2** and E384C antibody **4** by the partial reduction protocol as purified by cation exchange chromatography. The peaks at 6.5 min correspond to unreacted Ab (Drug Antibody Ratio, DAR = 0), 9.1 min to the DAR1 species and 11.7 min to the desired DAR2 product, respectively.

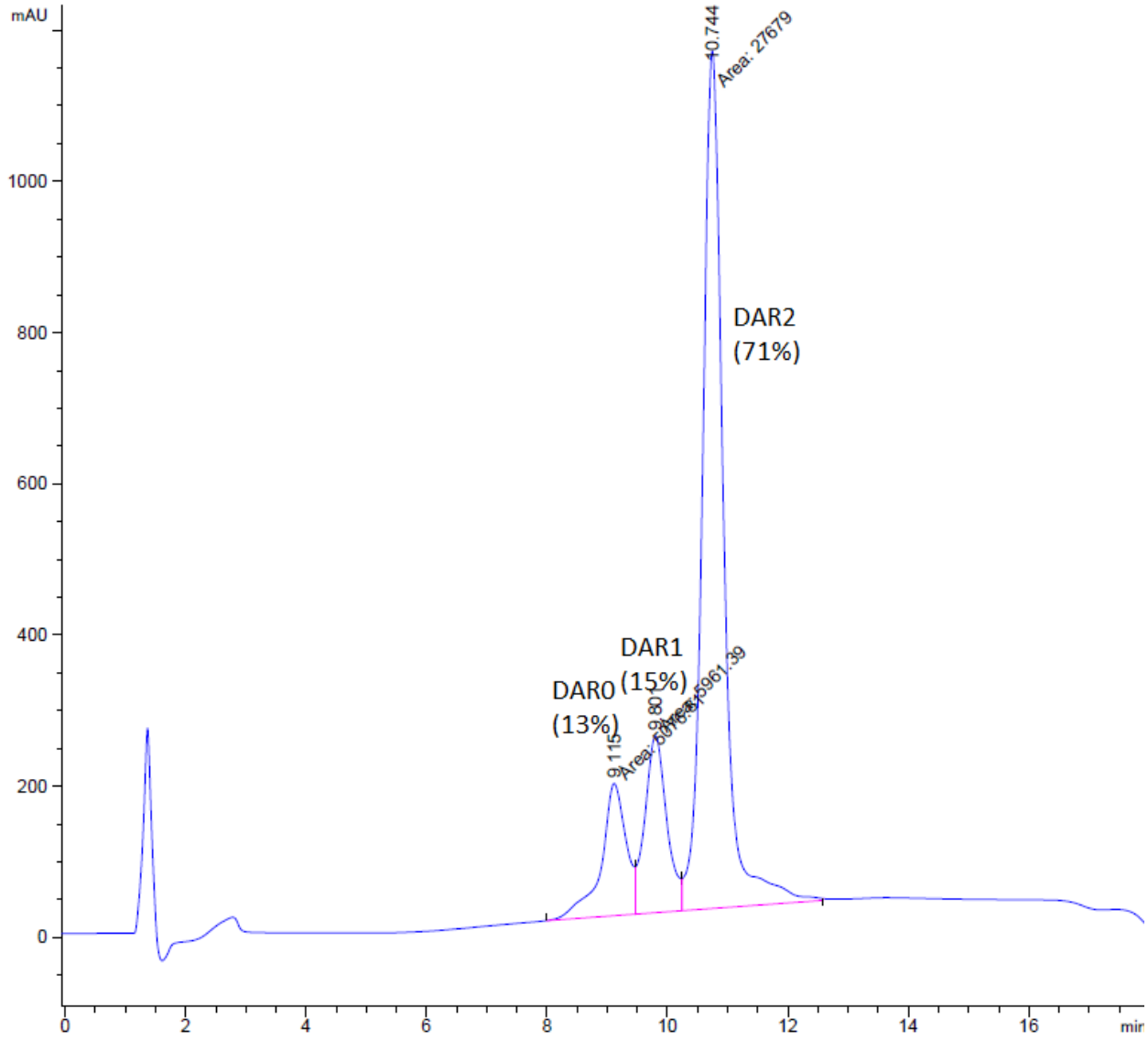


Figure S4. Conjugate reaction of peptide-linker **2** and E384C antibody **4** by the redox protocol as analyzed by hydrophobic interaction chromatography. The peaks at 9.1 min correspond to unreacted Ab (DAR0), 9.8 min to the DAR1 species and 10.7 min to the desired DAR2 product, respectively.

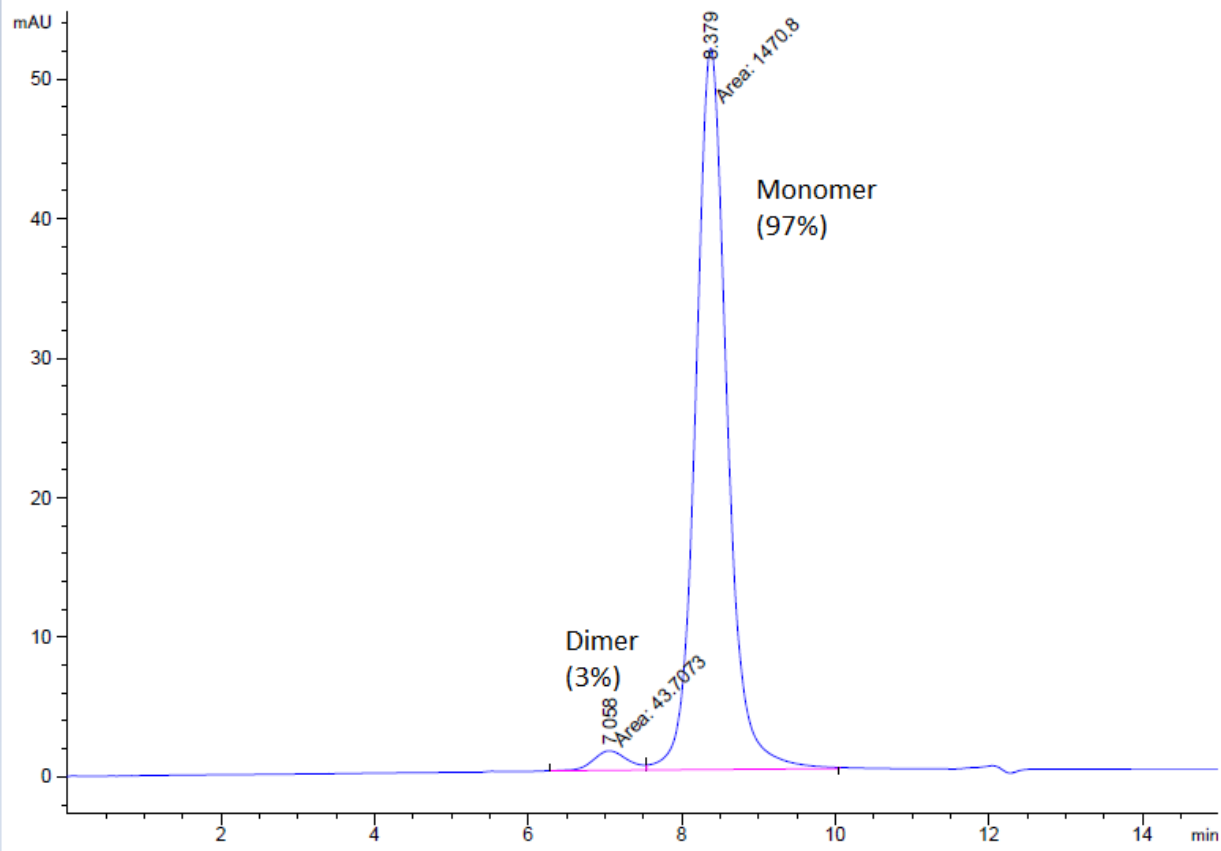
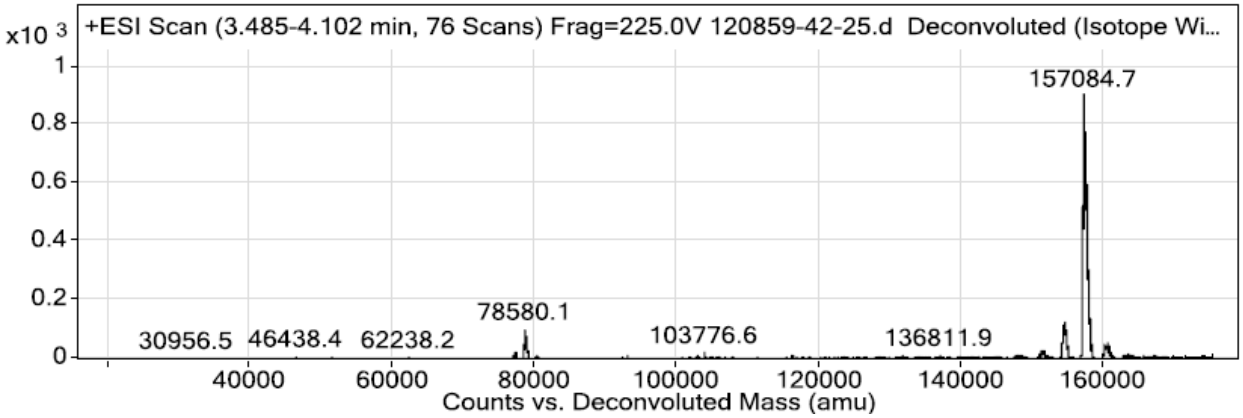
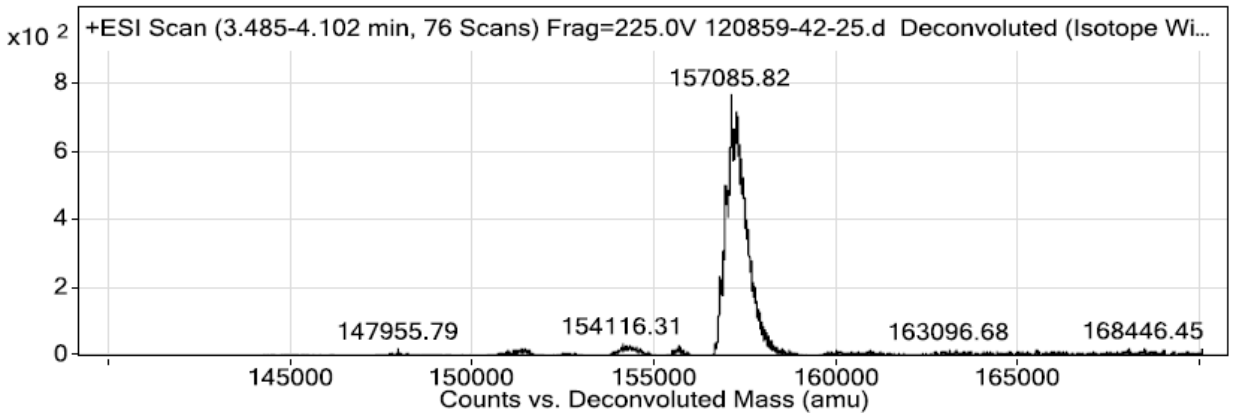


Figure S5. Size-exclusion chromatography of purified E384C conjugate **5**. The major peak corresponds to desired conjugate in 97% purity.

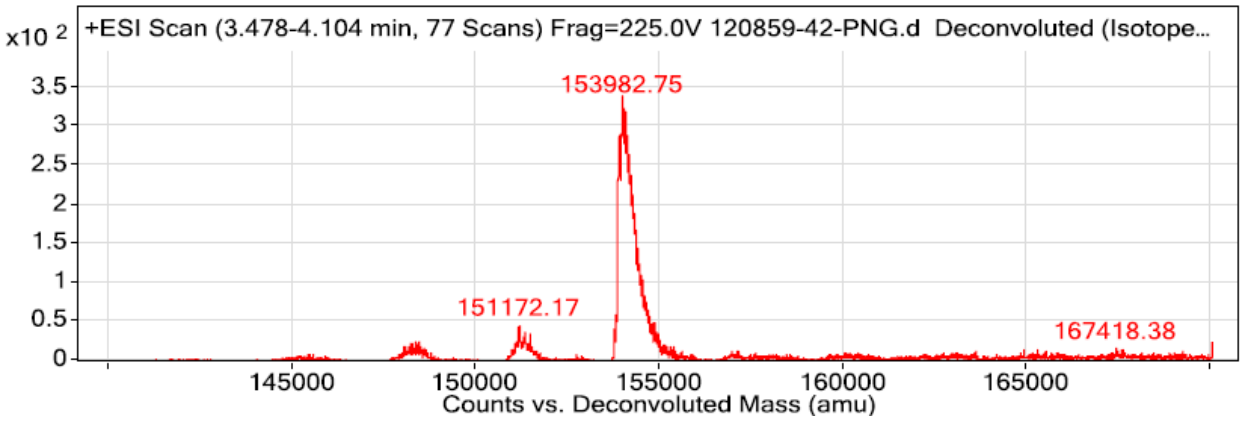
S6A



S6B



S6C



S6D

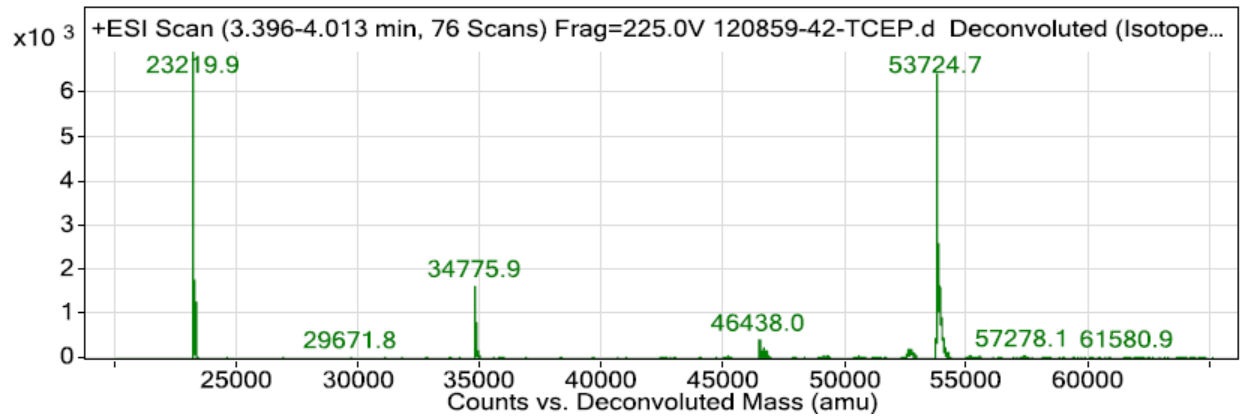


Figure S6. LC-TOF-MS of purified E384C conjugate **5**. (A) Fully intact conjugate (DAR2) MW observed: 157086, calc. 156756. (B) Expansion of conjugate **5** peak. (C) Conjugate **5** deglycosylated with PNGase (DAR2) MW observed: 153983, calc 153866. (D) Conjugate **5**

deglycosylated with PNGase and (d) fully reduced with TCEP (Heavy chain + 1) MW observed: 53725, calc: 53722.

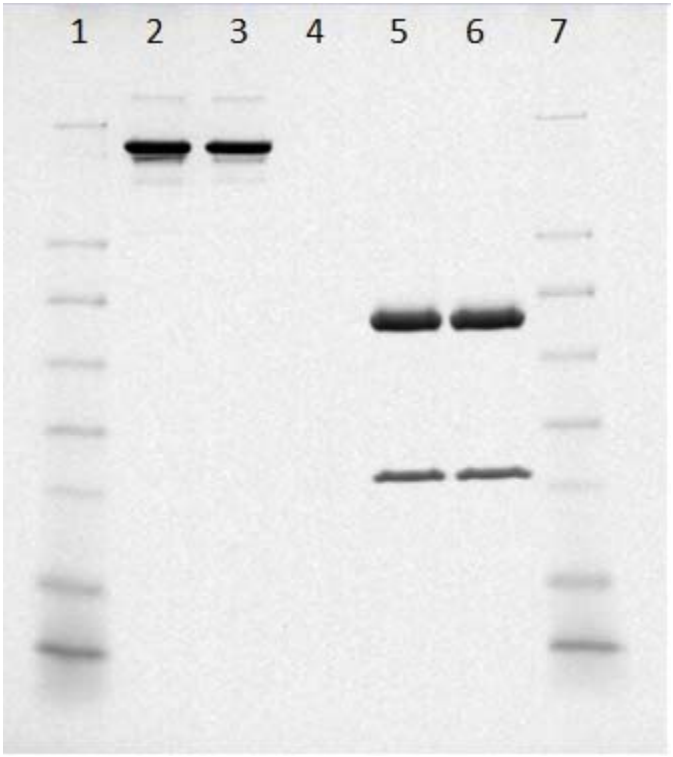
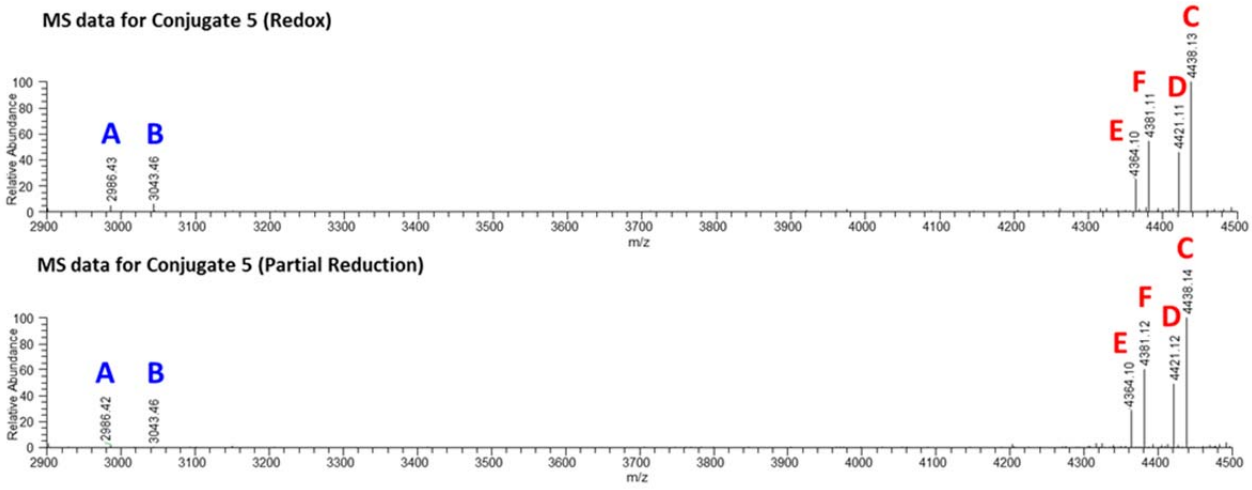


Figure S7. SDS-PAGE analysis of conjugate **5**. Lanes 1, 7: See-Blue Prestained protein ladder, Lanes 2, 3: Conjugate **5**, Lane 4: blank, Lanes 5, 6: Conjugate **5** reduced with DTT.

S8A

Peptide Category	Key	mAb Peptide/NAV 1.7 Peptide Description	Mass (Da)
Non-Conjugated mAb Peptides	A	DTLMISRTPEVTC$_{373}$*VVVDVSHEDPC$_{384}$*VK (mAb peptide w/ E384C)	2986.43
	B	DTLM*ISRTPEVTC$_{373}$*VVVDVSHEDPC$_{384}$*VK (mAb peptide w/ E384C)	3043.46
Conjugated mAb-NAV1.7 Peptides	C	C*IPD[Atz]DK (NAV1.7 peptide) | DTLM*ISRTPEVTC$_{373}$*VVVDVSHEDPC$_{384}$VK (mAb peptide with E384C)	4438.12
	D	C*IPD[Atz]DK (NAV1.7 peptide) | DTLM*ISRTPEVTC$_{373}$*VVVDVSHEDPC$_{384}$VK (mAb peptide w/ E384C)	4421.11
	E	C*IPD[Atz]DK (NAV1.7 peptide) | DTLMISRTPEVTC$_{373}$*VVVDVSHEDPC$_{384}$VK (mAb peptide w/ E384C)	4364.10
	F	C*IPD[Atz]DK (NAV1.7 peptide) | DTLMISRTPEVTC$_{373}$*VVVDVSHEDPC$_{384}$VK (mAb peptide w/ E384C)	4381.12

S8B



S8C

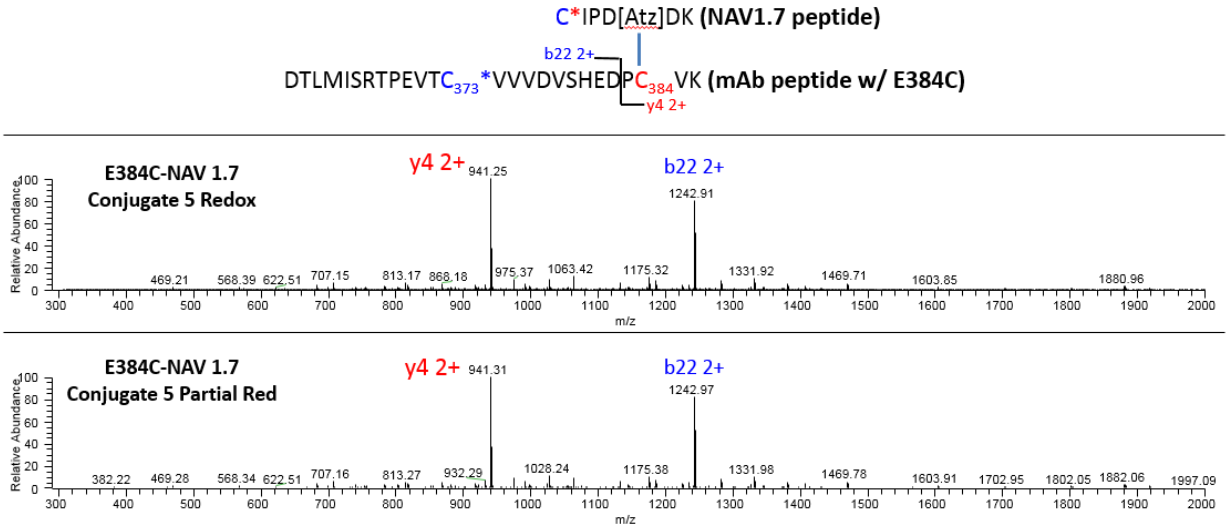


Figure. S8. Summary of peptide mapping data for Conjugate **5** prepared using redox and partial reduction methods. Briefly, conjugation was only observed on engineered E384C in the heavy chain of the anti-DNP Ab. No significant difference was observed in the MS data between redox and partial reduction methods for alkylation at this site of conjugation. (A) Various peptide structures describe non-conjugated Ab peptide (A, B) and conjugated forms of the $Na_V1.7$ inhibitory peptide conjugated to the anti-DNP Ab (C-F). Asterisks on cysteines and a methionine indicate alkylation products from iodoacetimide used to prevent disulfide formation during proteolytic digestion (*carboxyamidomethylation, * S-carbamoylmethylcysteine). (B) Observed masses for the non-conjugated and conjugated peptide structures, A-F. (C) The LC-MS/MS spectra (m/z=1092.28, 4+ ion) for Conjugate **5** preparations confirming conjugation to C_{384} and not C_{373}. The b22 $^{2+}$ ion matches the expected peptide fragment ion for the N-terminal piece of the mAb peptide with alkylated C_{373}, and the corresponding y4 $^{2+}$ ion fragment includes the C-terminal conjugated $Na_V1.7$ inhibitory peptide.

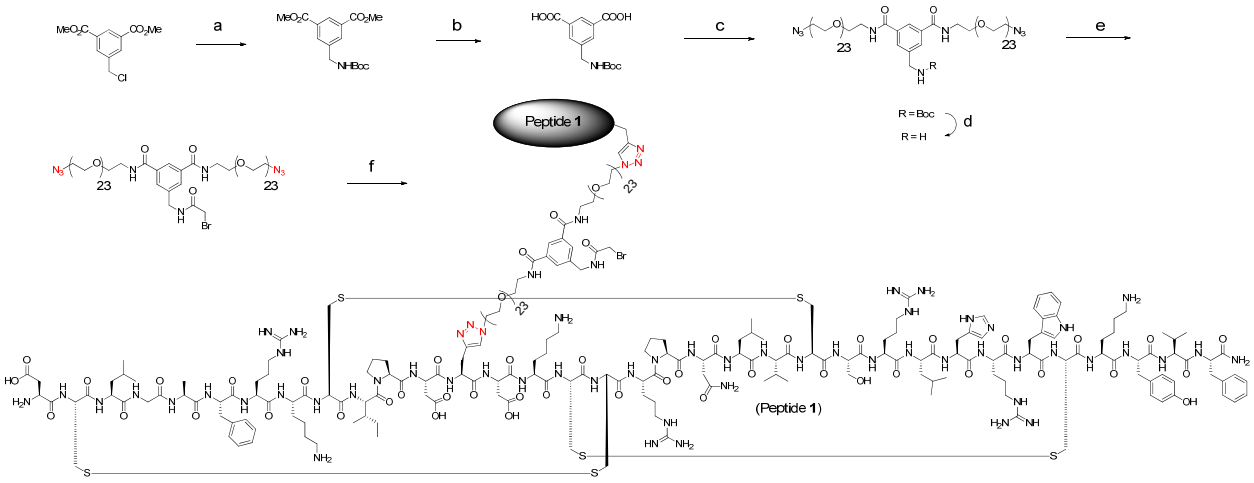


Figure S9. Scheme for synthesis of dimeric linker-peptide for making conjugates **10** and **11**. (a) NaN_3, acetone:water (3:1), reflux, 91%; *n*-Bu_3P, diethyl ether, then di-*t*-butyl-di-carbonate, -50 °C, 75%, 2 steps; (b) LiOH, MeOH, 45 °C, 75%; (c) 1-chloro-N,N,2-trimethylprop-1-en-1-amine, DCM, then azido-PEG_{23}-amine, 54%; (d) HCl (4.0M in dioxane), 90%; (e) bromoacetyl bromide, di-isopropylethylamine, DCM, 58%; (f) Peptide **1**, TBTA (Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine), sodium ascorbate, $CuSO_4$, water, 44%.

Table S1. Characterization data for peptide-linkers and conjugates **5-20**

Compd	Site	Linker	Peptide Load	Peptide-linker Calc. MW	Peptide-linker Observed ESI-MS MW	Conjugate Observed LC-TOF MW	Conjgate Purity by SEC[b]
5	E384C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	157085	97%
6	E384C[a]	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	63346	99%
7	E384C	PEG_{11}	1	4726.3	1576.3 $(M+3H)^{3+}$	152620	93%
8	E384C	PEG_3	2	4373.9	1458.7 $(M+3H)^{3+}$	156383	96%
9	E384C	PEG_{23}	2	5255.0	1752.2 $(M+3H)^{3+}$	158147	96%
10	E384C	Bis-PEG_{23}	2	10548.2	1759.2 $(M+6H)^{6+}$	158443	99%
11	E384C	Bis-PEG_{23}	4	10548.2	1759.2 $(M+6H)^{6+}$	168627	99%
12	N526C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	156793	99%
13	T487C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	157017	95%
14	E99C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	156761	99%
15	D88C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	156989	94%
16	S156C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	157045	98%
17	A154C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	157074	98%
18	E473C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	156971	97%
19	E189C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	156965	98%
20	D377C	PEG_{11}	2	4726.3	1576.3 $(M+3H)^{3+}$	157436	97%

[a]Fc version of E384C Ab; [b]SEC (size-exclusion chromatography) method: Tosoh QC-PAK GFC 300 7.8 mm x 15 cm column on Agilent 1260 bio-inert LC with quaternary pump and degasser, mobile phase 0.17 M potassium phosphate, 0.21 M KCl, 15% (v/v) IPA, pH 7.0 at flow rate 0.5 mL/min for 15 min.

Table S2. Pharmacokinetic parameters for data shown in Figure 5.

Compound	Dose (mpk)	$t_{1/2}$ (hr)	AUC (ng*hr/mL)	CL/F (L/hr/kg)	C_{max} (ng/mL)	T_{max} (hr)
Conjugate **5**	10	80	512160	0.0177	6082.6	24
Peptide **22**	5	0.605	8275	0.587	4157.6	1.25

$t_{1/2}$ = half-life, AUC = total area under the plasma drug concentration-time curve, CL/F = Apparent total clearance of the drug from plasma, T_{max} = (observed) time after drug administration at which peak plasma concentration occurs

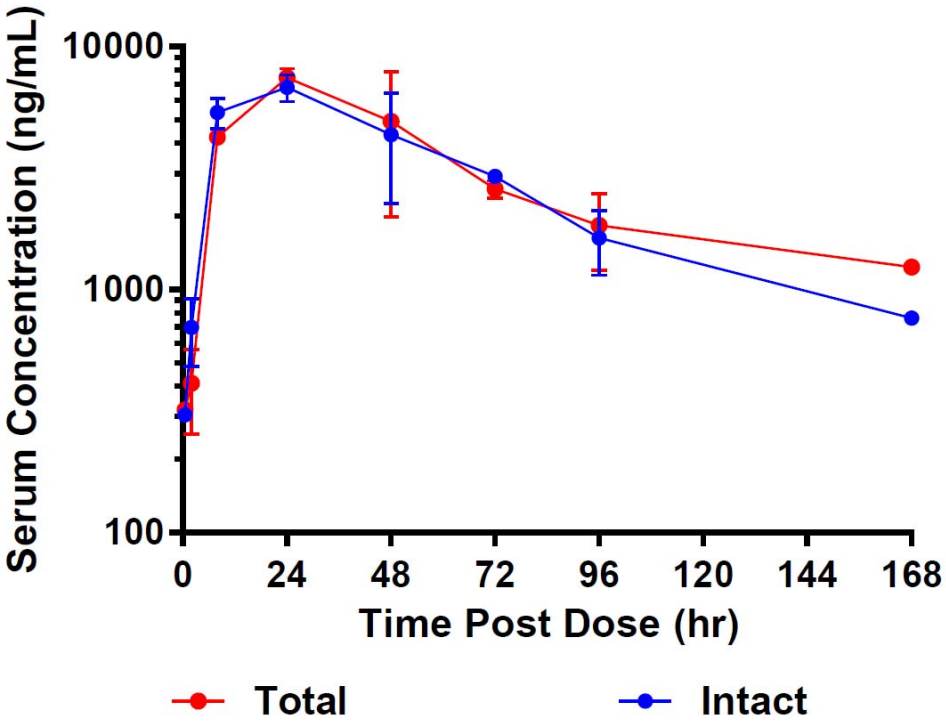


Figure S10. Concentration-time profile of total and intact conjugate **5** observed in the mouse pharmacokinetic study over a 7 day period post administration (10 mg/kg, s.c.). The intact/total ratio on day 7 = 0.62.

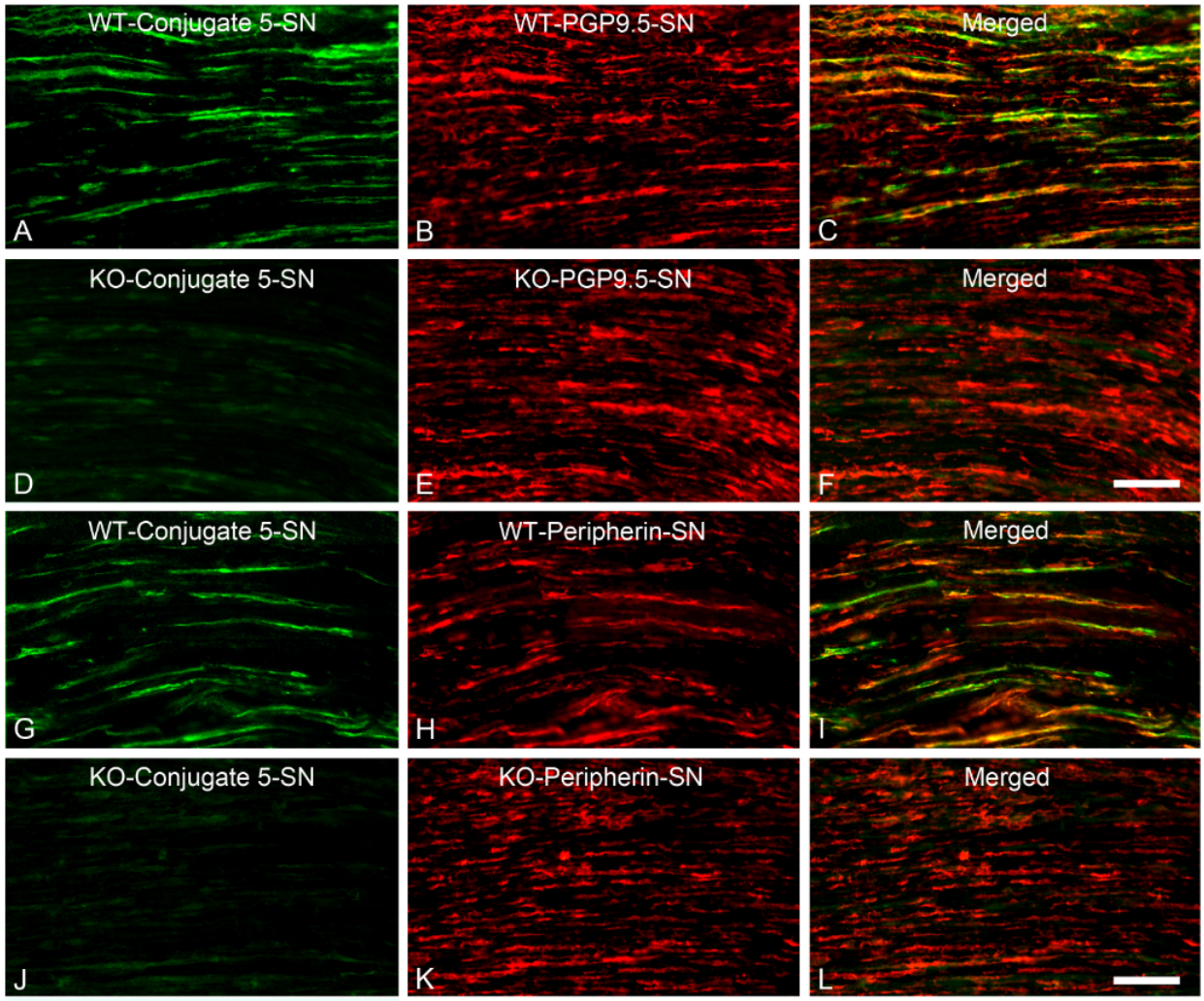


Figure S11. $Na_V1.7$ peptide-antibody conjugate labels neuronal processes. Conjugate **5** immunoreactivity co-localizes with the neuronal marker PGP9.5 in sciatic nerve SN (A-C) of WT but not SN (D-F) of $Na_V1.7$ KO mice. Conjugate **5** immunoreactivity co-localizes with peripherin, a C-fiber marker, in SN (G-I) of WT but not SN (J-L) of $Na_V1.7$ KO mice. Left panels denote conjugate **5** staining in green, middle panels denote PGP9.5 or peripherin staining in red, and right panels overlay green and red signals with yellow color indicating co-localization in saggital sections. Scale bars are 50 microns.

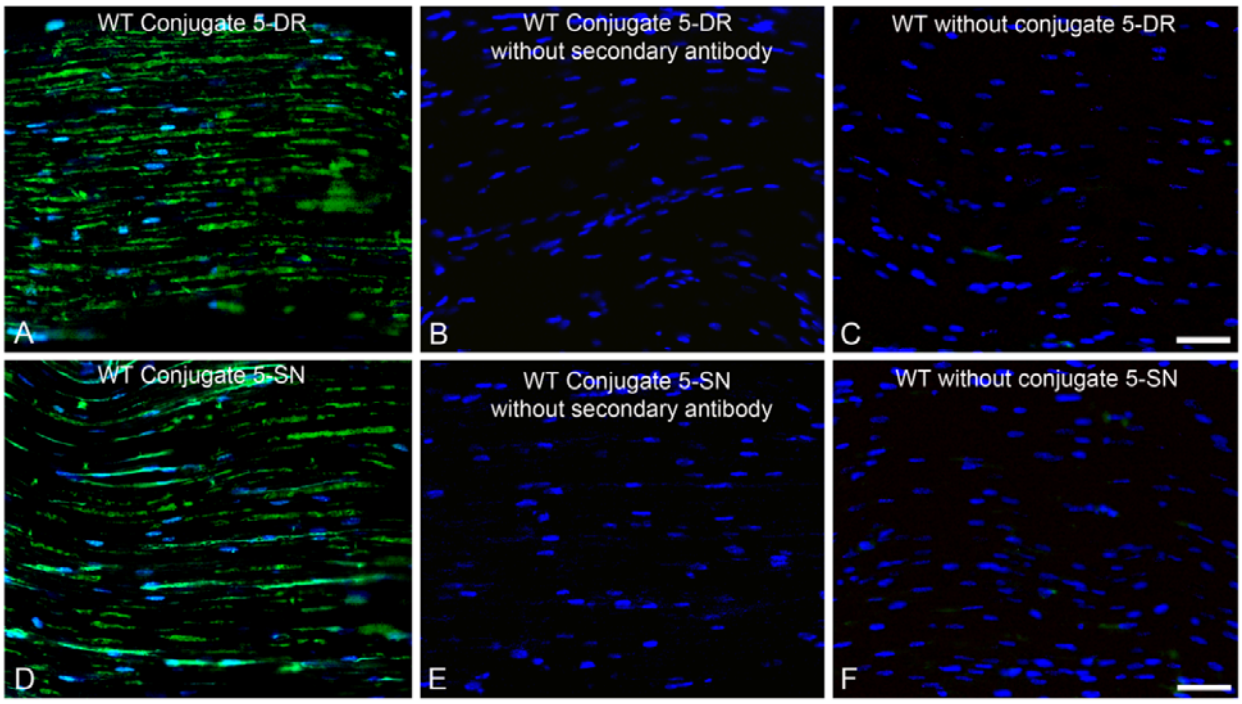


Figure S12. Controls showing conjugate **5** biodistribution to nerves. (A) Dorsal root (DR) or (D) sciatic nerve (SN) sections from WT mice injected with 20 mpk conjugate **5** and detected with Alexa Fluor 488 goat anti-human IgG. (B) DR or (E) SN sections from WT mice injected with 20 mpk conjugate **5** without Alexa Fluor 488 goat anti-human IgG. No nerve labeling was observed. (C) DR or (F) SN sections from WT mice with no conjugate **5** injected and stained with Alexa Fluor 488 goat anti-human IgG. No nerve labeling was observed. Green denotes conjugate **5** staining, and blue denotes cell nuclei staining in sagittal sections. Scale bars are 50 microns.

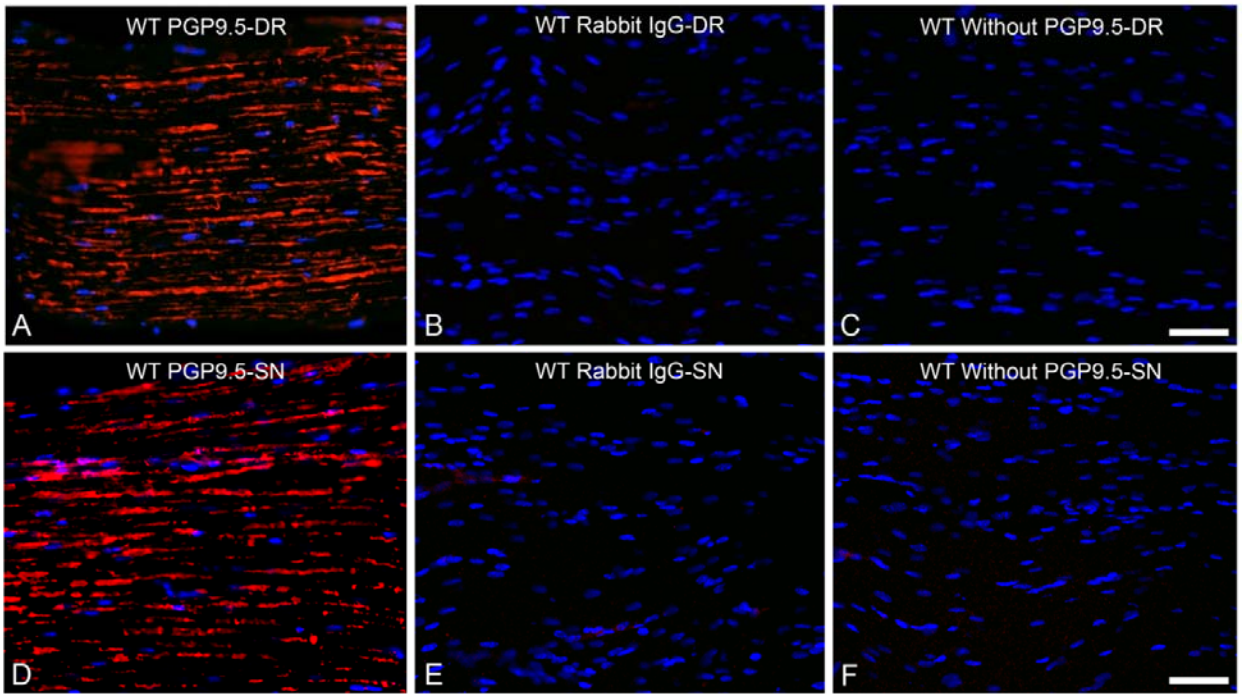


Figure S13. Controls showing specificity of PGP9.5 labeling. (A) Dorsal root (DR) or (D) sciatic nerve (SN) sections from WT mice stained with 1:500 rabbit anti-PGP9.5. (B) DR or (E) SN sections from WT mice stained with 1:500 rabbit IgG. No nerve labeling was observed. (C) DR or (F) SN sections from WT mice with no PGP9.5 primary antibody. No nerve labeling was observed. Red denotes PGP9.5 staining, and blue denotes cell nuclei staining in sagittal sections. Scale bars are 50 microns.

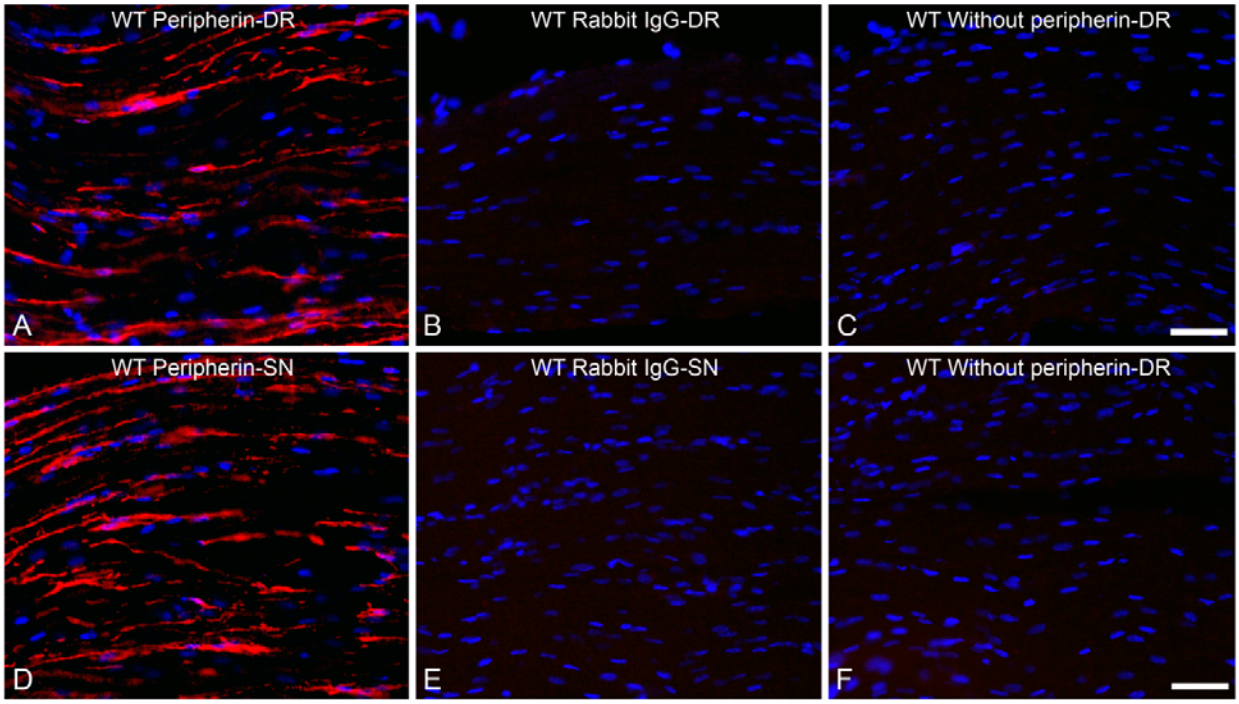


Figure S14. Controls showing specificity of peripherin labeling. (A) Dorsal root (DR) or (D) sciatic nerve (SN) sections from WT mice stained with 1:300 rabbit anti-peripherin. (B) DR or (E) SN sections from WT mice stained with 1:300 rabbit IgG. No nerve labeling was observed. (C) DR or (F) SN sections from WT mice with no peripherin primary antibody. No nerve labeling was observed. Red denotes peripherin staining, and blue denotes cell nuclei staining in sagittal sections. Scale bars are 50 microns.

SI References

1. Honegger, A and Plückthun, A. (2001) Yet another numbering scheme for immunoglobulin variable domains: An automatic modeling and analysis tool. *J. Mol. Biol*. *309*, 657-670.