SUPPORTING INFORMATION

Evaluation of a Hapten Conjugate Vaccine Against the “Zombie Drug” Xylazine

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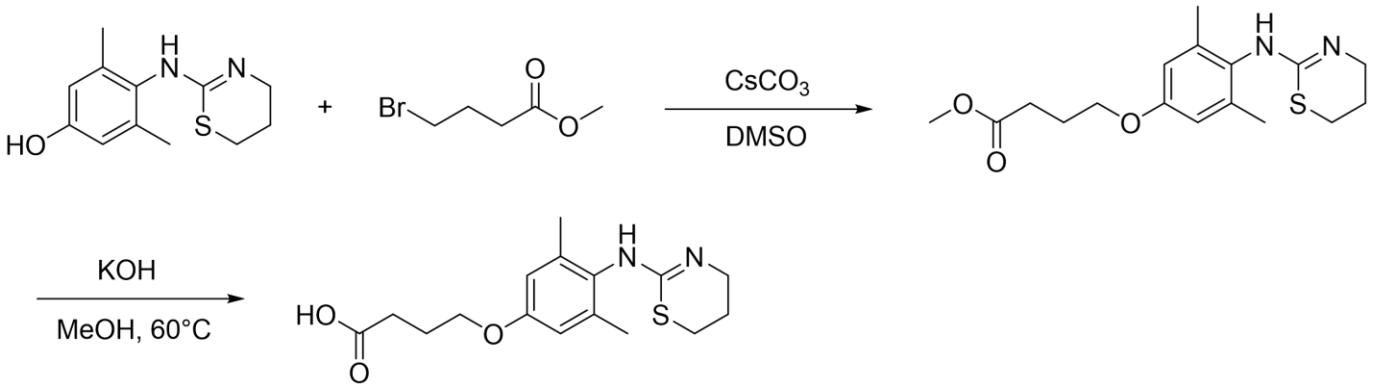
I. Chemicals and instruments

4-Hydroxy xylazine was purchased from Expert Synthesis Solutions (ESS0030), xylazine hydrochloride from TCI Chemicals and xylazine-d_6 from Cayman Chemical Company. All other chemicals and reagents were purchased from Aldrich and Combi-Block and used without further purification, unless otherwise stated. All solvents were American Chemical Society (ACS) grade or better and used without further purification. Analytical thin layer chromatography (TLC) was performed with glass backed silica gel (0.25 mM thick, 60 Å) plates purchased from Sigma and visualized under UV irradiation at 254 nm and/or by staining with potassium permanganate solution followed by heating. Flash automated column chromatography was performed using a CombiFlash Rf + Luman (Teledyne Isco) purification system with flash silica RediSep Rf columns for normal phase (NP) or RediSep Rf Gold C_{18} HP columns for reverse phase (RP). Analytical LCMS was performed on an Agilent ESI-ToF (LC/MSD ToF) with an Agilent Zorbax 300SB-C8 (4.6 x 50 mm), 5 µm column at a flow rate of 0.5 mL/min (Solvent A: 0.1% formic acid in H_2O, Solvent B: 0.1% formic acid in acetonitrile) for ten minutes (0-6 min: 5-95% Solvent B, 6-10 min: 95% Solvent B). High resolution mass spectra (HRMS) were obtained in the Scripps Centre of Mass Spectrometry. HPLC spectra were recorded on an Agilent Systems 1260 using a Poroshell 120 EC-C8 column.

II. Hapten synthesis



Scheme S1. Xylazine hapten synthesis.

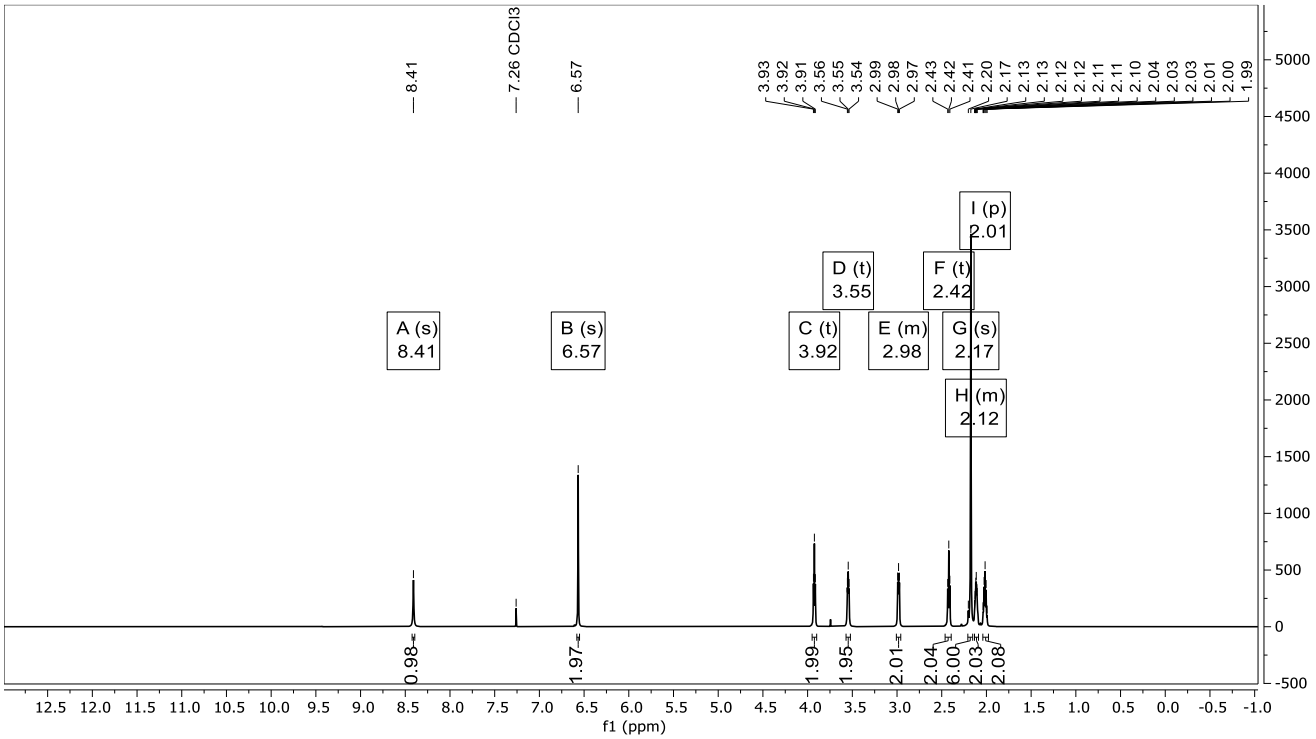
A solution was prepared by combining 50 mg of 4-hydroxy xylazine and 100 mg of $CsCO_3$ (3 equivalents) in 4 mL of DMF. To this mixture, methyl 4-bromobutanoate (27 µL, 1.2 equivalents) was added. The reaction proceeded at room temperature for 3 hours. Following standard extraction and wash procedures, flash chromatography purification was carried out using RediSep® Rf Normal-phase Silica Flash Columns (60 Å pore size, 35-70 microns particle size, 230 to 400 mesh) with a gradient of hexane and EtOAc as the mobile phase.

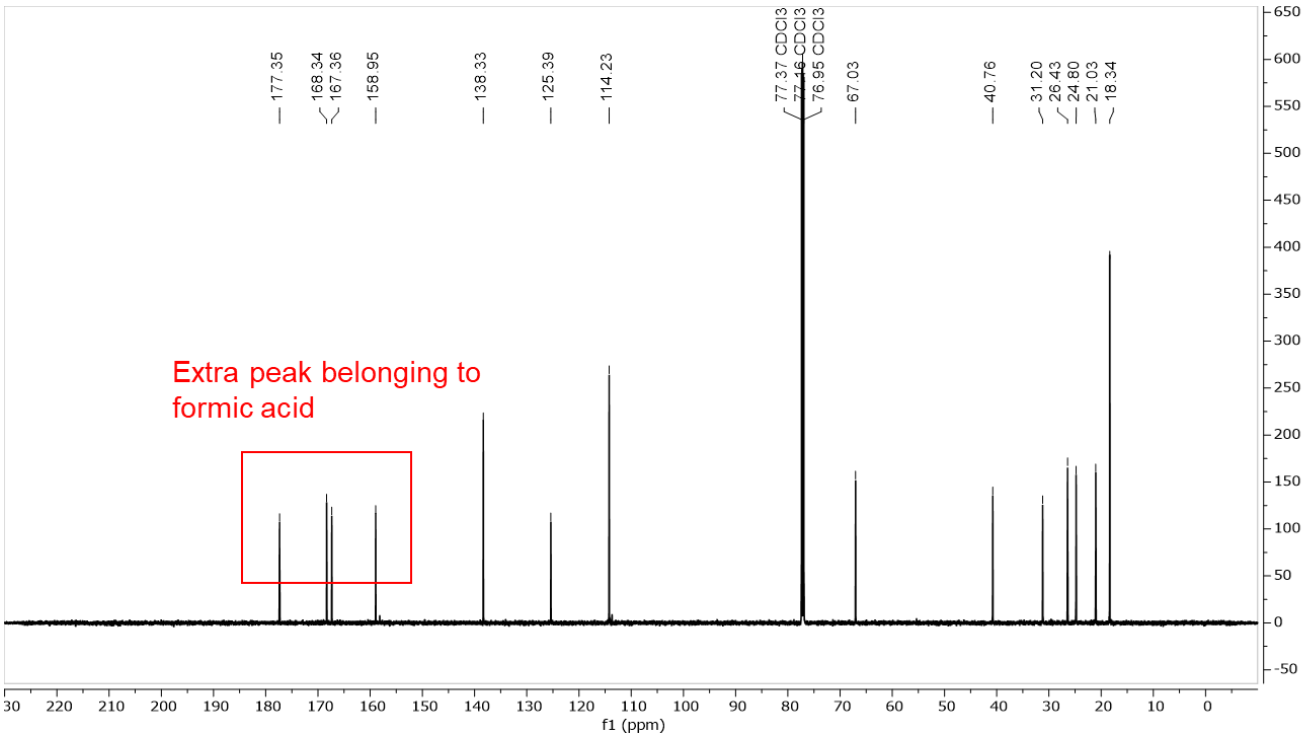
The collected product was dissolved in 5 mL of methanol, and 5 mL of 2M KOH solution was added. The reaction was allowed to continue at 50 degrees overnight. The solvent was then evaporated using a Rotavap, and the residue was loaded onto a Reversed-phase chromatography column (RediSep Rf Gold® Reversed-phase C18) using a gradient of H_2O (0.1% formic acid) and CH_3CN (0.1% formic acid) as the mobile phase. A white solid was obtained with a total yield of 41%.

^{1}H NMR (600 MHz, $CDCl_3$) δ 8.41 (s, 1H), 6.57 (s, 2H), 3.92 (t, *J* = 6.3 Hz, 2H), 3.55 (t, *J* = 5.6 Hz, 2H), 3.01 – 2.96 (m, 2H), 2.42 (t, *J* = 7.2 Hz, 2H), 2.17 (s, 6H), 2.13 – 2.09 (m, 2H), 2.01 (p, *J* = 6.8 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 177.35, 168.34, 167.36, 158.95, 138.33, 125.39, 114.23, 67.03, 40.76, 31.20, 26.43, 24.80, 21.03, 18.34. HRMS (ESI): calcd for $C_{16}H_{22}N_2O_3S$ ($[M+H]^+$), 323.1429, found 323.1428.

III. NMR spectrum

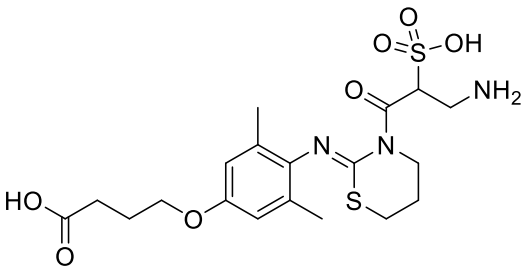
Xylazine hapten:





IV. Bioconjugation

Due to the presence of a nucleophilic amine structure in the molecule, the hapten could not be preactivated in DMF for extended periods. Instead, activation was achieved by mixing the hapten with EDC/NHS-sulfo in H_2O for 15 minutes before adding the carrier protein solution. The reaction then proceeded at room temperature for 2 hours. Mass studies revealed the occurrence of a side reaction between the hapten and NHS in the presence of EDC as a result of an elongated activation, resulting in the formation of the Lossen rearrangement side product, as depicted below. The resulting amine could further react with another NHS molecule, leading to the formation of a series of polymers at higher EDC and NHS concentrations and longer incubation times. This phenomenon is due to the activation effect of formic acid on the thiourea. If purified without formic acid, this phenomenon was not observed.

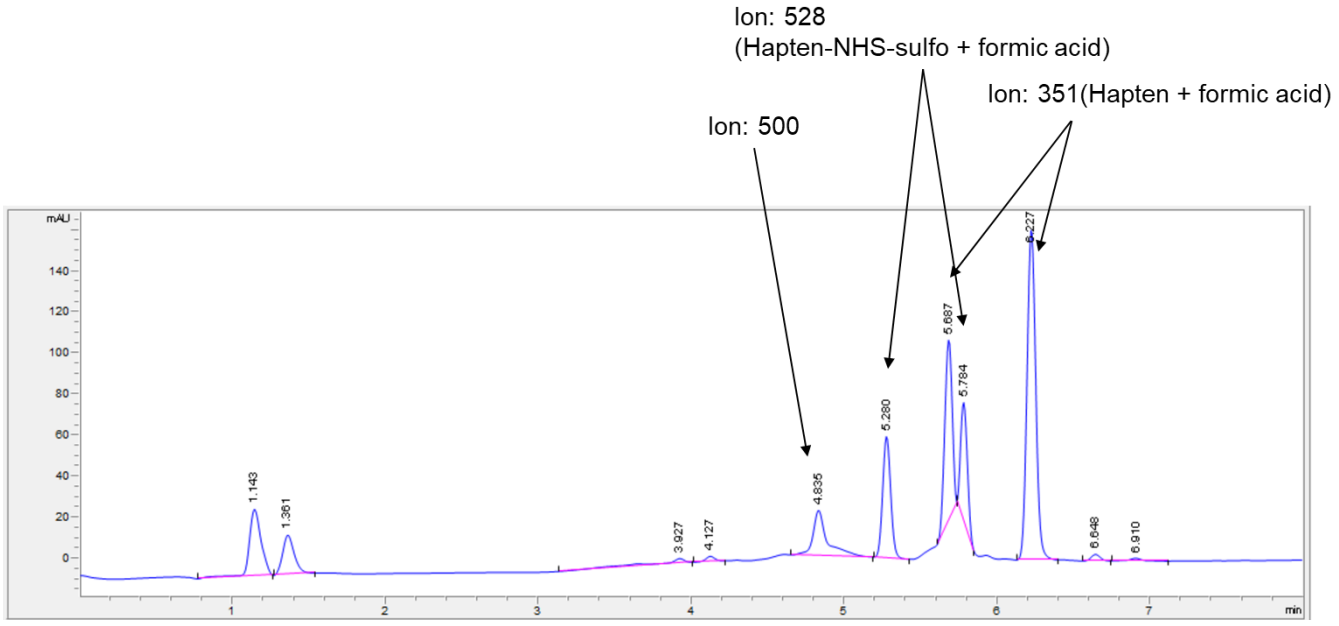


Exact Mass: 473.13

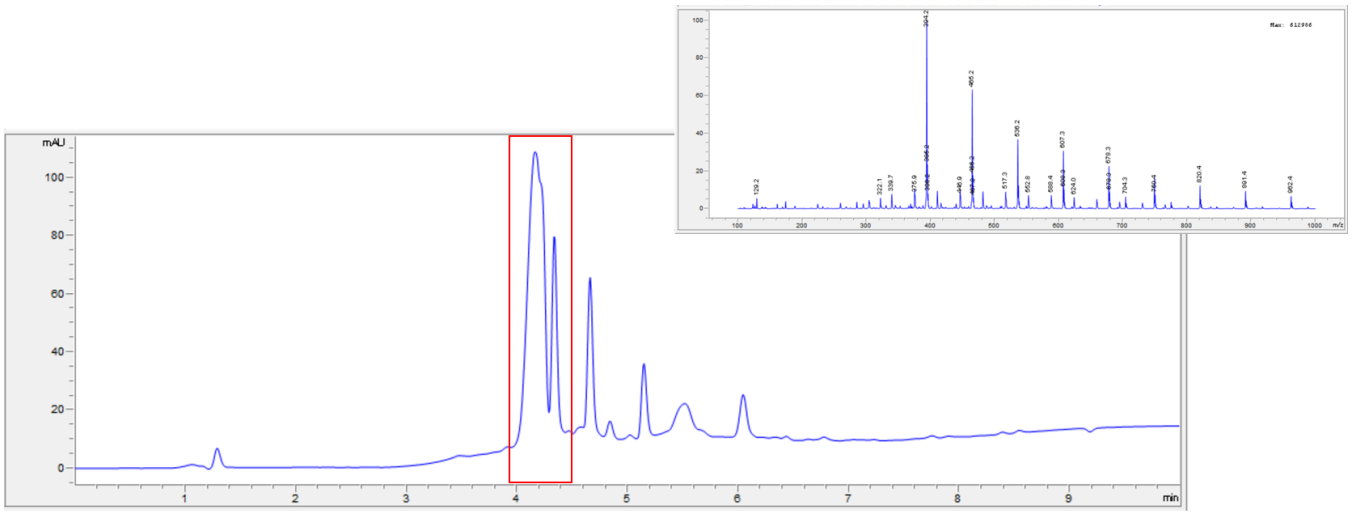
To address this, the amount of EDC was minimized to 2 equivalents of the hapten, ensuring that the side reaction was controlled to less than 10% of the total conversion. The final reaction procedure for 1 mg of hapten involved mixing 1 mg of EDC and 1 mg of NHS-sulfo with the hapten and allowing the reaction to proceed in 200 µL of H_2O for 15 minutes. Subsequently, 1 mg of protein, diluted in PBS buffer, pH = 7.4, was added to the mixture. After two hours of conjugation at room temperature, LC-MS analysis was employed to assess the percentage of side reaction. The solution was then subjected to extensive dialysis against PBS buffer, pH = 7.4.

V. Side reactions of bioconjugation monitored in LC-MS

1. Condensation with formic acid during activation.

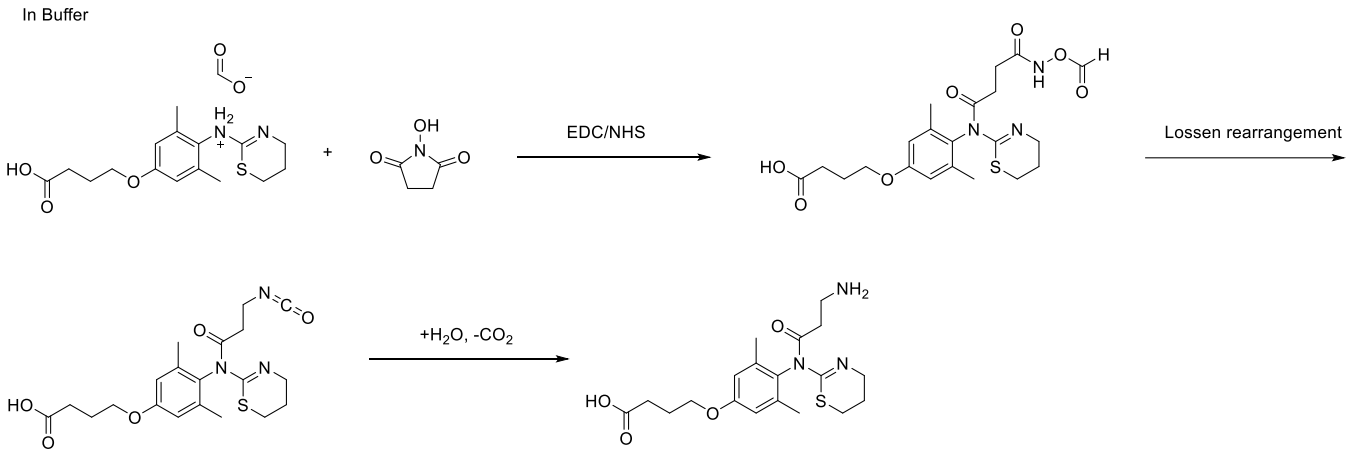


2. Lossen-rearrangement polymerization.



Impurity

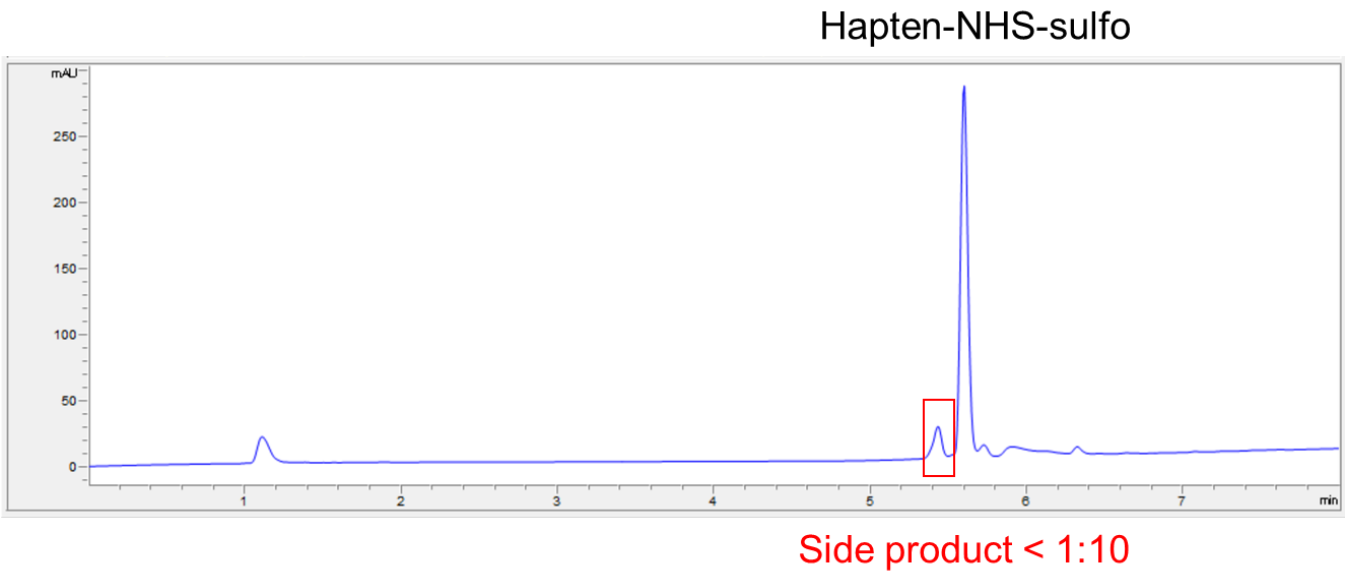
Proposed mechanism:



A similar study that supports this phenomenon can be seen in paper.[1]

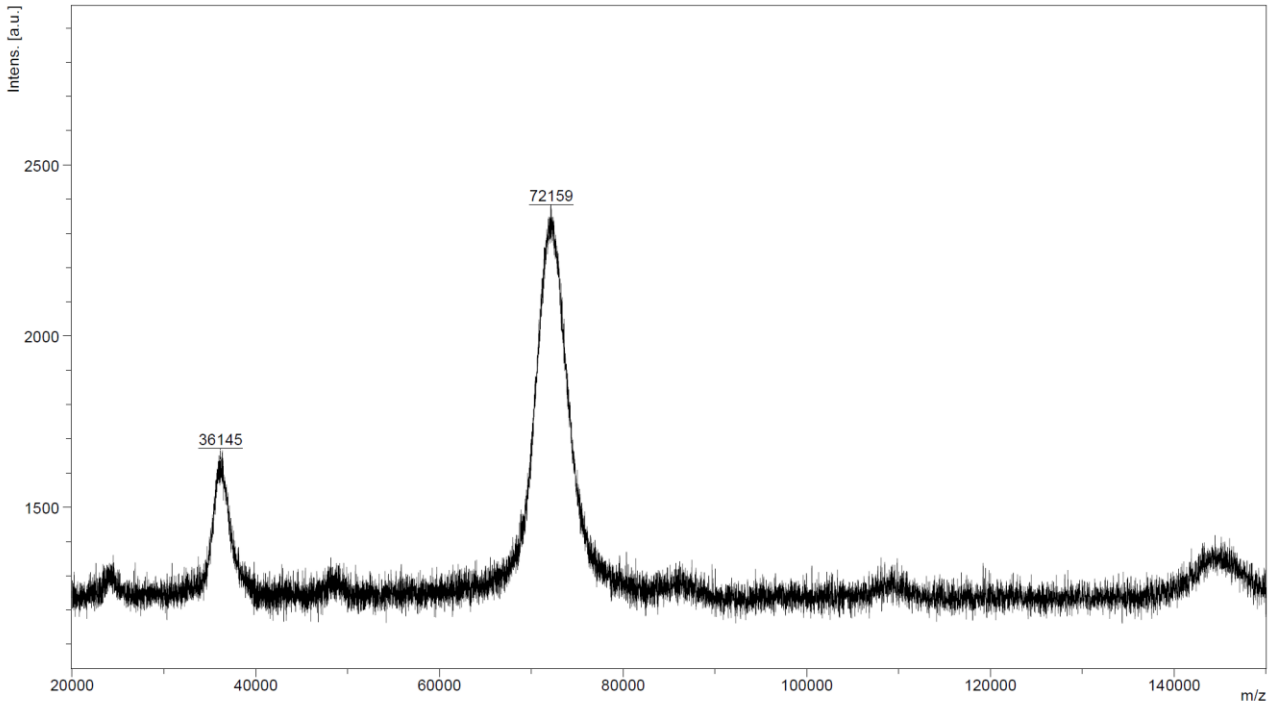
3. Optimization of conditions to minimize the side reaction and conjugate impurity.

Solution of 1mg EDC, 1mg NHS-sulfo and 1mg hapten (with formic acid adduct) was pre-activated for 15 minutes in H_2O, followed by addition of 1mg BSA and 2 hours at room temperature.

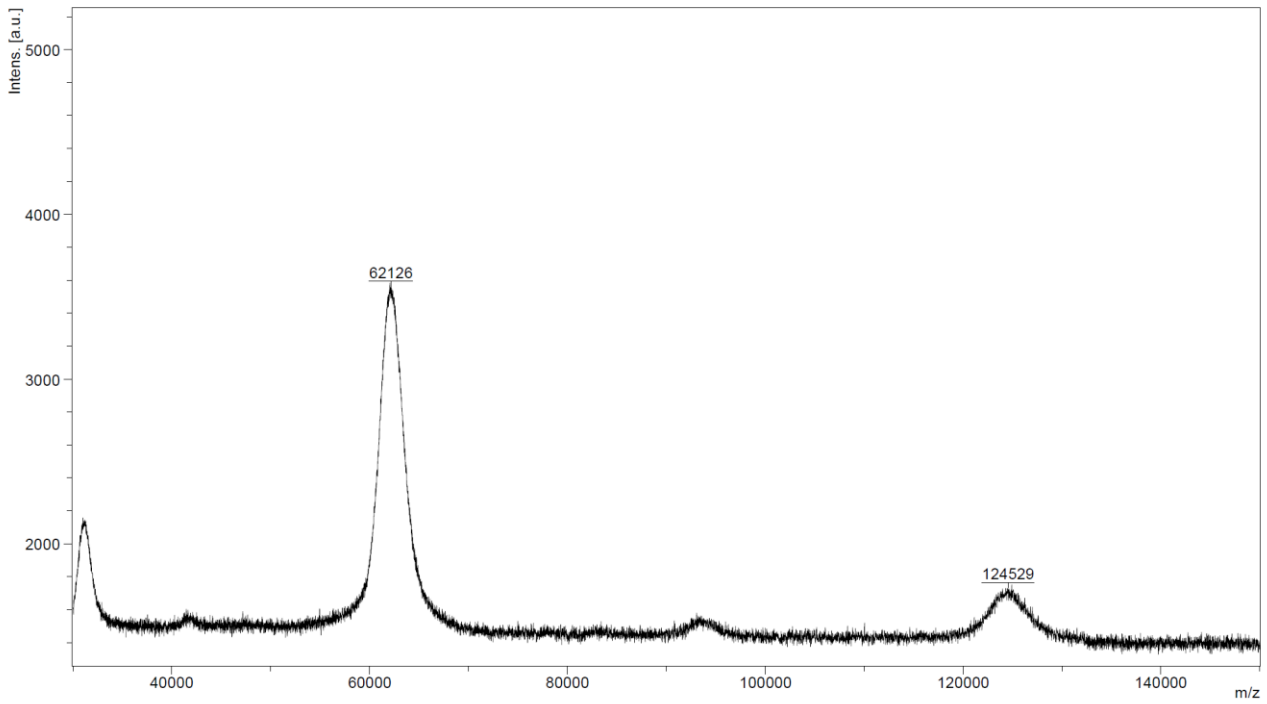


VI. MALDI-TOF

BSA conjugate



CRM_{197} conjugate



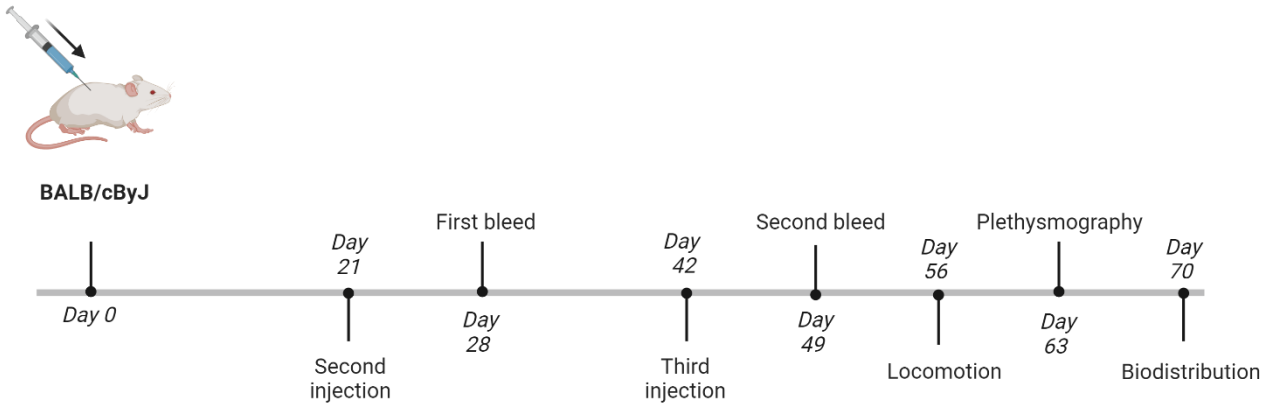
VII. Vaccine preparation

Solutions of BSA/KLH/CRM_{197} conjugates were concentrated using Amicon® Ultra Centrifugal Filters 10K MWCO at 5,000 rpm until protein concentrations reached >0.5 mg/mL. Conjugate protein concentrations were verified by bicinchoninic acid (BCA) assays using the appropriate protein standard BSA, KLH, TT, or CRM_{197} and the concentrated conjugate solutions were stored at 4 °C until formulated. For each mouse, 50 µg of hapten conjugates were formulated with 500 mg of alum (alhydrogel) and 50 µg of CpG 1826 and then gently rotating at room temperature for 1 hour prior to immunizations to ensure sufficient absorption of the conjugate onto the alum surface.

VIII. Animals

Seven week old male BALB/cByJ mice (n = 6/vaccine group) were obtained from Jackson Laboratories and allowed to acclimate for approximately 1 week before vaccination. Mice were group housed in an AAALAC-accredited vivarium containing temperature- and humidity-controlled rooms, with mice kept on a reverse light cycle (lights on: 9PM to 9AM). All experiments were performed during the dark phase. General health was monitored by both the scientists and veterinary staff of The Scripps Research Institute. All animal studies were performed in compliance with The Scripps Institutional Animal Care and Use Committee (Protocol#08-0127) and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

IX. Immunization



X. ELISA

To measure the antibody response in mouse serum, 96-well plates (Corning 3690) were coated with 25 ng of hapten-BSA conjugate in PBS pH 7.4 (1 µg/mL, 25 µL per well) and incubated overnight at 4 °C. The plates were then washed with dH_2O and blocked with 5% skim milk in PBS, pH 7.4 at room temperature for 45 minutes. Mouse serum samples were added to the first column of the plate, and a 1:1 serial dilution in 1% BSA-PBS, pH 7.4 was performed across each row starting with a dilution of 1:200. After incubation at 37 °C for 2 hours, the plates were washed 10 times with dH_2O, then incubated 1 hour at 37 °C with horseradish peroxidase-conjugated secondary antibody (donkey anti-mouse IgG, Jackson ImmunoResearch 715–035-151) diluted 1:10,000 in 1% BSA-PBS, pH 7.4 . Plates were again washed 10 times with dH_2O before being developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermo Scientific). The TMB substrate (40 µL) was incubated for 8-10 minutes at room temperature, and the reaction was quenched with 2 M aq. H_2SO_4 (40 µL). Absorbance at 450 nm was recorded, and the absorbance values were normalized to the highest absorbance value per assay using GraphPad PRISM 8. Data was fit using the log(inhibitor) vs normalized response–variable slope equation to determine midpoint titer values.

For hapten density measurements, varying concentrations of BSA-conjugates were used to coat 96-well plates (4µg/mL, 2µg/mL, 1µg/mL, 0.5µg/mL, 0.25µg/mL, 0.125µg/mL) and the same protocol was followed *vide supra*. Absorbance measurements in the plateau region were plotted against the antigen coating concentrations to determine hapten density.

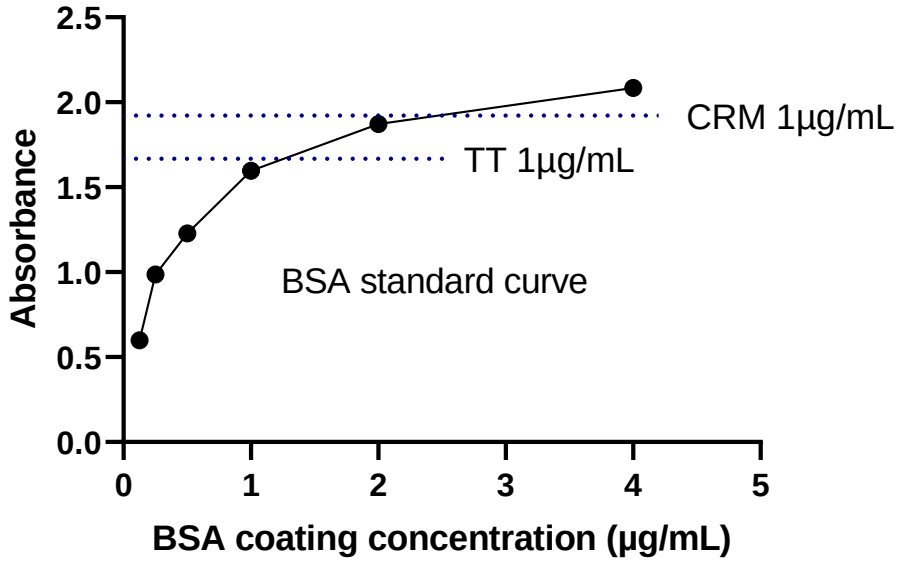


Figure S1. Hapten density measurements. BSA standard curve and the matching results for TT and CRM_{197}.

XI. Surface plasmon resonance

A competitive binding assay was conducted using SPR on a Biacore 3000 instrument (GE Healthcare Life Sciences) equipped with a research-grade CM5 sensor chip.[2] Ligands were immobilized on the chip surface using standard NHS/EDC coupling chemistry with BSA in the reference flow cell (FC1) and hapten-BSA conjugate in FC2. Mouse serum was diluted in running buffer (HBS-EP+ buffer: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% v/v Surfactant P20) and titrated on the hapten-BSA immobilized flow cell minus the reference (FC2-1) to generate a response of ~100 RU following the dissociation phase. For competitive binding studies, serum diluted at the predetermined concentration was incubated with analyte (xylazine) at room temperature for at least 30 minutes on a 600 rpm shaker. Following pre-incubation, samples were injected for 5 minutes followed by a 2.5 minutes dissociation phase. The chip surface was then regenerated by injection of 10 mM Glycine-HCl (pH 1.5) for 30 seconds, followed by a stabilization period before the next assay. Due to the carryover of the analyte, the assay was performed at a sequence of increasing inhibitor concentration, and a blank injection of 5% DMSO in HBS-EP+ buffer was performed after each serum sample. Inhibitor concentrations ranged from 100 µM to 0 nM with two-fold dilution for a total of 12 points.

XYLAZINE	TT 1 (1:20K)	TT 2 (1:20K)	CRM 1 (1:10K)	CRM 2 (1:10K)	KLH 1 (1/25K)	KLH 2 (1/25K)
0	210.74	148.62	114.62	114.42	104.4	124.51
9.8	185.49	119.59	107.14	100.02	82.22	88.65
19.5	175.48	113.14	105.73	96.30	74.51	77.72
39	161.45	101.17	99.08	88.59	66.37	67.35
78	144.08	87.39	91.82	79.40	56.51	56.07
156	122.05	71.67	80.41	65.53	47.72	45.28
312	103.94	57.36	69.84	58.34	37.96	36.61
625	81.71	44.43	58.24	48.69	31.86	27.97
1250	62.94	32.47	47.31	39.03	24.3	22.01
2500	45.03	23.38	37.50	29.74	18.58	16.39
5000	32.61	16.35	29.12	22.12	14.87	12.63
10000	23.19	11.65	21.85	17.62	11.59	9.71
IC_{50} (nM)	**276.30**	**133.40**	**486.20**	**277.40**	**92.14**	**46.69**

XII. Locomotor activity

Mice were acclimated to the testing room with a white light source in their home cages for at least 10 minutes. Subsequently, mice were placed in individual plastic cages (267 × 483 × 203 mm), enclosed with clear ventilated acrylic top, for a habituation period of 1 hour. Movement was tracked and recorded with overhead cameras using ANY-Maze video tracking software (Stoelting Co., Wood Dale, IL). Following the habituation period, mice were injected IP with vehicle or drug (1, 3, 6 mg/kg) and returned to the cage for a 1 hour testing period. The distance traveled was binned into 3-minute intervals and plotted against time.

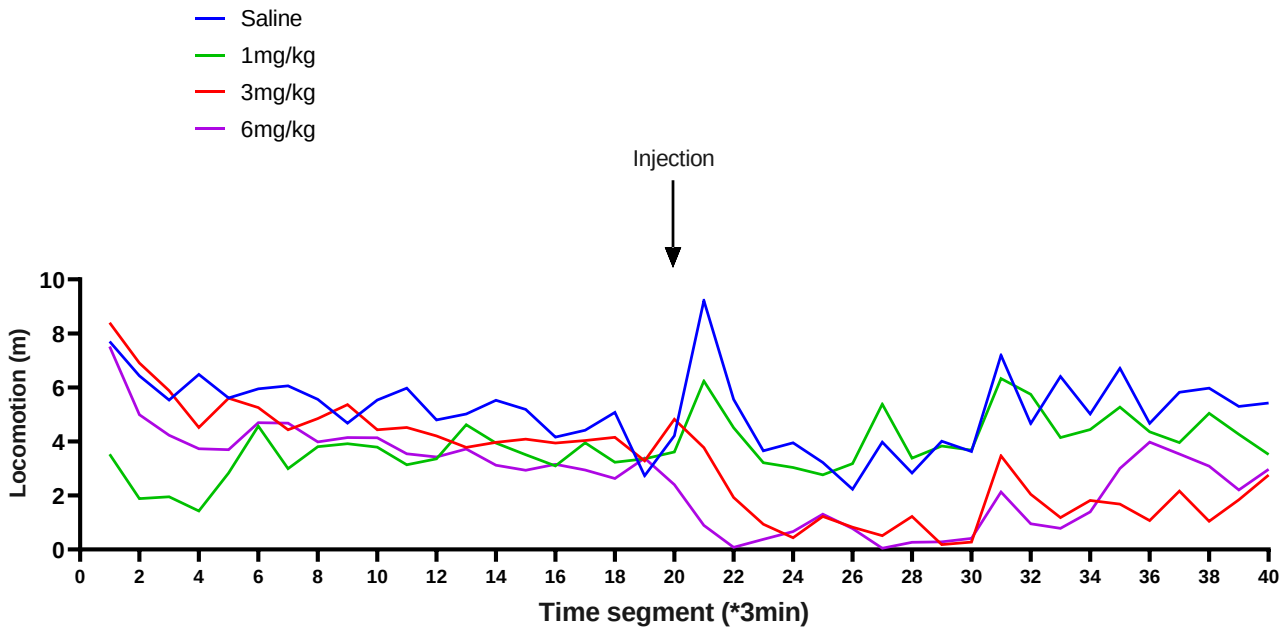


Figure. S2 Longitudinal locomotion results of xylazine at different drug concentrations. Data is analyzed in 3-minute segments, totaling 40 segments over 120 minutes (n = 4)

XIII. Plethysmography

Respiration was measured in freely moving mice using whole-body mouse plethysmography chambers (EMKA Technologies, France) under a 5% CO_2 atmosphere. One day before the experiment, mice were habituated to the chambers for 30 minutes while breathing air. On the day of the experiment, baseline respiration was recorded for each mouse over a 20-minute period before drug challenge IP. Using the IOX software by EMKA, data was recorded and binned in 5-minute intervals. Changes in minute volume were used to assess respiratory effects after acute drug administration. For each mouse, the percent change in minute volume was calculated as the percentage or pre-drug baseline. Statistics were analyzed using Two-Way ANOVA (repeated measure, each row represents a different time points), together with imbedded multiple comparison, within each row comparing columns with Bonferroni's post-test. Data were graphed and analyzed using GraphPad Prism 8, setting $p < 0.05$ as the critical value. All data are displayed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

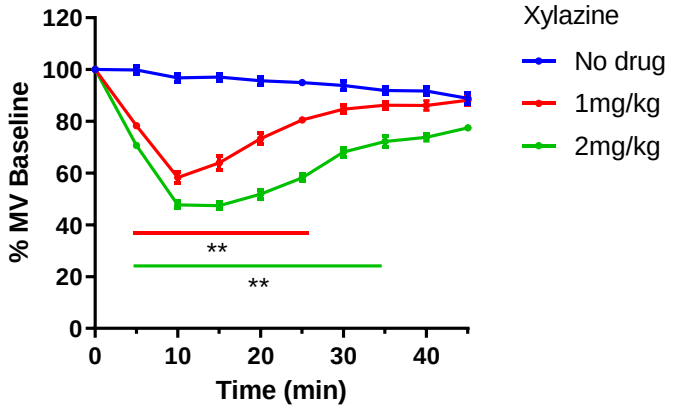


Figure. S3 Dose-response curves for xylazine-induced respiratory depression. Significance between xylazine groups and the no drug control group is denoted by asterisks (n = 8).

XIV.Blood-Brain biodistribution

In this study, blood and brain samples were collected from mice to investigate drug biodistribution. Specifically, 15 minutes after IP drug injection, mice were fully anesthetized with isoflurane and rapidly decapitated using a sharp guillotine. Trunk blood was collected, and the brain was surgically separated, weighed (typically 0.4-0.5 g), and added to a tube containing zirconium oxide beads (Next Advance ZrOB05). PBS, pH 7.4 was added to the brain sample at a 1:1 weight ratio (i.e., 1 mL PBS for 1 g brain tissue), and the sample was homogenized using a Bullet Blender homogenizer (Next Advance). The samples were then stored at −80 °C until analysis.

To generate a standard curve, 100 µL of blank serum or brain homogenate samples were spiked with 50 µL of 50 ng/mL deuterated xylazine as an internal standard and 50 µL of xylazine at concentrations of 63, 125, 250, 500, and 1000 ng/mL in ACN and samples were mixed thoroughly through intensive vortexing. The sample pH was adjusted by adding 100 µL of 50 mM sodium carbonate (Na_2CO_3), and extraction was performed by vortexing extensively with 700 µL of EtOAc. After centrifugation at 3,000 rpm for 5 minutes, 500 µL of the top solvent layer was removed, transferred to a new tube, and evaporated to dryness. The resulting residue was reconstituted in 100 µL of MeOH before analysis by LC-MS. The standard curve was produced by plotting drug concentrations divided by the factor of two (ratio of sample volume over spiked drug volume), which is 31, 63, 125, 250, and 500 ng/mL, against the corresponding ratio of drug/internal standard signal.

For experimental samples, frozen samples were thawed at room temperature, and 100 µL of serum or brain homogenate samples were spiked with 50 µL of 50 ng/mL deuterated internal standard and 50 µL of pure PBS. Following the same sample processing methods as previously stated, the drug concentration in the samples was calculated by referencing the standard curve using the ratio of drug/internal standard signal. The values in the brain samples should be multiplied by two due to the 1:1 PBS dilution.

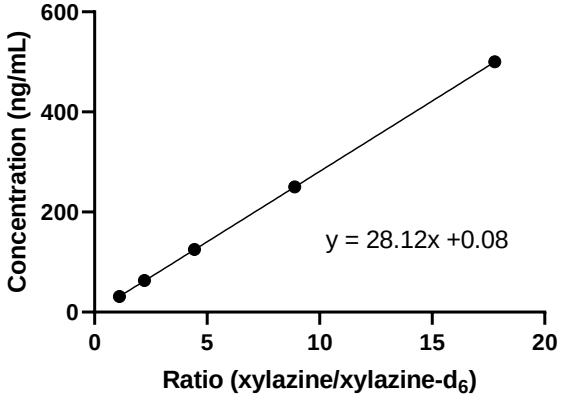


Figure. S4 Standard curve of blood-brain biodistribution. Linear regression fit, $r^2 = 0.99$.

Reference

1. K. A. Totaro, X. L. Liao, K. Bhattacharya, J. I. Finneman, J. B. Sperry, M. A. Massa, J. Thorn, S. V. Ho and B. L. Pentelute, *Bioconjugate Chem*, 2016, **27**, 994-1004.
2. P. T. Bremer, A. Kimishima, J. E. Schlosburg, B. Zhou, K. C. Collins and K. D. Janda, *Angew Chem Int Edit*, 2016, **55**, 3772-3775.