Supporting Information:

Development of a Clinically Viable Heroin Vaccine

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Materials and Methods

Hapten Synthesis and Characterization. All starting materials and reagents were purchased from Combi-Blocks or Sigma-Aldrich. Organic solvents were anhydrous or distilled prior to use. NMR spectra were recorded on a Bruker 600 MHz instrument and peaks are reported in ppm, using the solvent peak as a chemical shift reference. High resolution mass analysis was performed on an Agilent ESI-TOF-MS system. All compounds showed ≥95% purity according to the 254 nm UV trace acquired on an Agilent LCMS equipped with an Agilent Zorbax 300SB-C8 4.6 X 50 mm 5 µm column. LCMS method parameters were as follows: flow rate 0.5 mL/min, solvent A: H_2O+0.1% COOH, solvent B: MeCN+0.1% COOH, gradient 0-4 min: 5-95% B, 4-7 min: 95% B. Compound **5** (HerSH) was synthesized and conjugated as previously described.[1-3]

HerCOOH (**2**). Previously reported heroin butyl amine **1·TFA** (26 mg, 0.048 mmol) was dissolved in 0.5 mL DCM. Mono-*tert*-butyl succinate (9 mg, 1.1 eq), HATU (20 mg, 1.1 eq) and Et_3N (22 µL, 3.3 eq) were added and the solution was stirred for 40 min at rt. The reaction mixture was diluted with DCM, washed once with sat. sodium bicarbonate solution, and the organic layer was concentrated. Purification proceeded via pTLC in 8% MeOH in DCM to yield *tert*-butyl protected HerCOOH (28 mg, 80%). The *tert*-butyl ester was deprotected with 0.5 mL 1:1 TFA to DCM for 18 h at rt to quantitatively produce HerCOOH **2**. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.88 (d, *J* = 8.2 Hz, 1H), 6.86 – 6.82 (m, 1H), 6.69 (d, *J* = 8.3 Hz, 1H), 5.77 (d, *J* = 10.4 Hz, 1H), 5.45 (d, *J* = 10.2 Hz, 1H), 5.25 – 5.21 (m, 1H), 5.20 – 5.16 (m, 1H), 4.32 – 4.26 (m, 1H), 3.51 (d, *J* = 13.7 Hz, 1H), 3.48 – 3.40 (m, 1H), 3.35 – 3.25 (m, 1H), 3.24 – 3.15 (m, 3H), 3.14 – 3.03 (m, 1H), 2.98 – 2.79 (m, 1H), 2.76 – 2.65 (m, 2H), 2.61 – 2.49 (m, 2H), 2.48 – 2.33 (m, 1H), 2.29 (s, 3H), 2.17 – 2.09 (m, 1H), 2.13 (s, 3H), 1.89 – 1.74 (m, 2H), 1.71 – 1.55 (m, 2H). ^{13}C NMR (151 MHz, CDCl3) δ 176.06, 174.59, 170.75, 168.68, 149.65, 132.99, 130.84, 129.32, 127.65, 125.38, 123.84, 120.07, 87.29, 67.00, 58.60, 54.51, 47.15, 46.46, 41.40, 38.34, 37.89, 32.51, 31.07, 29.97, 26.15, 21.79, 21.01, 20.57. ESI-TOF-MS (*m*/*z*): $[M+H]^+$ *calc*. 527.2388, *obs.* 527.2385.

6AMCOOH (**3**). The *tert*-butyl ester of **2** (4 mg, 0.0069 mmol) was deacetylated using tributyltin hydroxide (10 mg, 7 eq) in 0.25 mL DCE at 70 °C for 2 h. The product was purified via pTLC in 10% MeOH in DCM to yield the *tert*-butyl ester of 6AMCOOH (1.22 mg, 33%). The *tert*-butyl ester was deprotected with 0.5 mL 1:1 TFA to DCM for 18 h at rt to quantitatively produce 6AMCOOH **3**. ^{1}H NMR (600 MHz, MeOD) δ 6.64 (d, *J* = 8.2 Hz, 1H), 6.58 (d, *J* = 8.2 Hz, 1H), 5.78–5.73 (m, 1H), 5.56–5.49 (m, 1H), 5.21 (s, 1H), 5.16 (d, *J* = 6.9 Hz, 1H), 4.26 (s, 1H), 3.49–3.39 (m, 1H), 3.34–3.22 (m, 4H), 3.06–2.94 (m, 2H), 2.87 (d, *J* = 19.7 Hz, 1H), 2.62 (t, *J* = 6.3, 2H), 2.47 (t, *J* = 6.5 Hz, 2H), 2.27 (t, *J* = 8.4, 1H), 2.15 (s, 3H), 2.09 (d, *J* = 13.8 Hz, 1H), 1.86–1.75 (m, 2H), 1.68–1.58 (m, 3H). ^{13}C NMR (151 MHz, MeOD) δ 176.29, 174.98, 172.23, 146.77, 141.26, 131.49, 129.56, 126.80, 122.61, 121.12, 118.88, 87.74, 68.55, 60.31, 55.30, 47.98, 42.70, 39.49, 38.77, 33.95, 33.04, 30.73, 30.02, 27.87, 22.15, 20.65. ESI-TOF-MS (*m*/z): $[M+H]^+$ *calc*. 485.2282, *obs.* 485.2281.

HerdBA (**4**). H-β-Ala-OEt (31 mg, 0.2 mmol), Boc-β-Ala-OH (38 mg, 1 eq), Cl-HOBt (34 mg, 1.05 eq), EDC-Cl (46 mg, 1.2 eq) and Et_3N (64 µL, 38 mg) were dissolved in 1 mL DCM and stirred for 16 h at rt. A workup was performed by washing the DCM solution with 1 M HCl, sat. sodium bicarbonate and brine to yield the crude Boc-di-β-Ala-OEt, which was deprotected with 1 mL 1:1 MeOH/1 M LiOH over 0.75 h at rt. Extraction

with EtOAc yielded the product Boc-di-β-Ala-OH (35 mg, 67%, 2 steps). Previously reported heroin butyl amine **1** (5.5 mg, 0.013 mmol) was coupled to Boc-di-β-Ala-OH (5.1 mg, 1.5 eq) using Cl-HOBt (3.3 mg, 1.5 eq), EDC-Cl (5.0 mg, 2 eq) and Et_3N (3 eq, 5.4 µL) in 0.5 mL DCM at rt for 4 h. Purification by pTLC in 10% MeOH in DCM yielded the Boc intermediate which was deprotected in 0.5 mL 1:1 TFA to DCM over 1 h at rt, affording the corresponding amine (4.1 mg, 55%). The same coupling, purification and deprotection procedure was then repeated with mono-*tert*-butyl succinate to produce HerdBA **4** (1.4 mg, 32%). ^{1}H NMR (600 MHz, Acetone-d_6) δ 6.89 (d, J = 8.2 Hz, 1H), 6.74 (d, J = 8.3 Hz, 1H), 5.71 (d, J = 10.2 Hz, 1H), 5.65 (d, J = 9.3 Hz, 1H), 5.29 (d, J = 6.9 Hz, 1H), 5.26 – 5.19 (m, 1H), 4.57 – 4.39 (m, 1H), 3.64 – 3.54 (m, 2H), 3.53 – 3.37 (m, 4H), 3.34 – 3.21 (m, 4H), 3.12 – 2.97 (m, 4H), 3.09 – 2.98 (m, 1H) 2.66 – 2.52 (m, 2H), 2.53 – 2.44 (m, 2H), 2.42 – 2.32 (m, 2H), 2.26 (s, 3H), 2.09 (s, 3H), 1.98 – 1.85 (m, 4H), 1.68 – 1.56 (m, 2H). ESI-TOF-MS (*m*/*z*): $[M+H]^+$ *calc*. 669.3130, *obs.* 669.3126.

***Standard Mouse Immunization Protocol*.** 6-8 week old male Swiss Webster mice n = 6/group (Taconic Farms) were immunized i.p. with 50 µg immunoconjugate with or without 30 µg CpG ODN 1826 (75 µL in pH 7.4 PBS) adsorbed onto alum (75 µL, 0.75 mg, shaken for 30 min) at weeks 0, 2 and 4. Bleeds were taken at weeks 3 and 6. KLH carrier protein was obtained from Thermo Pierce and DT and TT were obtained from Statens Serum Institute. Adjuvants alum and cGAMP were obtained from InvivoGen as 10 mg/mL Alhydrogel suspension and 2'3'-cGAMP, respectively. CpG ODN 1826 (5'-TCCATGACGTTCCTGACGTT-3') was obtained as a phosphorothioated oligonucleotide from Eurofins. Weights and general health of mice were monitored by both the scientists conducting the studies, and the veterinary staff of Scripps Research Institute. All mice continued to gain weight similar to naïve counterparts following the immunization procedures, and no mice were required to be excluded due to any adverse effects.

***Conjugations*.** For thiol conjugations, carrier proteins (1.9 mg/mL in pH 7.4 PBS) were maleimide activated with 250 wt% sulfo-GMBS (Thermo Pierce) for 2 h at rt. Following purification via Zeba 7k MWCO desalting column (Thermo Pierce), 100 wt% HerSH was added to the proteins as a DMSO solution (10% DMSO final), and the conjugations proceeded for 5 h at rt. This procedure is similar to previously described procedures.[1-3]

Carboxylate haptens HerCOOH and HerdBA were dissolved in anhydrous DMF (15 mM) and were activated with 6 eq EDC, 6 eq Et_3N and 2 eq NHS. Upon complete conversion to the NHS ester by LCMS (typically 2-6 h), 100 wt% activated hapten was added to 1.6 mg/mL carrier protein in pH 7.4 PBS to give a final pH of 10 and 10 vol% DMF. Conjugations proceeded for 2 h at rt at which point LCMS indicated NHS esters had been consumed while noticeable 3-acetyl hydrolysis had occurred. In the 6AM v Heroin hapten study, HerCOOH and 6AMCOOH (15 mM) were activated in 10% H_2O in DMF with 6 eq EDC and 6 eq NHS over 2 h. After a 4 h rt conjugation, no 3-acetyl hydrolysis was observed by LCMS. This conjugation method was used for all monkey studies.

Following all hapten couplings, the resulting conjugates were dialyzed exhaustively at 4 °C over a 24 h period. Conjugates were diluted 2:1 with glycerol and stored at -20 °C until immunization.

***ELISA*.** Half-area, high-binding, 96-well plates were coated for 18 h at 4 °C with 1 µg/mL Heroin-BSA conjugate in pH 6.4 PBS or 1 µg/mL unconjugated carriers in pH 7.4 PBS. Heroin-BSA conjugates were prepared in the same manner as conjugates for immunization. Blocking was achieved at rt with 5% skim milk in pH 6.4 PBS or pH 7.4 PBS for unconjugated carriers. Mouse or monkey sera diluted 1:100 into 1% BSA in pH 7.4 PBS were used for twelve 1:1 serial dilutions across the plate followed by 2.5 h incubation at r.t. Washing before and after the addition of secondary was performed 5X with pH 7.4 PBS. Donkey anti-mouse IgG, Fcγ specific HRP secondary antibody (Jackson ImmunoResearch); goat anti-monkey, Fcγ specific HRP (Novus Biologicals) and goat anti-monkey IgM, Fcμ specific HRP (Sigma Aldrich) were used for detection at 0.1 µg/mL, incubated for 18 h at 4 °C. Plates were developed with TMB substrate (Thermo Fisher) and 2M H_2SO_4 as a stopping solution.

***Antinociceptive Behavioral Evaluation*.** At least 4 days following the week 6 bleed, mice were tested for cumulative, s.c.-administered heroin response in primarily supraspinal (hot plate) and spinal (tail immersion) behavioral tests as previously described.[3] Both tests were performed at 54 °C. Briefly, the hot plate test was measured by placing the mouse in an acrylic cylinder (14 cm diameter x 22 cm) and timing latency to perform one of the following nociceptive responses: licking of hindpaw, shaking/withdrawal of hindpaw, or jumping.

Immediately following a response, with typical baseline latency between 8-15 s and a 35 s cutoff to prevent tissue damage, mice were removed from the plate. The tail immersion test was administered by lightly restraining mice in a small pouch constructed from absorbent laboratory underpads and dipping 1 cm of the tip of the tail into a heated water bath, with the time to withdrawal timed. Typical baseline response was 1-2 s and a cutoff of 10 s was used to prevent tissue damage. Since tail immersion is a more reflexive behavior, testing order was always hot plate first followed by tail immersion, and then heroin (10 mL/kg in normal saline) was immediately administered after completing both tests. Roughly 15-20 min following each injection, the tests were repeated, and if mice did not reach full antinociception for both tests, the animals continued to receive further cumulative drug injections and repeated testing until cutoff times were reached. Upon completion of all testing, mice were administered a cocktail of 1 mg/kg naloxone and naltrexone in saline to prevent subsequent consequences of potential overdose.

Statistics. Computational and statistical analyses were performed in GraphPad Prism 6. All values are reported as means ± SEM. Antinociceptive data were transformed from time to % maximum possible effect (%MPE), which is calculated as: %MPE = (test – baseline) / (cutoff – baseline) * 100. These data were then fit using a log(agonist) vs. normalized response non-linear regression. These produced ED_{50} values for each antinociception test for the individual treatment groups, allowing calculation of potency ratios. Differences in potency were determined by one-way ANOVA with Tukey's post-hoc test. For ELISA, absorbance values were normalized to the maximum absorbance value per sample and fit with log(inhibitor) vs. normalized response – variable slope to generate the midpoint titer value. Titers were compared via a one-way ANOVA with Dunnett's or Fisher's post-hoc test. Half-life was calculated by fitting the data at C_{max} with an exponential one phase decay model, while AUC was calculated using the AUC model provided in the software.

Study Approval (Mice). All studies conducted utilizing mice were performed using protocols reviewed and approved by the Scripps Institutional Animal Care and Use Committee, and were in concordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in an AAALAC-accredited facility, with proper temperature and humidity controlled environments, standard 12 h light cycle (9AM-9PM), and all studies conducted during the dark cycle (1PM-5PM).

Vaccines (Monkeys). Heroin-TT immunoconjugate was prepared as previously described for the mice. Each immunization per monkey consisted of 400 µg Her-TT, 600 µg phosphorothioated CpG ODN 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (Eurofins) and 5 mg alum as 10 mg/mL Alhydrogel suspension (Invivogen) injected i.m. into the quadriceps at a final total volume of approximately 1 mL. TT was obtained from either Statens Serum Institute or MassBiologics. No adverse reactions to the vaccine injection were observed and all vaccinated monkeys maintained healthy body weights throughout the entire experimental period (Figure S7).

Subjects (Monkeys). Studies were conducted in a total of 4 adult male rhesus monkeys (*Macaca mulatta*) of either Indian or Chinese origin. All monkeys had an extensive experimental history of opioidergic and monoaminergic compound exposure. Their diet consisted of food biscuits (Lab Diet High Fiber Monkey Biscuits; PMI Feeds, St Louis, MO) and fresh fruit delivered in the afternoons after behavioral sessions to minimize the effects of biscuit availability and consumption on food-maintained operant responding. Water was continuously available in each monkey's home chamber, which also served as the experimental chamber. A 12-h light/dark cycle was in effect (lights on from 0600 to 1800 h). Environmental enrichment, which consisted of movies displayed on a monitor in the housing room and foraging boards loaded with nuts, seeds or diced vegetables, was also provided after behavioral sessions. Facilities were licensed by the United States Department of Agriculture and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The Institutional Animal Care and Use Committee approved all experimental and enrichment protocols. Animal research and husbandry were conducted according to the Guide for the Care and Use of Laboratory Animals.

Assay of schedule-controlled responding (SCR)

Behavioral Procedure. As described previously,[4] each home cage was equipped with a customized operant response panel, which had a response key that could be transilluminated red, and a pellet dispenser (Med Associates, ENV-203–1000, St. Albans, VT) that delivered food pellets (1-g banana-flavored pellets; Grain-

based Precision Primate Tablets; Test Diets, Richmond, IL) to a receptacle below the operant panel. The panel was controlled by a MED-PC interface and an IBM compatible computer programmed in MEDSTATE Notation (MED Associates). Experimental sessions were conducted 5 days per week and consisted of five 30-min cycles. Each cycle consisted of two components: a 25-min time-out period followed by a 5-min response period. During the time-out period, no stimulus lights were illuminated, and responding had no scheduled consequences. During the response period, the right key was transilluminated red, and subjects could respond for up to 10 food pellets under a fixed-ratio 30 (FR30) schedule of reinforcement. If all 10 food pellets were earned before 5 min had elapsed, the lights were turned off, and responding had no scheduled consequences for the remainder of that response period. All monkeys were trained until they responded at rates ≥ 1.0 response/s during all five cycles, and we have shown previously that monkeys respond at relatively stable rates across successive response periods in this procedure.[5-8] Test sessions were usually conducted on Tuesdays and Fridays, and training sessions were conducted on Mondays, Wednesdays, and Thursdays. In addition, test sessions were conducted only after a training session during which the monkeys responded at rates ≥1.0 response/s for all five cycles. During training sessions, monkeys received either no injection or saline injections at the beginning of each cycle. During test sessions, test compounds were administered in a cumulative dosing procedure, in which test drug doses were administered 15 min before each response component and each dose increased the total cumulative drug dose by either one-fourth or one-half units.

Initially, dose-effect functions were determined for 3,6-diacetylmorphine (heroin; 0.01-0.78 mg/kg, IM) and oxycodone (0.01-1.0 mg/kg, IM) alone and each drug was tested twice. In addition, both heroin (0.032-1.8 mg/kg, IM) and oxycodone (0.032-1.8 mg/kg, IM) dose-effect functions were determined twice following pretreatment with the opioid antagonist naltrexone (0.0032-0.032 mg/kg, IM; 30 min; positive control). Subsequently, the heroin-TT conjugate vaccine was administered at weeks 0, 2, 4, 11, and 18. Heroin and oxycodone dose-effect functions were then re-determined at weeks 6, 9, 10, 13, 14, 15, 16, 20, 21, 24, 25, and 26. Mu-opioid agonists were tested up to doses that decreased percent control rates of operant responding below 50%.

Data Analysis. Raw response rates from each test cycle were converted to percent of control using the average rate from the previous training day in that monkey as the control value. ED_{50} values were determined for each mu agonist alone in each monkey. ED_{50} values were calculated by log-linear interpolation (one below and one above 50% effect). Individual ED_{50} values were averaged to yield mean ED_{50}s ± SEM. Individual potency ratios were determined by dividing the test ED_{50} value by the baseline ED_{50} value. Individual potency ratios were averaged to yield mean ratios ± SEM. Significance for each post-vaccine ED_{50} was evaluated versus the baseline ED_{50} by a repeated measures one-way ANOVA with Dunnett's post-hoc test. Heroin and oxycodone ED_{50}s were compared by a repeated measures two-way ANOVA with Bonferroni's post-hoc test.

Pharmacokinetic (PK) Studies

Sample Collection. Blood samples (1–2 mLs) were collected in Vacutainer tubes containing 3.0mg of sodium fluoride and 6.0mg sodium ethylenediaminetetraacetic acid before, and 3, 10, 30, 56, 100, 180, and 300 min after 0.32 mg/kg, IM heroin administration in monkeys seated in primate restraint chairs. Heroin pharmacokinetic studies were conducted both before heroin-TT conjugate vaccine administration and at week 8. Samples were immediately centrifuged at 1000g for 10min. The plasma supernatant was transferred into a labeled storage tube and frozen at -80°C until analyzed. Quantitative analysis of heroin, 6-monoacetylmorphine and morphine was based upon a previously described method.[9]

Sample Extraction. A seven-point calibration curve of 0.5-50 ng/mL for heroin and 6-monoacetylmorphine and 10-1000 ng/mL for morphine in 0.5 mL of drug free serum, a blank control and a double blank control were prepared. The internal standard consisted of 0.5 µg/mL (12.5 ng total) of monoacetylmorphine-d_6 and 10 µg/mL (250 ng total) of morphine-d_3. Samples were mix with 1mL of saturated carbonate/ bicarbonate buffer (1:1, N:N, pH 9.5) and 3mL of chloroform:2-propanol (9:1) for 5 minutes and then centrifuge at 2500 rpm for 5 minutes. The top aqueous layer was aspirated. The organic layer was then transferred to a clean test tube and evaporated to dryness at 40°C under a constant stream nitrogen. The samples were reconstituted in mobile phase and then placed in auto-sampler vials for ultra-performance liquid chromatography tandem mass-spectrometry (*UPLC-MS/MS*).

Instrumental Analysis. *UPLC-MS/MS* Waters analysis was performed with a Waters Acquity Xevo TQD LC-MS/MS system with MassLynx version 3.5 software (Milford, MA, USA). The electrospray ionization probe was operated in positive ion mode. Chromatographic separation was performed on a Selectra® PFPP column, 100 x 2.1 mm, 3.0µm (United Chemical Technologies, USA). The mobile phase consisted of A: water with 10 mM ammonium formate and B: methanol with 10 mM ammonium formate. The following gradient was used: 0.0-1.5. min starting at 5% B, with a linear gradient to 40% B, then increasing to 100% B, with a linear gradient for 1.5 min and holding for 0.5 mins and then returning at 3.6 min to 95% B. The source temperature was set at 150°C. The cone was at 60V and had a flow rate of 0.6 mL/ min. The ionspray voltage was 4.00 kV, with the ion source flow rate of 650 mL/min. The acquisition mode used was multiple reaction monitoring (MRM). The following transition ions (m/z) were monitored in MRM mode with their corresponding collection energies (eV) in parentheses were: Heroin: 370 >58 (28) and 370 >165 (40); 6-monoacetylmorphine: 328 >60 (36) and 328 >165 (38); 6- monoacetylmorphine-d_6: 334 >165 (40) and 334 >211 (28); morphine: 386 >152 (56) and 286 >210 (26); and morphine-d_3: 389 >156 (56) and 286 >185 (28). The total run time for the analytical method was 5.0 minutes.

Drugs and Reagents. 3,6-Diacetylmorphine HCl, oxycodone HCl, and (–)-naltrexone HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). All drugs were dissolved in sterile water. Drug doses were calculated and expressed using the salt forms listed above. Primary reference materials for morphine-d_3, 6-monoacetylmorphine-d_6, heroin, morphine and 6-monoacetylmorphine were purchased from Cerilliant Corporation (Round Rock, Texas). Chloroform, deionized water, hydroxamine HCl, 2-propanol, sodium bicarbonate and sodium carbonate were purchased form Fisher Scientific (Hanover Park, Illinois).

Surface Plasmon Resonance. Determination of IC_{50} of 6-AM for antisera derived from heroin-TT vaccinated monkeys and mice. We employed a competitive assay using surface plasmon resonance (SPR) spectrometry to determine the IC_{50} of 6-AM compound against all antisera. The competitive assay was conducted on a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden) equipped with a research-grade CM4 sensor chip. The ligands, BSA and 6AMCOOH and HerCOOH-BSA conjugate, were immobilized separately to flow cell 1 and 2 using NHS/EDC coupling chemistry according to manufacturer's instruction. The dilution factors for all tested anti-sera were normalized to give a similar binding response on 6AM-BSA immobilized chip surface. Each diluted serum was pre-incubated with a twelve-point concentration series of 6AM or heroin compound, ranging from 10 µM to 0 µM (3-fold dilution series) at room temperature for 30 minutes before Biacore injection. A separate comparison was made directly between a single concentration of 6AM (10 µM), heroin, morphine, oxycodone and methadone (100 µM) in triplicate. Sequentially, each pre-incubated mixture was then injected over both flow cells for 5 minutes and were dissociated for 3 minutes in running buffer (HBS-EP+ buffer, pH7.4, GE Healthcare) before the surface was regenerated with Gly-HCl, pH 2.0. All competitive binding experiments were conducted at 25 °C. The BSA immobilized flow cell was used as reference and the binding response from BSA flow cell was subtracted out from the response from 6-AM-BSA immobilized flow cell. The binding data were collected in the sensorgrams (Figure S8) and the IC_{50} for each sample was calculated: RU values were normalized to the RU value produced from chip binding without competitor and the resulting data was fit using the log(inhibitor) vs normalized response – variable slope using Graphpad Prism v 6. The competitive IC_{50} value is highly representative of actual antibody K_d for drug binding as established previously.[10] The IC_{50} curve was run in triplicate against HerCOOH and 6AMCOOH-BSA immobilized ligands with no difference in IC_{50} observed between the two ligands. See Figure S8 for raw sensorgrams.

Supplementary Figures and Tables

	Conjugate	Conjugate Mass (Da)	Hapten Mass (Da)	Hapten to carrier molar ratio
Carrier Study	Activated DT	62996	2496	15.1
	Activated TT	156857	3357	20.3
	HerSH-DT	67716	4720	9.2
	HerSH-TT	160023	3166	6.1
Linker Study	Activated TT	159805	6305	38.2
	HerSH-TT	171934	12129	23.5
	HerdBA-TT	161279	7779	12.0
	HerCOOH-TT	164209	10709	21.1
Dose Study	HerCOOH-TT	163496	9996	19.7
	HerCOOH-BSA	76961	10461	20.6
Adjuvant/ Priming Study	HerCOOH-TT	160087	6587	13.0
6AM vs. Her Study	6AMCOOH-TT	162491	8991	19.3
	HerCOOH-TT	161295	7795	15.3
	HerCOOH-BSA	76307	9807	19.3
	6AMCOOH-BSA	79990	13490	28.9
Monkey Study	HerCOOH-TT	164432	10932	21.5

Table S1. Mass values and copy numbers of immunoconjugates
Conjugates were dialyzed into DI water for 2 h and analyzed by MALDI-ToF using a sinapinic acid matrix.

GMBS	165.15
HerSH	515.64
HerCOOH	508.57
HerdBA	650.73
6AMCOOH	466.54
BSA	66500
DT	60500
TT	153500

Table S2. Mass values for starting materials used in conjugate preparation (Da)

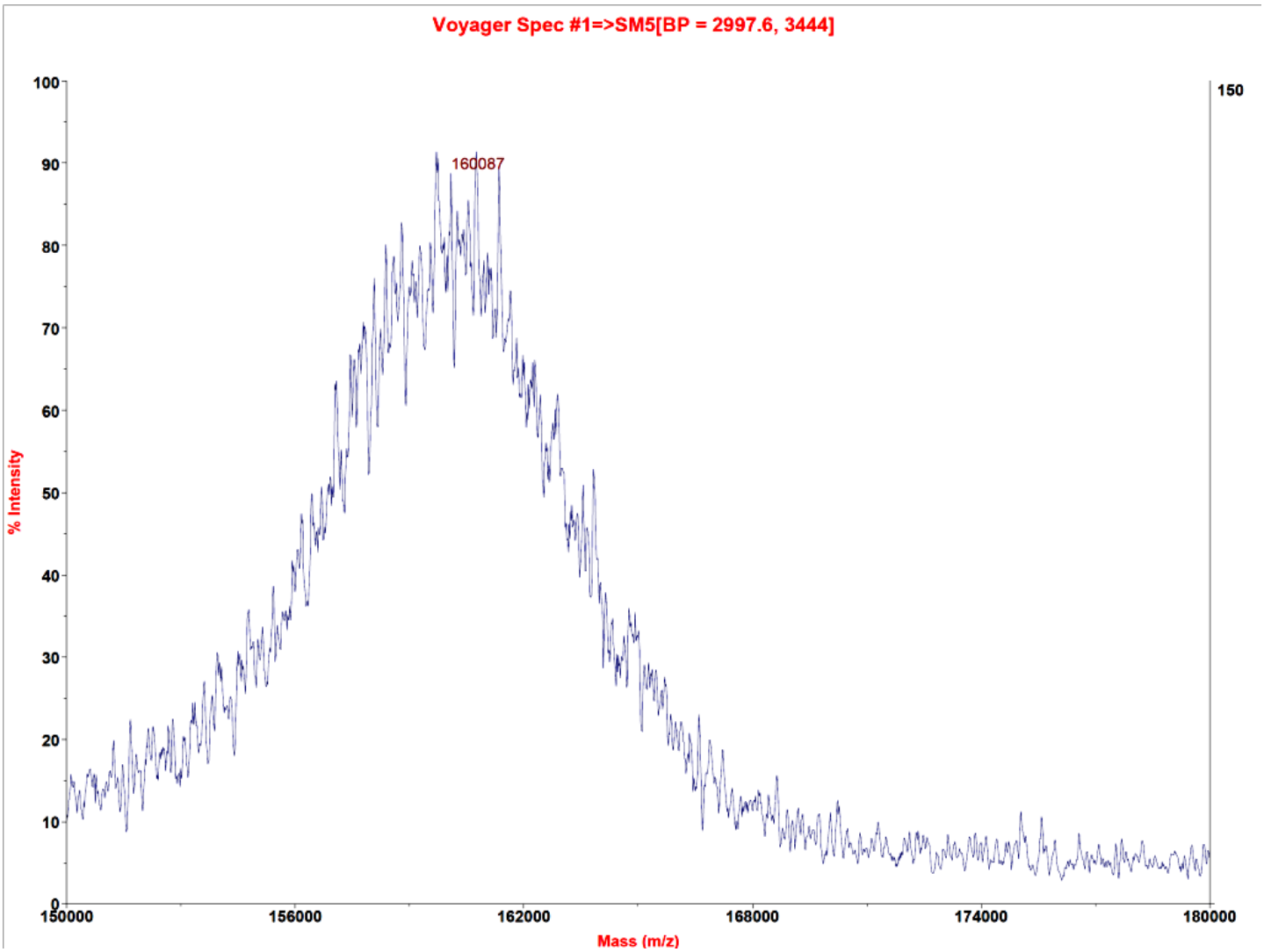

Figure S1. Representative mass spectrum of HerCOOH-TT.

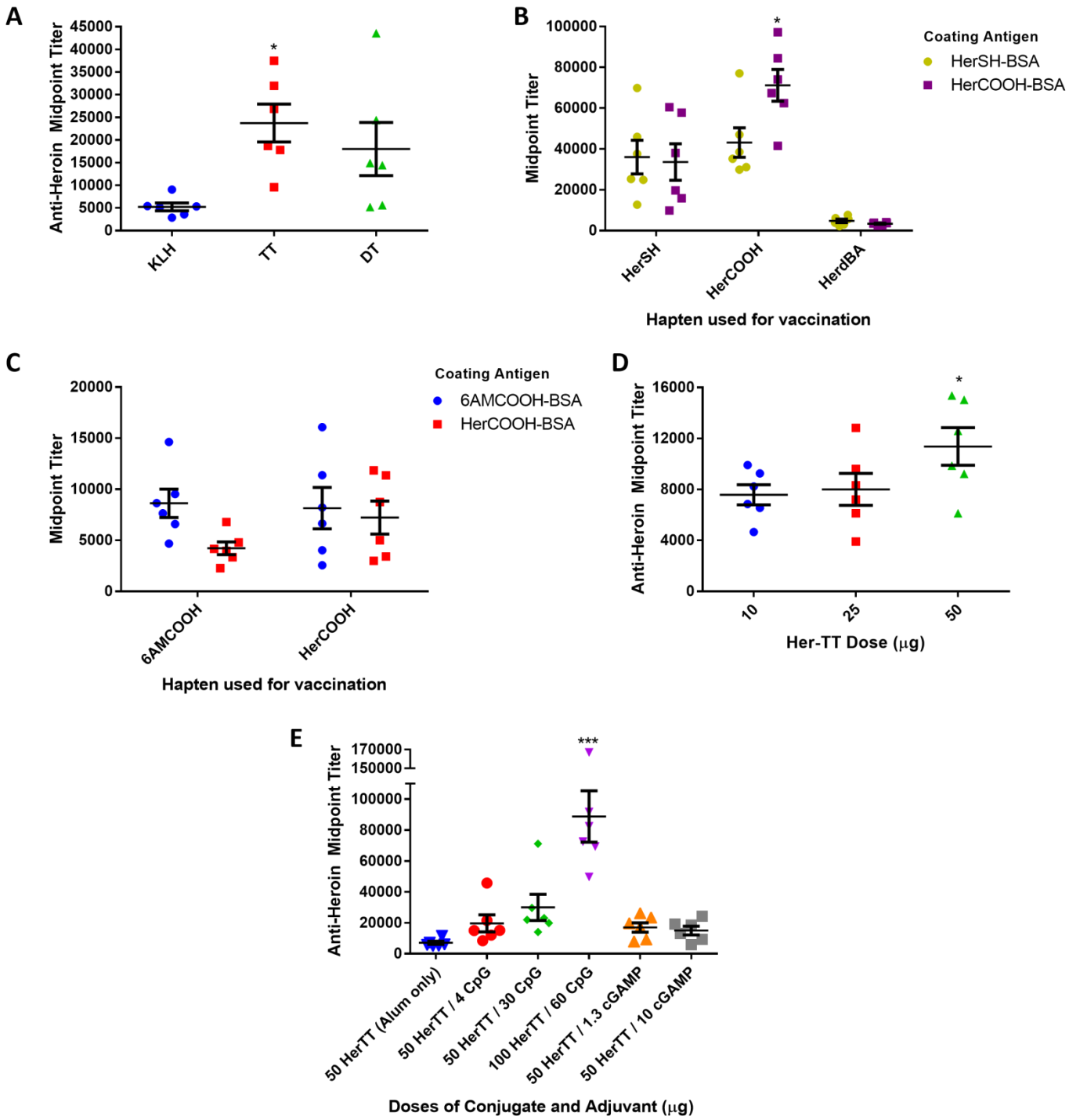

Figure S2. Anti-heroin titers in response to immunization with various heroin vaccine formulations. (**A**) Comparison of HerSH with various carrier proteins. **P* < 0.05 by one-way ANOVA with Dunnett's post-hoc test vs. KLH (**B**) Comparison of HerSH and HerCOOH haptens as heroin-TT conjugate vaccines. **P* < 0.05 by unpaired t-test. (**C**) Comparison of HerCOOH and 6AMCOOH haptens. No significant differences were detected. (**D**) Evaluation of various doses of HerCOOH-TT on vaccine efficacy without CpG ODN. **P* < 0.05 by one-way ANOVA with Fisher's post-hoc test vs. the 10 μg dose. (**E**) Effect of various adjuvants on vaccine-generated anti-heroin titers. ****P* < 0.001 by one-way ANOVA with Dunnett's post-hoc test vs. alum alone. For all panels, unless otherwise noted, mice (*n* = 6) were vaccinated with 50 μg heroin conjugate formulated with 30 μg CpG ODN 1826 and 0.75 mg alum at weeks 0, 2 and 4. Serum was collected at week 6 from individual mice and analyzed by ELISA for IgG midpoint titers against HerCOOH-BSA or the specified coating antigen. Titer mean ± SEM are reported for each group.

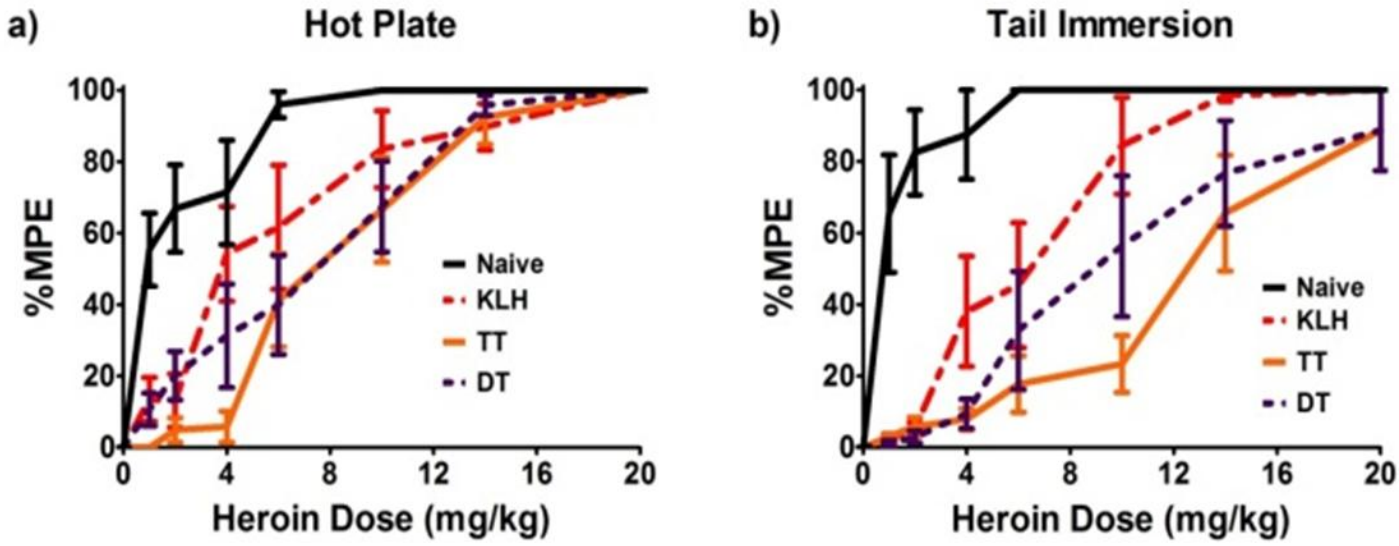

Figure S3A. Raw heroin antinociception curves corresponding to Figure 3A.

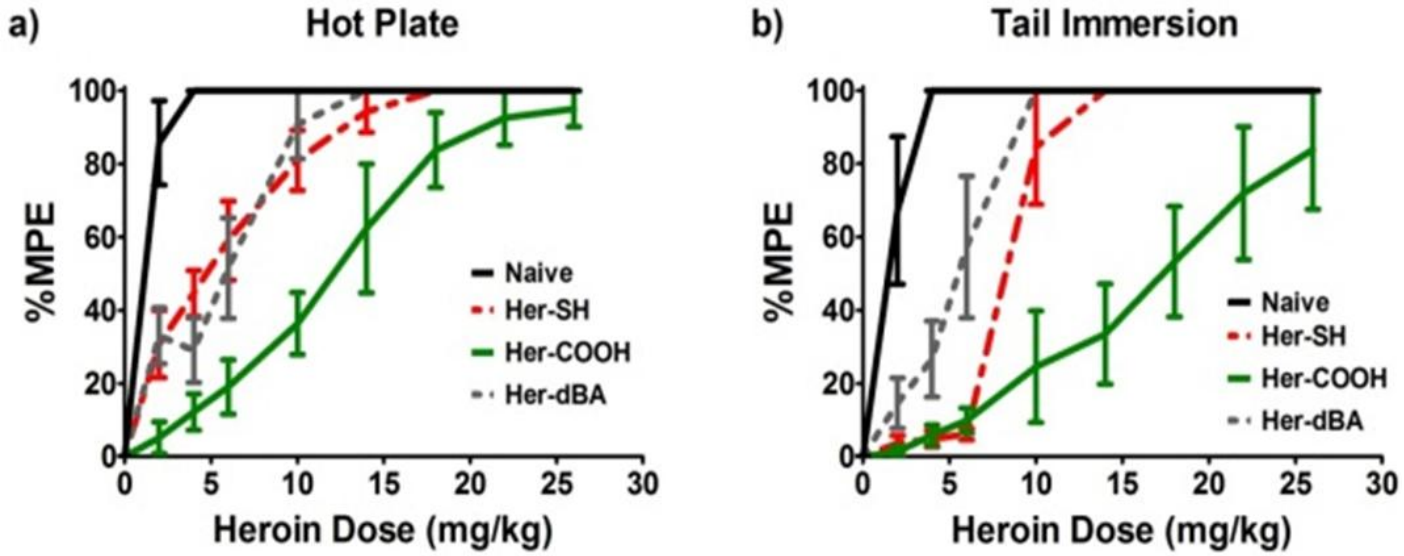

Figure S3B. Raw heroin antinociception curves corresponding to Figure 3B.

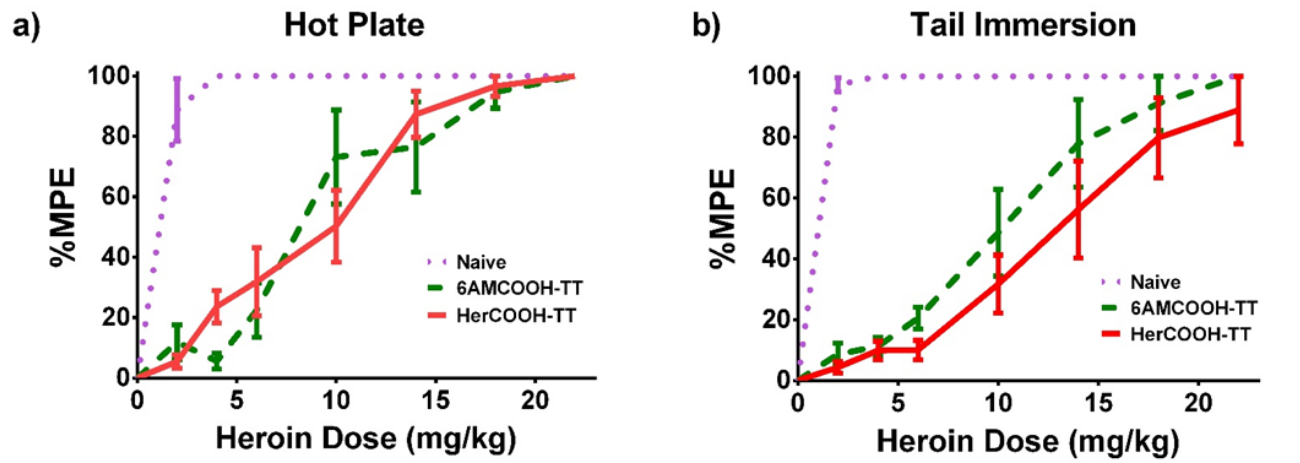

Figure S3C. Raw heroin antinociception curves corresponding to Figure 3C.

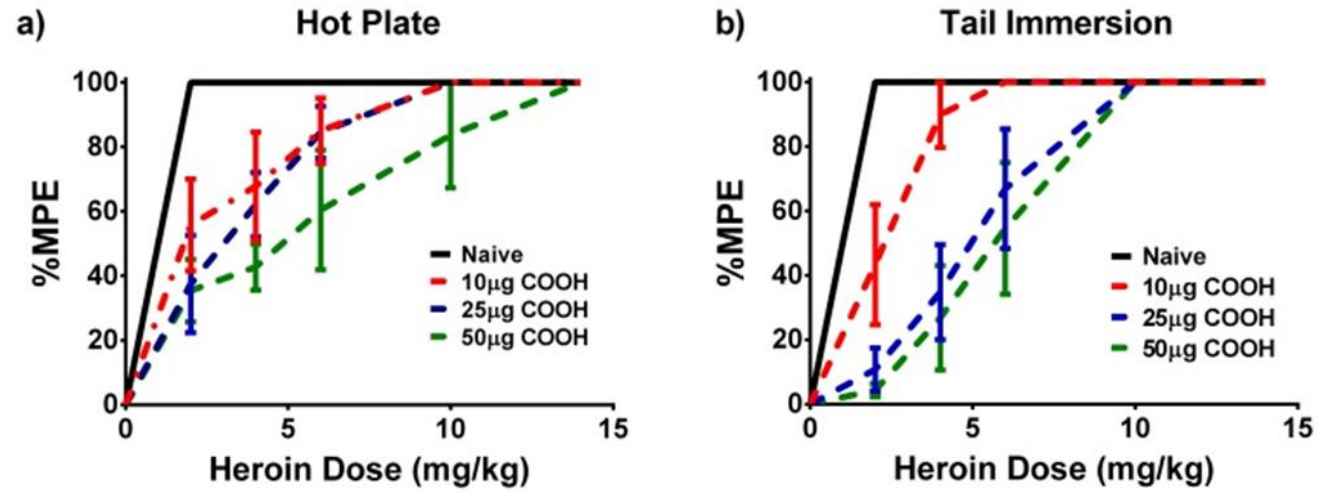

Figure S3D. Raw heroin antinociception curves corresponding to Figure 3D.

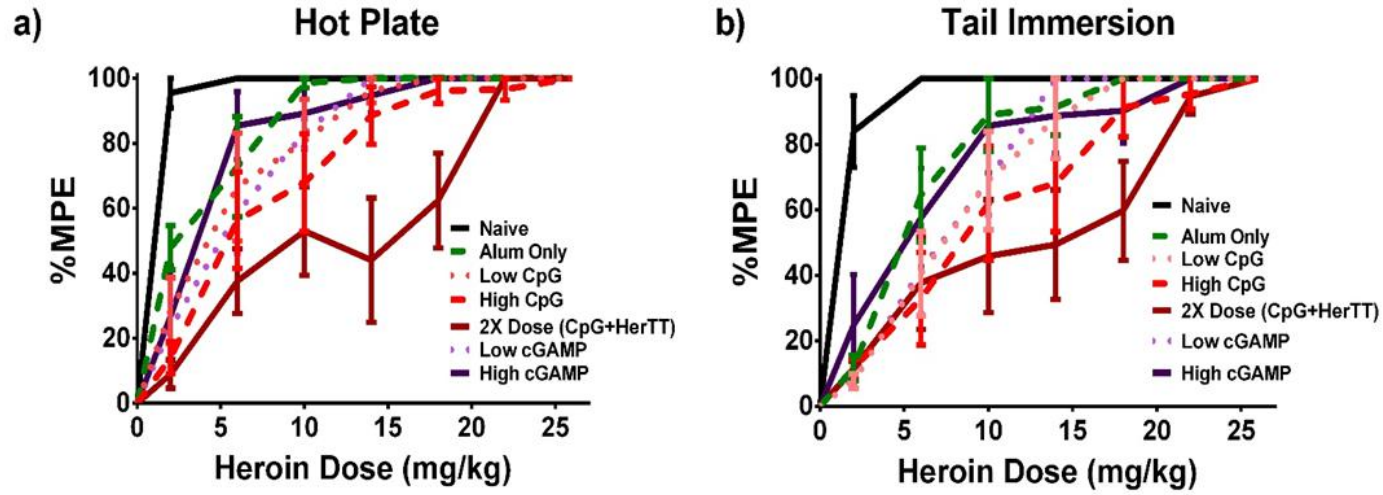

Figure S3E. Raw heroin antinociception curves corresponding to Figure 3E.

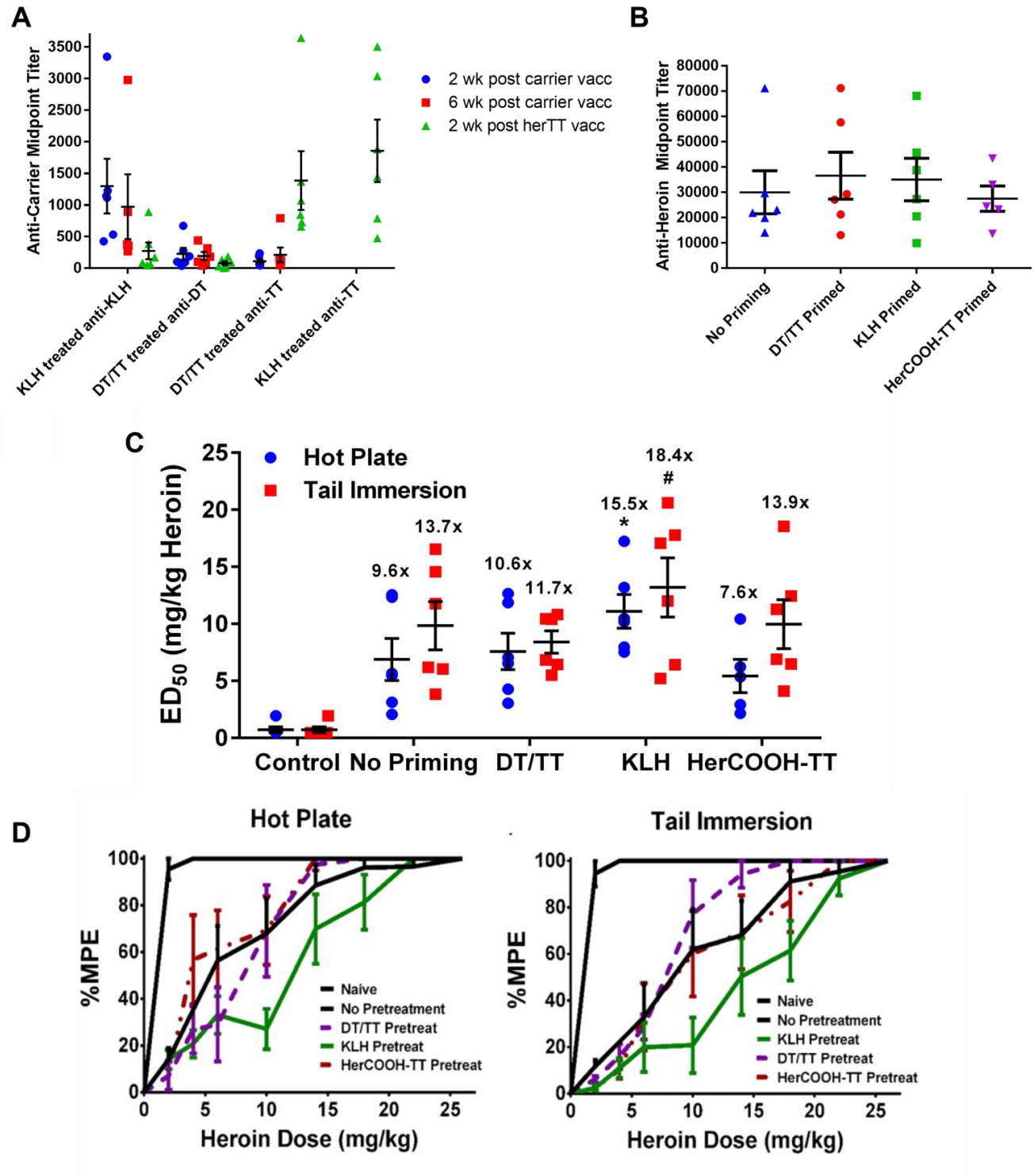

Figure S4. Carrier priming study establishes no effect of carrier immunization on subsequent response to the heroin vaccine. (**A**) Anti-carrier titers at weeks 6 (blue circles), 10 (red squares) and 16 (green triangles). Mice (n = 6 per group) were vaccinated with 5 µg TT/12.5 µg DT or 10 µg KLH in both cases with 0.75 mg alum i.p. at weeks 0, 2 and 4. (**B**) Anti-heroin titers following immunization with heroin vaccine. At week 10, 12 and 14, the same mice in addition to two other groups of n = 6 mice (vaccine naïve and HerCOOH-TT vaccinated) were immunized with 25 µg HerCOOH-TT + 15 µg CpG + 0.75 mg alum. (**C**) Cumulative heroin analgesic response in the hot plate and tail immersion antinociceptive tests. $^{\#}P < 0.05$ versus the unprimed dose group in the tail immersion test; $^{*}P < 0.05$ versus the unprimed dose group for the hot plate determined by a one-way ANOVA with Tukey's post-hoc test. Fold shifts compared to unvaccinated control mice are shown. (**D**) Raw curves corresponding to panel (C). All data are represented as mean ± SEM.

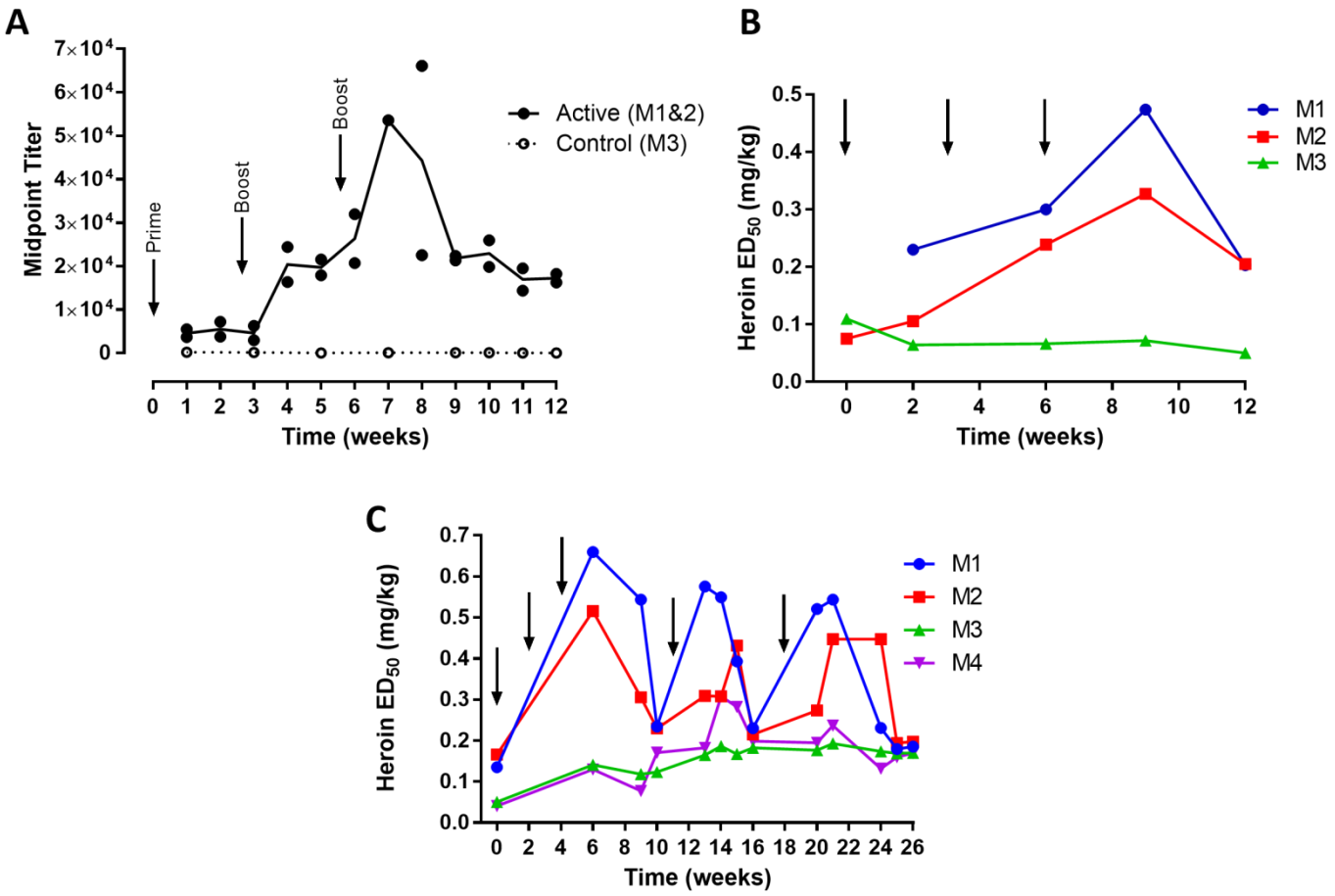

Figure S5. Heroin ED_{50} and anti-heroin titer timelines in rhesus macaques. (**A**) Anti-heroin titer timeline from rhesus monkey vaccine pilot study. Two monkeys (M1, M2) were vaccinated i.m. at the indicated time points with 330 µg HerCOOH-TT, 400 µg CpG ODN 2006 and 4 mg alum. One control monkey (M3) was vaccinated in the same manner but with 100 µg unconjugated TT per injection. Serum was collected and analyzed by ELISA against HerCOOH-BSA for IgG titers in duplicate. (**B**) Individual heroin ED_{50} values in pilot monkey vaccine study. Schedule-controlled responding (SCR) was performed throughout the pilot study to determine heroin ED_{50}s. The initial ED_{50} for M1 was excluded as an exceptionally high outlier. (**C**) Individual heroin ED_{50} values in the extended heroin vaccine study. Seven months following the pilot study, n = 4 rhesus monkeys were vaccinated i.m. at the indicated time points with 400 µg HerCOOH-TT, 600 µg CpG ODN 2006 and 5 mg alum. Schedule-controlled responding (SCR) was performed throughout the study to determine heroin ED_{50}s.

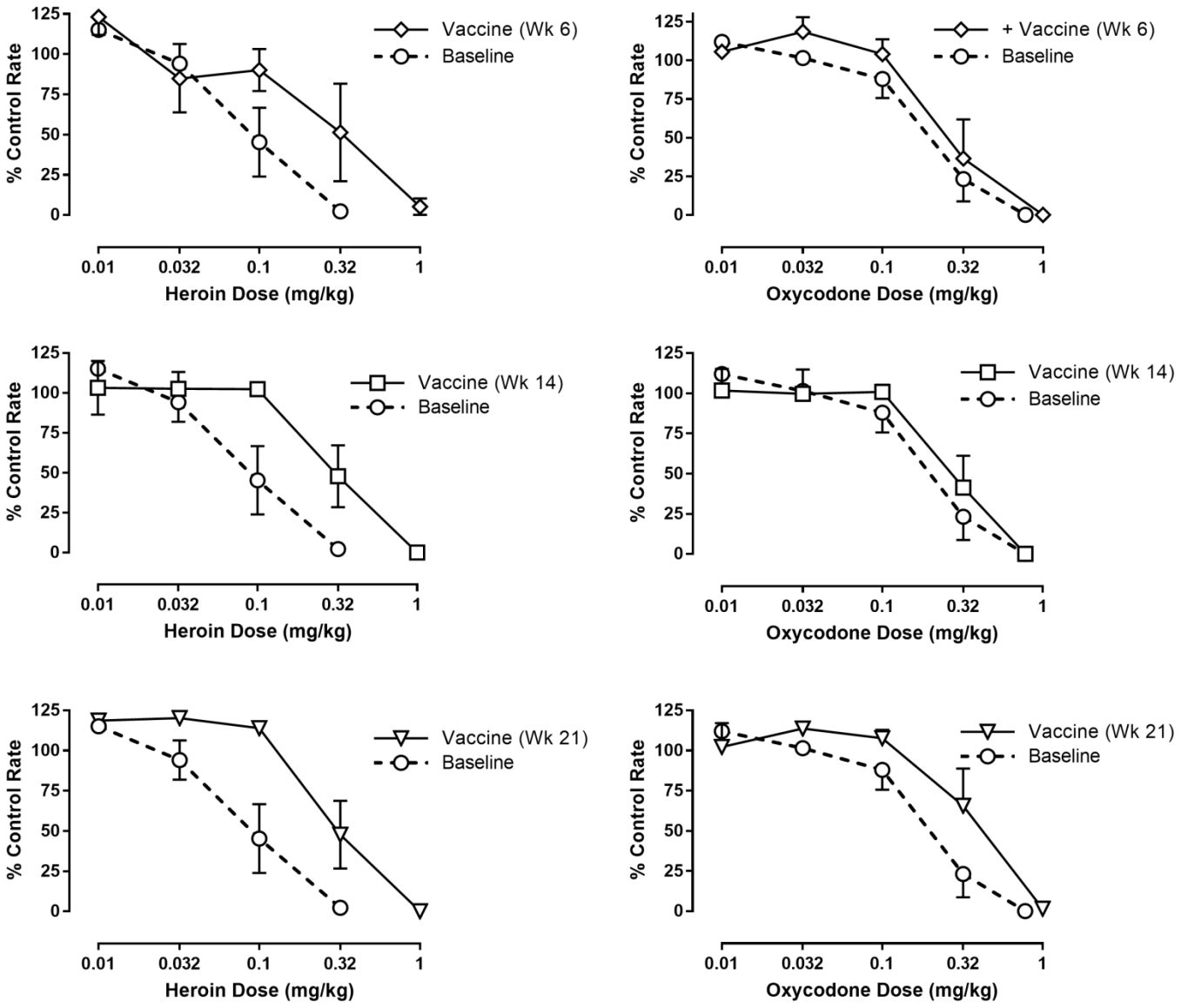

Figure S6. Heroin and oxycodone dose-effect curves in *n* = 4 rhesus macaques. SCR was used to determine the dose effect curves (DEC) throughout the 26 week study. Prior to vaccination, baseline DECs were determined for comparison to post-vaccination DECs. As mentioned in Figure 6A, DEC-derived heroin ED_{50} values were significantly different compared to the heroin baseline ED_{50} and compared to oxycodone ED_{50}s at weeks 6, 14 and 21, while oxycodone ED_{50}s were never statistically different from the baseline ED_{50} value. All data are reported as mean ± SEM.

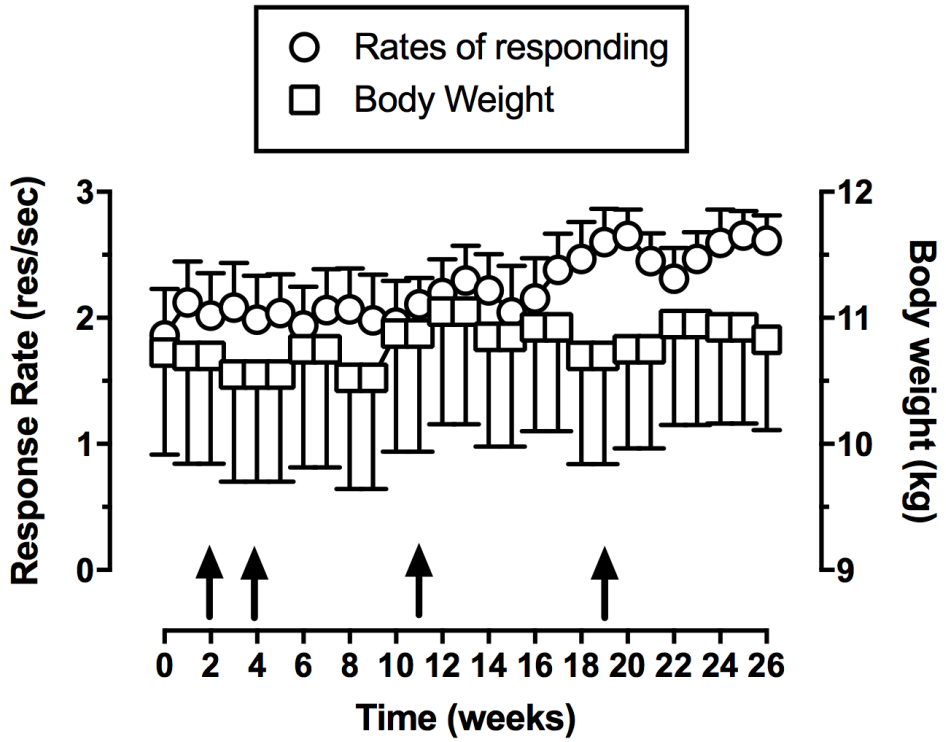

Figure S7. Vaccinated rhesus macaques maintained normal rates of responding and healthy weights throughout the study. Abscissa: time in weeks of the experimental period. Left ordinate: rates of operant responding in the assay of schedule-controlled responding (SCR) in responses per second. Right ordinate: body weight in kilograms. All points represent the mean ± SEM of four rhesus monkeys for all baseline behavioral SCR sessions for a given week. Heroin vaccine administration did not significantly decrease either rates of operant responding or body weight at any time during the 26-week experimental period. Furthermore, no injection site reactions were noted at any time.

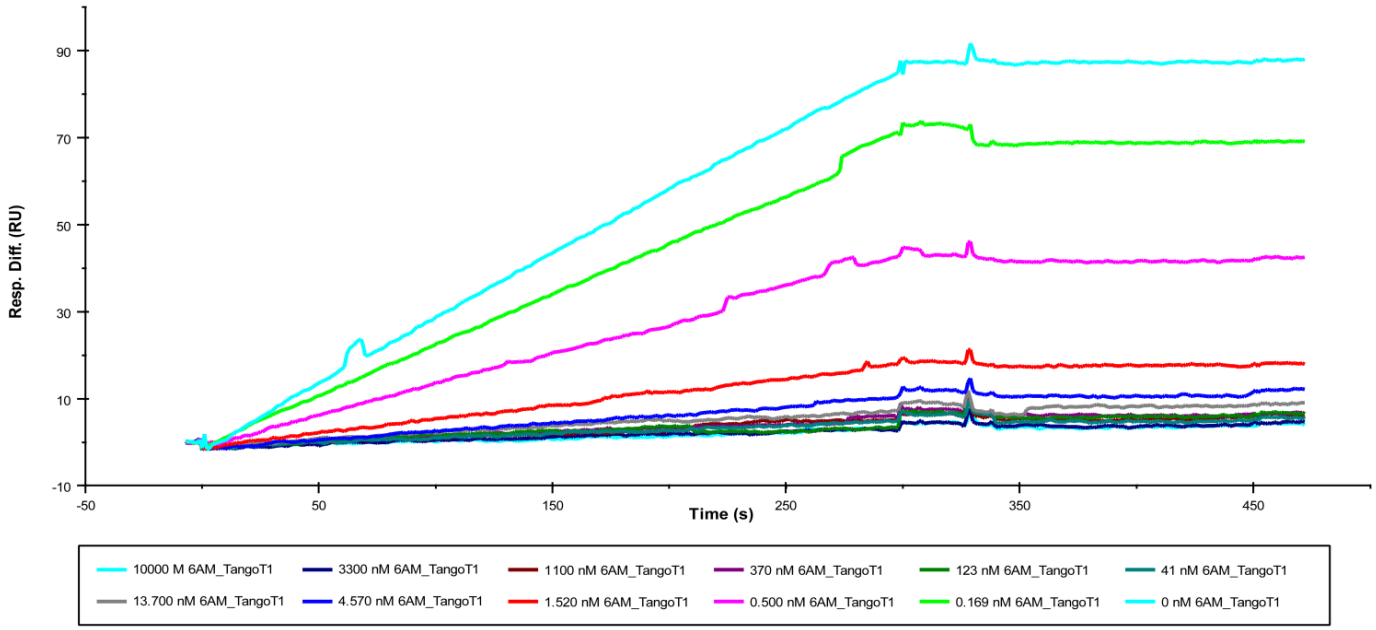

Figure S8. Representative sensorgram for determination of antiserum competitive IC_{50}s by SPR. Overlaid plots of sensorgrams obtained at 25 °C for the monkey antiserum binding to immobilized 6AMCOOH-BSA conjugate with free 6AM compound as a competitor at the specified concentrations.

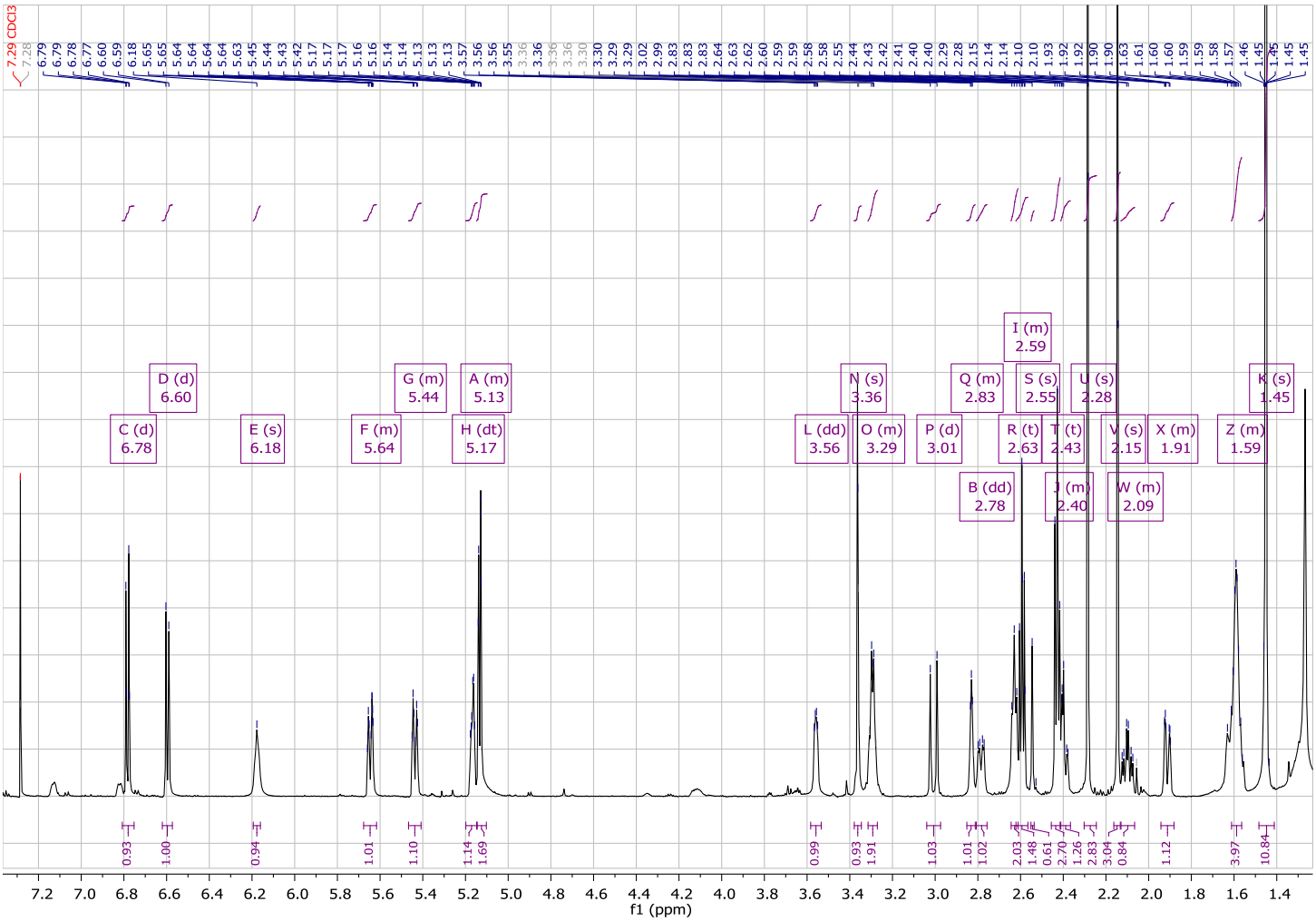

Figure S9. ^{1}H NMR spectrum of HerCOOH as the *tert*-butyl ester.

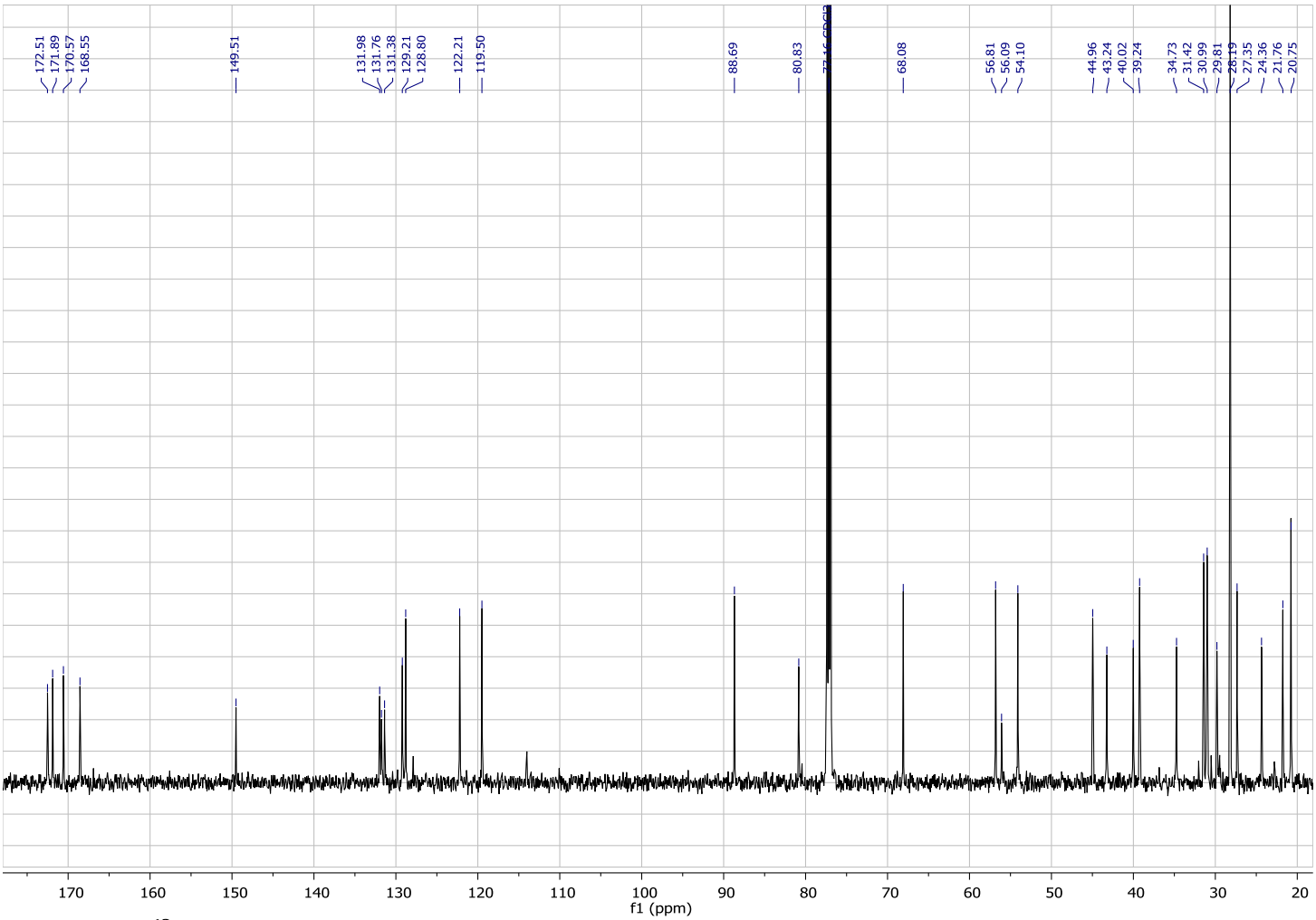

Figure S10. ^{13}C NMR spectrum of HerCOOH as the *tert*-butyl ester.

Supplementary References

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