Supporting Information
For

A Bioconjugate Leveraging Xenoreactive Antibodies to Alleviate Cocaine-Induced Behavior

Nicholas T Jacob, Kensaku Anraku, Atsushi Kimishima, Bin Zhou, Karen C Collins, Jonathan W Lockner, Beverly Ellis, Kim D Janda*

Department of Chemistry, Department of Immunology and Microbial Sciences, The Skaggs Institute for Chemical Biology, The Worm Institute for Research and Medicine (WIRM), The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Email address: kdjanda@scripps.edu

Materials and Methods:

General Methods. Unless otherwise stated, all reagents were procured from Sigma-Aldrich chemical company. All reations were carried out under nitrogen in anhydrous solvents unless otherwise stated. Analytical thin layer chromatography (TLC) was performed on Merck precoated analytical plates, 0.25 mm thick, silica gel 60 F254. Preparative TLC (PTLC) separations were performed on Merck analytical plates (0.50 mm thick) precoated with silica gel 60 F254. Flash chromatography separations were performed on Aldrich silica gel (60 Å pore size, 40-63 μm particle size, 230-400 mesh). ^{1}H and ^{13}C NMR spectra were obtained using a Bruker 500 or 600 MHz instrument unless otherwise noted. Chemical shifts were reported in parts per million (ppm, δ) referenced to the residual 1H resonance of the solvent ($CDCl_3$, 7.26 ppm) or (CD_3OD, 3.31 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77.0 ppm) or (CD_3OD, 49.0 ppm). The LC/MS analysis was performed using an Agilent G-1956D single quadrupole mass spectrometer equipped with an 1100 series LC system from Agilent Technologies. HPLC separations were performed on a Vydac 218TP C18 reversed phase preparative (10−15 μm) HPLC column using a gradient of acetonitrile and water. Protein concentration was determined by BCA assay (Pierce BCA Protein Assay Kit) with analysis on a plate reader (Molecular Devices SpectraMax 250) at 562 nm36. High resolution mass spectra were obtained in the Scripps Center for Mass Spectrometry. Electrospray Ionization (ESI) mass were obtained on a ThermoFinnigan LTQ Ion Trap, and characterization of protein conjugates was carried out using comparative MALDI-MS, using sinapinic acid as the matrix on an Applied Biosystems VoyagerDE STR MS.

Enzyme Expression. Expression was carried out as previously reported.[1] Briefly, the enzymes Galactose-4-epimerase (GalE) and α1,3-Galactosyltransferase (α1,3GalT) were overexpressed in the *E. coli* strain BL21(DE3) (Invitrogen) and purified using TALON® metal affinity resin (Clontech). Cell pellets were lysed with 5 mL of 0.2 g/L lysozyme (Sigma) in 20 mM PBS (pH 7.0) containing 1% Triton X-100, disrupted by gentle pipetting, incubated at 37°C on rotator for 20 min, treated with DNase I (New England Biolab), and incubated for 20 min at 37°C. Cell lysate was centrifuged at 16,000 rcf at 4°C for 20 min and supernatant added to 2 mL of TALON resin (Clonetech) pre-equilibrated with 20 mM PBS (pH 7.0) containing 0.3 M NaCl. The column was washed with 10 mL of 20 mM Sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl and the protein was eluted using a step gradient with increasing concentrations of imidazole (20, 50, 100, and 200 mM) in 20 mM Sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl. Protein purity in each 5mL fraction was visualized by SDS-PAGE. Fractions containing pure enzyme were pooled and concentrated to ~0.3-0.5 mL using an Amicon 4 centrifugal filter (MWCO 10-kDa) followed by dialysis against PBS. The final enzyme solution was added to equal volume of glycerol to make a 50% glycerol solution for storage at -20°C. Protein concentration was determined by BCA (Pierce). Overall protein yield from a 50 mL culture was ~1.5 mg for GalE and ~1.4 mg for α1,3GalT.

Immunoconjugation protocol. Dialyzed OVA, TT or BSA in 0.5M Borate buffer (pH 9.0) was added to **1** to give a final concentration of 1.25 mg/mL in 0.5 M Borate buffer (pH 9.0) and the solutions mixed at rt for 24 h. Hapten density for each conjugate was determined by MALDI-TOF mass analysis and compared to the MW of unmodified protein, per the formula: copy number = (MW αGal BSA – MW carrier) / (MW αGal – MW EtOH). The resulting protein conjugates were dialyzed into PBS (pH 7.4) at 4 °C. Compound **5** (5 mg) was dissolved in DMF:H_2O (9:1). To this was added 0.06 mmol sulfo-NHS and 0.06 mmol EDC. Mixture stirred at rt for ~3.5 h. This solution was concentrated to one-third of volume under reduced pressure, and added to 1 equivilent of carrier or **1**-carrier in PBS (pH 7.4). This mixture was stirred at 4 ºC overnight. The resulting solution was dialyzed into PBS (pH 7.0).

Animal Care and Use. All studies were performed in compliance with the Scripps Institutional Animal Care and Use Committee (La Jolla, CA), and were in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. Mice with disrupted α1,3galactosyltransferase genes[2] (6-10 week old) 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-9AM). All experiments were performed during the dark phase, generally between 1PM-4PM. General health was monitored by both the scientists and veterinary staff at The Scripps Research Institute. Mice were vaccinated against **1** as previously described.[1] For immunization with **6** or **7**, 50 μg conjugate in ~50 μL sterile PBS (pH 7.4) was combined with 50 μL of Alhydrogel® (10 mg/mL, Invivogen). The resulting suspension was mixed and subsequently administered to mice intraperitoneally. Each mouse was bled via retro-orbital sinus on indicated days. Blood samples were then centrifuged at 10,000 rpm for 10 min to collect sera for further analysis.

ELISA. Microtiter plates (Costar 3690) were incubated with coating antigen-BSA in PBS (4 μg/mL, 25 μL) for 18 h at 37 °C. 5% nonfat milk in PBS was added to block nonspecific binding for 30 mins at 37 ºC, then removed. Mouse sera was dilute in 2% BSA (1:100 for **1**, 1:50 for **5**) then serially diluted across the plate before incubation in a moist chamber (24 h, 4 °C). The plate was washed with dH_2O before incubation with HRP-conjugated secondary (goat anti-mouse IgG (Abcam) or goat anti-mouse IgM (SouthernBiotech)) in a moist chamber for 2 h at 25 °C. The plates were further washed with dH_2O before being developed with using TMB (Thermo Pierce) and the absorbance at 450 nm measured on a microplate reader (SpectraMax M2e Molecular Devices). Titers were calculated as the dilution corresponding to 50% of the maximum absorbance from a plot of the absorbance versus log(dilution) using Prism 6 (GraphPad). Subtyping end-point ELISA was carried out using an SBA Clonotyping System-HRP (SouthernBiotech, Birmingham, AL) according to manufacturers instructions using **1**-BSA coated microtiter plates. Subtyping of 10 normal human serum samples was carried out using the same procedure, using mouse anti-huIgG1 (ab99774, Abcam, Cambridge, MA), mouse anti-huIgG2 (ab99779, Abcam), mouse anti-huIgG3 (9210-05, SouthernBiotech), and mouse anti-huIgG4 (ab99826, Abcam).

Surface Plasmon Resonance. The Ki for mouse serum binding to **1** was determined by a competitive binding assay via surface plasmon resonance using a Biacore 3000 instrument (GE Healthcare) equipped with a research-grade CM5 sensor chip. The ligand, **1**-BSA conjugate, was immobilized using NHS, EDC coupling reaction. The surface of all flow cells were activated for 7 min with a 1:1 mixture of 0.1 M NHS and 0.1 M EDC at a flow rate of 5 μL/min. The ligand resuspended in 10 mM sodium acetate (pH 4.0) was immobilized at a density of 2,000 RU on flow cell 2; whereas flow cell 1 was immobilized with BSA at the same density to serve as a reference surface. All the surfaces were blocked with a 7 min injection of 1.0 M ethanolamine-HCl (pH 8.5). Mouse serum was diluted in running buffer (HBS-EP+ buffer) and titrated on both coated flow cells, so as to give a response of ~ 100 RU at the end of the injection with 5 min of injection and 2.5 min dissociation at a flow rate of 30 μL/min. Mouse serum at optimized binding concentration was incubated with **1** for 1 h at room temperature prior to injection. To collect binding data, the compound mixture was injected over the two flow cells at a flow rate of 30 μL/min at 25 °C for 5 min and was dissociated in buffer for 2.5 min before regeneration. The chip surface was regenerated by injection of 10 mM Gly-HCl (pH 2.2) for 30 seconds before the next injection. The response at the end of dissociation phase for each cycle of binding analysis was used to calculate the IC_{50} value for each compound by GraphPad Prism 6 software.

Blood-Brain LC-MS. Mice (n > 5/group) were administered 20 mg/kg cocaine in a 10 mL/kg volume of physiological sterile saline via intraperitoneal injection. After 15 mins, mice were fully anesthetized using isoflurane, rapidly decapitated, and trunk blood was collected. Whole blood samples were centrifuged at ~10000 rcf for 10 min, and the serum collected. To a 300 μL aliquot of serum sample, 120 μL of 0.6M aq. NaF was added. To a 100 μL aliquot of this solution was added 100 μL of 326 nM cocaine-D_3 in acetonitrile and vortex mixed for 30 s. After equilibration, solid-phase extraction was conducted by using an Oasis® HLB μElution plate (Waters Corp, Milford, MA). The brain tissue was removed and immediately flash-frozen in a -78 ºC bath. To each frozen brain was added 1 mL of 0.6M aq. NaF, homogenized using a Bullet Blender® (Next Advance, NY), and then centrifuged at ~1000 rpm for 10 min. To a 150 μL aliquot of the supernatant was added 150 μL of 326 nM cocaine-D_3 in acetonitrile and mixture vortexed for 30 s. After the equilibration, solid-phase extraction was conducted by using Oasis® HLB μElution plate (Waters Corp, Milford, MA). Mass spectrometry analysis was conducted on an Agilent 6100 Quadruple LC-MS system equipped with an Agilent ZORBAX SB-C8 column. Samples were run using H_2O+0.1% formic acid and MeCN+0.1% formic acid as the mobile phase. The percentage of H_2O+0.1% formic acid was linearly increased from 10-95% and the percentage of MeCN+0.1% formic acid was linearly decreased from 90-5% over a 7 minute run (500 μL/mL). Cocaine concentration was quantified via extraction and integration of the 304.35 [$MW+H^+$] mass peak using MassHunter (Agilent). Integration was normalized to the internal standard, cocaine-D_3. The normalized integration was compared to a 7-point standard curve for cocaine, made using blank mouse serum that had been spiked with known concentrations (0 to 1 μM) of cocaine.

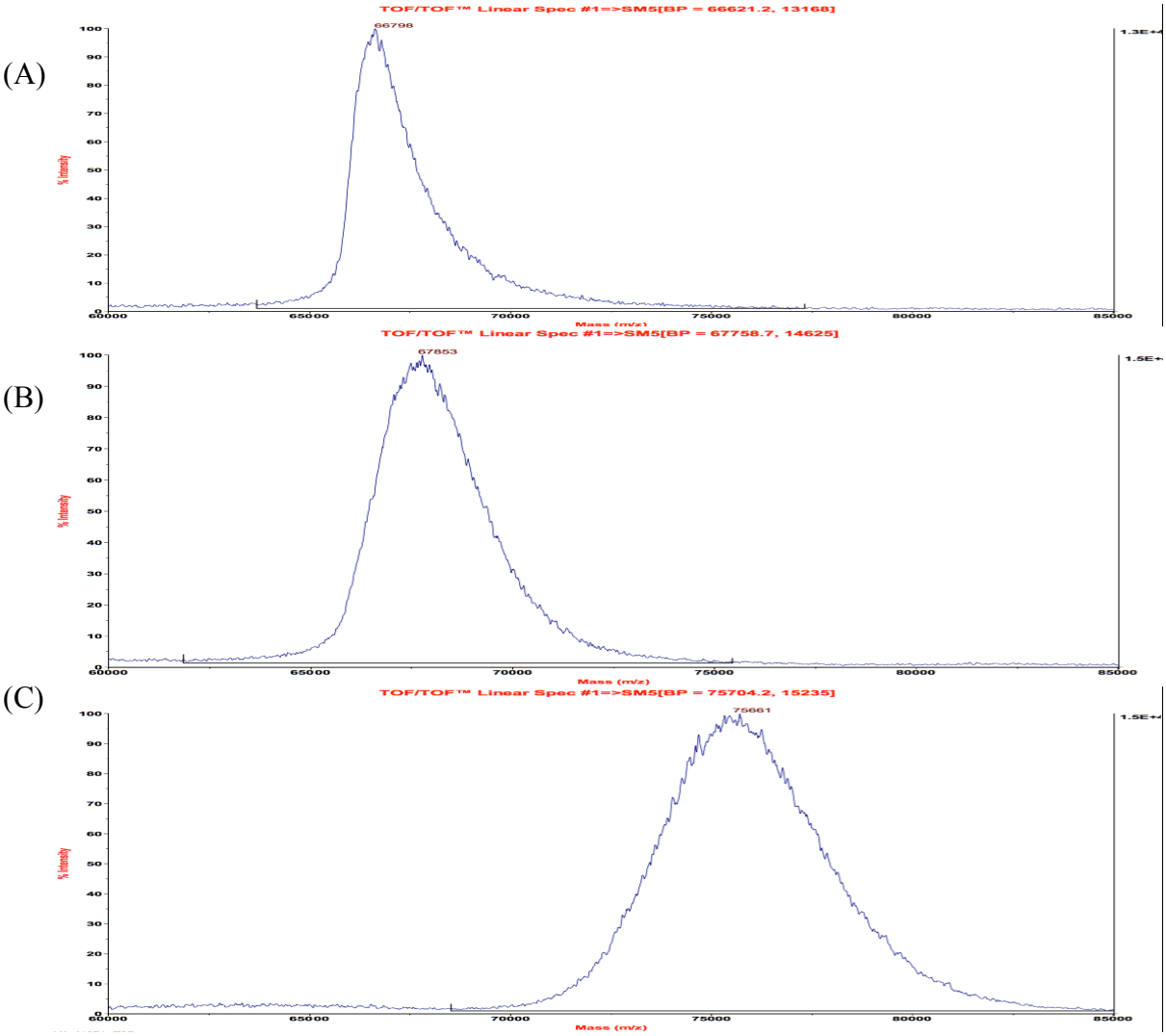
Open Field Hyperlocomotion. Animals were allowed to acclimate for one hour in a clean plastic cage (267 × 483 × 203 mm) with a clear ventilated acrylic top. Mice were then removed, injected IP with cocaine, and immediately returned to this cage to be recorded and tracked by an overhead camera for 90 min. Tests were conducted with a minimum 24 h washout period to clear cocaine before retesting. All behaviors were automatically scored using AnyMaze video tracking software (Stoelting) to avoid the introduction of bias and analyzed using Prism 6 (GraphPad).

RIA. Binding curves and competitive RIA were carried out on 5 kDa MWCO Equilibrium Dialyzer-96 plates (Harvard Apparatus, Holliston, MA) as described previously.[3] Briefly, pooled mouse serum was diluted in 2% BSA to a concentration that bound ~50% of levo-[benzoyl-3,4-^{3}H(N)]-cocaine tracer (PerkinElmer). Sample chambers were loaded with 75 µL of diluted serum and 75 µL of radiolabeled cocaine, and buffer chambers were loaded with 150 µL of unlabeled cocaine at indicated concentrations in 1% BSA. Dialysis plates were equilibrated at ambient temperature for 22 h, after which a 75 µL aliquot was removed from each side and diluted into 5 mL of scintillation fluid (Ecolite(+), MP Biomedicals, Santa Ana, CA). Radioactivity (dpm) was measured in a Beckman LS 6500 scintillation counter. Average dissociation constants and antibody concentrations were obtained using Microsoft Excel and GraphPad Prism 6.

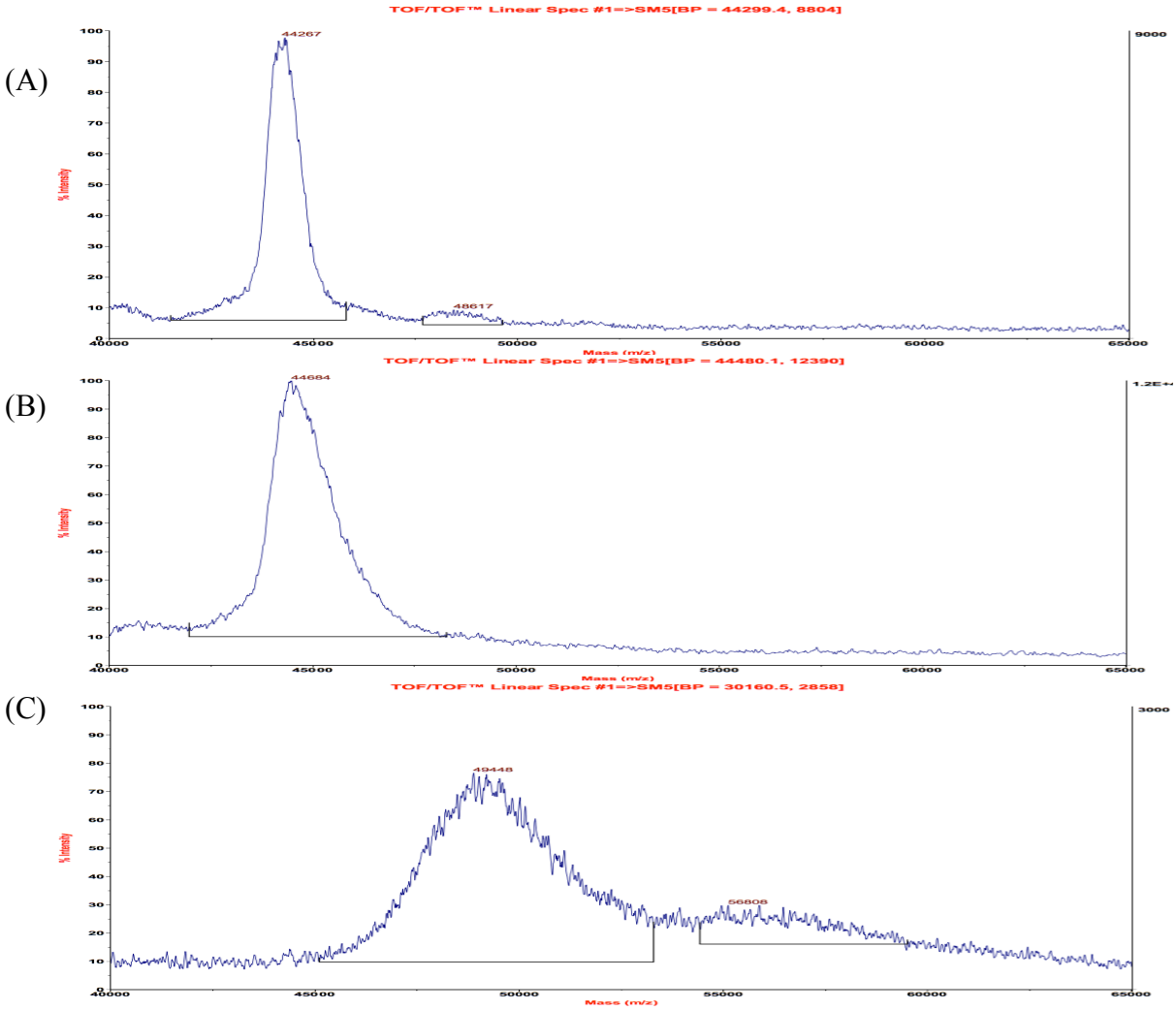
1 K. Anraku, S. Shun, N. T. Jacob, L. M. Eubanks, B. A. Ellis, K. D. Janda, *Org. Biomol. Chem*, 2017, **15**, 2979–2992.

2 A. D. Thall, P. Maly, J. B. Lowe. *J Biol Chem*, 1995, **270**, 21437-21440.

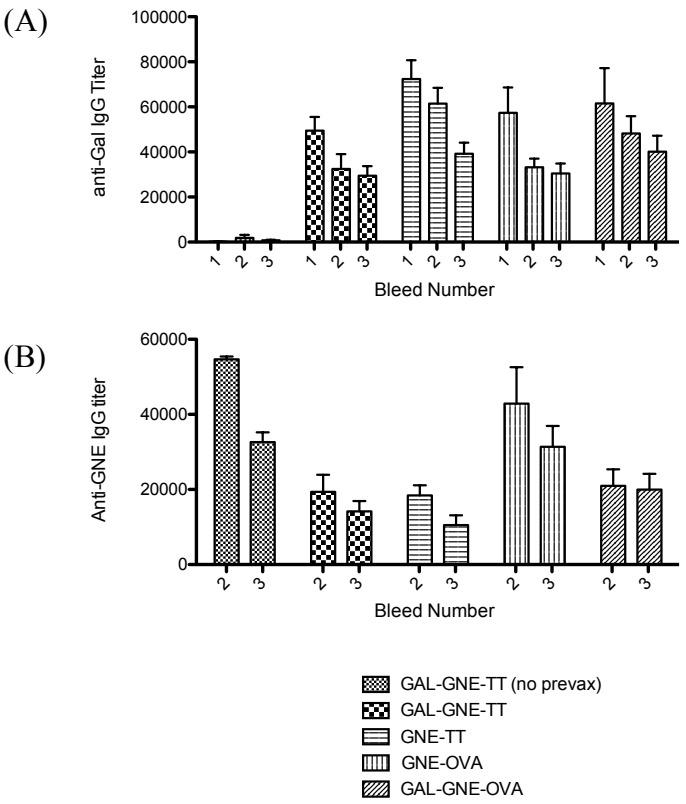
3 A. Kimishima, C. J. Wenthur, L. M. Eubanks, S. Sato, K. D. Janda, *Mol. Pharm*, 2016, **13**, 3884–3890.



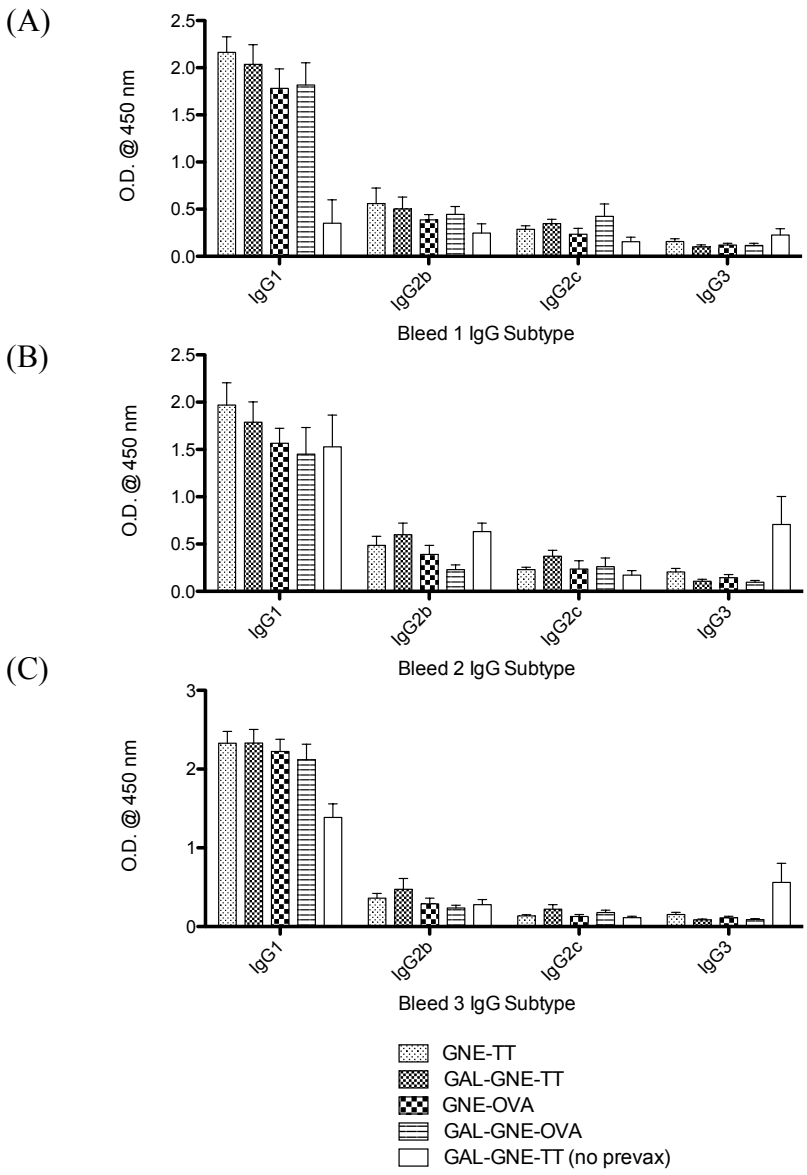
Supplementary Figure 1: MALDI-TOF spectra for TT conjugation. (A) Spectra for protein alone. (B) Spectra for **3**. (C) Spectra for protein **6**.



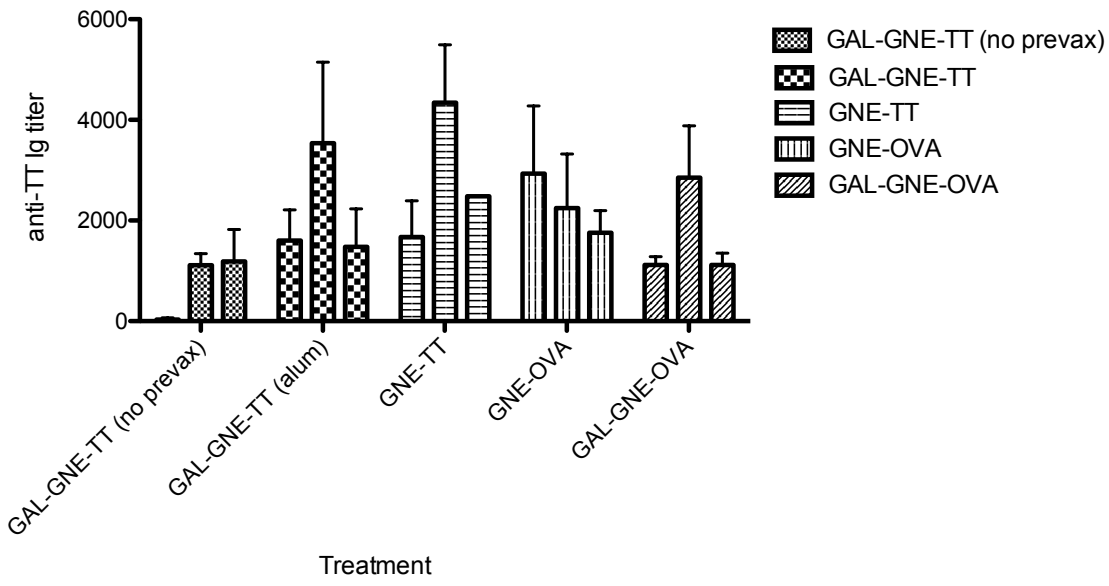
Supplementary Figure 2. MALDI-TOF spectra for OVA conjugation. (A) Spectra of protein alone. (B) Spectra of **4**. (C) Spectra of protein **7**.



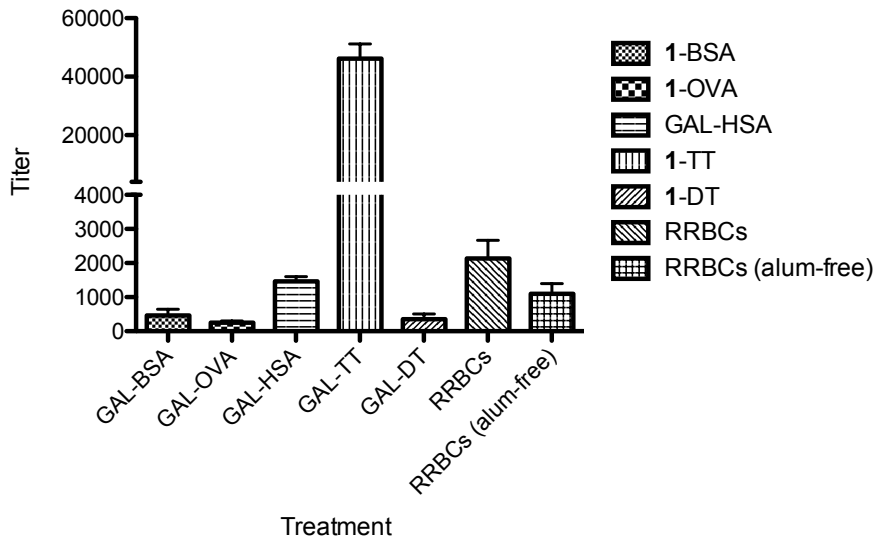
Supplementary Figure 3. (A) Anti-**1** IgG titers over bleeds. (B) Anti-**5** IgG titers for bleeds 2 and 3. Error bars represent SEM, $n \geq 5$.



Supplementary Figure 4: End-point O.D. values for isotyping of anti-**1** IgG. Error bars represent SEM, $n \geq 5$.

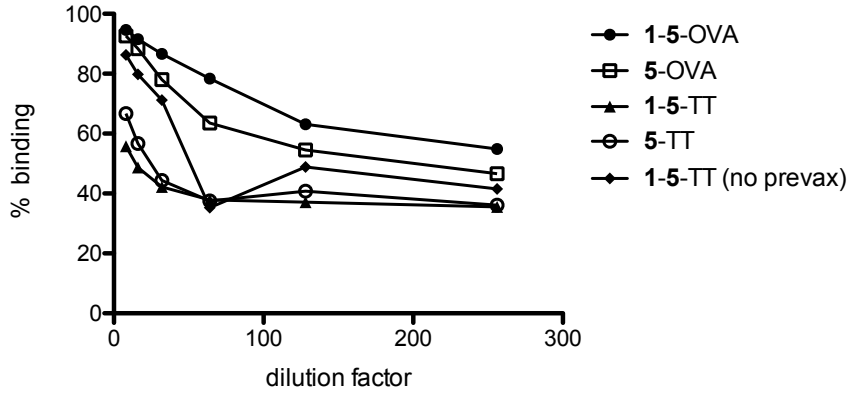


Supplementary Figure 5. Anti-TT titers measured by mid-point ELISA. Error bars represent SEM, $n \geq 5$.

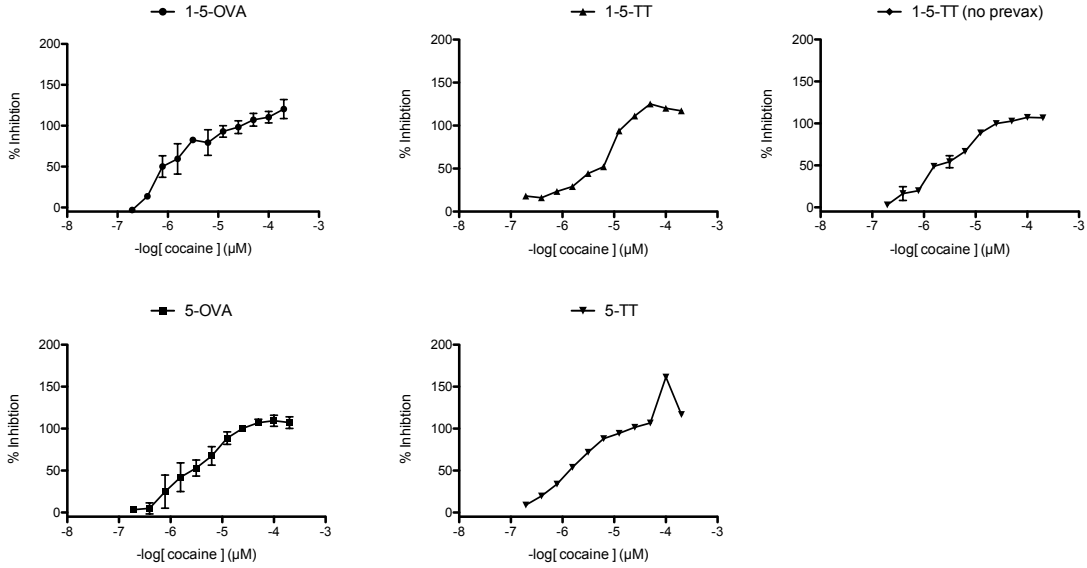


Supplementary Figure 6: Anti-**1** IgG titers from conjugates with differing carrier proteins. All treatments were formulated with alum unless otherwise noted. All conjugates of **1** synthesized as described. GAL-HSA procured from V-Labs, Inc (Covington, LA). RRBC – rabbit red blood cells. Error bars represent SEM, $n \geq 5$.

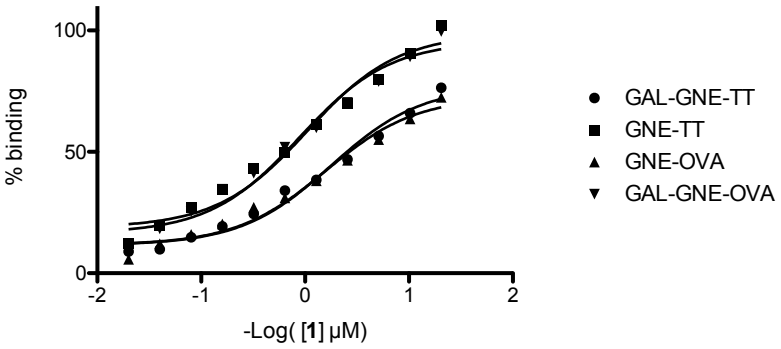
(A)



(B)



Extended Figure 1. (A) Binding curves of pooled serum samples for each treatment group for cocaine-^{3}H. Optimal binding for competitive assay is 50%. (B) Competitive binding curves for each group. IC_{50} values calculated using Prism 6. Error bars represent SEM, n = 2. The **1**-**5**-TT and **5**-TT groups required high concentrations of serum that did not allow for more than one replicate.

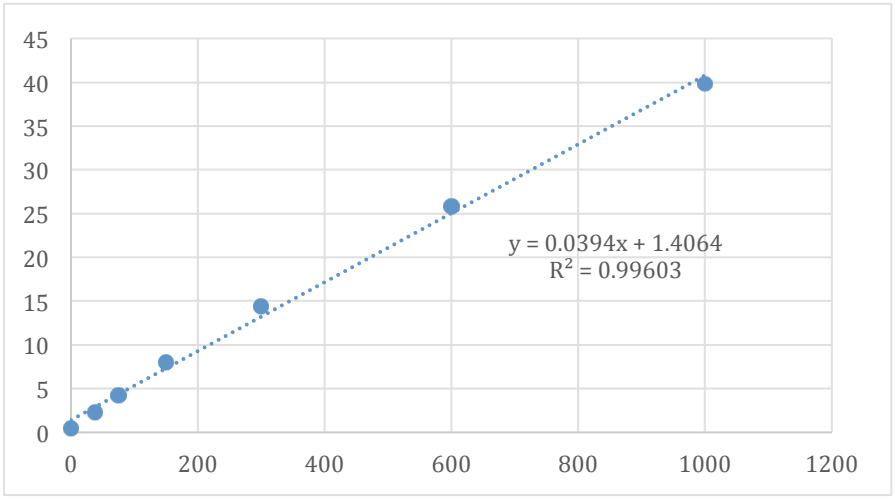


Extended Figure 2. Binding curves for determination of Ki for **1** of pooled serum samples by SPR.

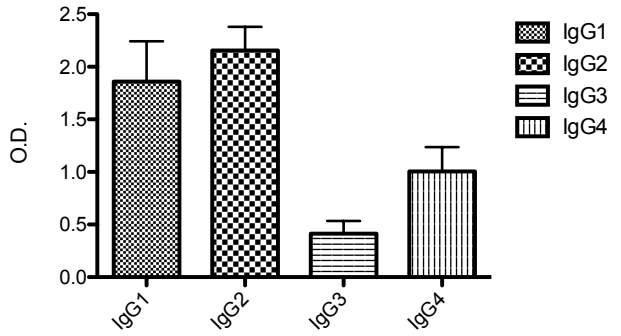
(A)

I.D.	Brain	cocaine	cocaineD	coca/cocaD	Cocaine (nM)		Blood	cocaine	cocaineD	coca/cocaD	Cocaine (nM)
2	PBS/ααTTGA	3253328	270740	12.01642905	269.2900773		PBS/ααTTGA	6119637	604632	10.12125888	221.1893117
3	αGal/TTGA	7580123	485199	15.62270944	360.8200367		αGal/TTGA	3362258	544790	6.171658804	120.9456549
4	αGal/ααTTGA	4083327	246953	16.53483456	383.9704204		αGal/ααTTGA	3424550	536086	6.388060871	126.4380932
5	αGal/GOA	5903271	416154	14.185304	324.3376649		αGal/GOA	8369475	460229	18.18545767	425.864408
6	αGal/ααGOA	4512514	355995	12.67577915	286.0248516		αGal/ααGOA	4596686	391665	11.73626952	262.1794293
8	αGal/TTGA	5053577	326782	15.4646737	356.8089771		αGal/TTGA	3284407	433191	7.581891129	156.7383535
10	αGal/GOA	6343063	322144	19.69014788	464.0545148		αGal/GOA	3719129	439718	8.45798671	178.974282
11	αGal/ααGOA	4731185	417033	11.34486959	252.2454211		αGal/ααGOA	4687882	342005	13.70705691	312.1994141
13	αGal/TTGA	7441915	333621	22.3064945	530.4592511		αGal/TTGA	2305292	235518	9.788177549	212.7354708
14	αGal/ααTTGA	5685178	413390	13.75257747	313.3547581		αGal/ααTTGA	2167267	166562	13.01177339	294.5526242
15	αGal/GOA	3786635	392312	9.652100879	209.2817482		αGal/GOA	2888152	218316	13.22922736	300.0717604
16	αGal/ααGOA	3771451	484742	7.780326442	161.7747828		αGal/ααGOA	3143916	212768	14.77626335	339.3366332
18	αGal/TTGA	7078501	407021	17.39099702	405.7004321		αGal/TTGA	2867179	355687	8.060960901	168.8974848
19	αGal/ααTTGA	4386511	637051	6.885651227	139.06729		αGal/ααTTGA	1875533	343283	5.463518438	102.9725492
20	PBS/ααTTGA	5657491	408218	13.85899446	316.0556969		PBS/ααTTGA	3797049	236182	16.07679247	372.3449866
21	αGal/GOA	4908184	360765	13.60493396	309.6074609		αGal/GOA	4320976	284318	15.1976871	350.0326674
22	αGal/ααGOA	3642035	355201	10.25344805	224.5443667		αGal/ααGOA	1757341	275641	6.375470267	126.1185347
23	PBS/ααTTGA	4989993	436176	11.44031996	254.6680194		PBS/ααTTGA	3521484	199626	17.64040756	412.0306488
25	αGal/TTGA	5237868	425873	12.29913143	276.4652648		αGal/TTGA	2675858	318066	8.412901725	177.829993
26	αGal/ααTTGA	7679948	473265	16.22758497	376.1722073		αGal/ααTTGA	2984374	459940	6.488615906	128.9902514
27	PBS/ααTTGA	13555478	778201	17.41899329	406.4109973		PBS/ααTTGA	3353447	286587	11.70132281	261.2924571
28	αGal/GOA	5791016	580802	9.970723241	217.3686102		αGal/GOA	3698250	316672	11.67848752	260.7128812
29	αGal/ααGOA	5836638	755926	7.721176411	160.273513		αGal/ααGOA	1829620	187235	9.771784122	212.3193939
31	αGal/TTGA	9812507	616263	15.92259636	368.4313797		αGal/TTGA	1599538	305935	5.228358965	97.00403465
32	αGal/ααTTGA	9865146	541558	18.21623169	426.6454743		αGal/ααTTGA	1816546	321053	5.658087605	107.9108529
33	αGal/GOA	6736115	550127	12.24465442	275.0825994		αGal/GOA	3355875	200254	16.75809222	389.6368584
34	αGal/ααGOA	7954796	482193	16.49712045	383.0132093		αGal/ααGOA	2792718	204315	13.66868806	311.2255852
35	PBS/ααTTGA	7767635	449194	17.29238369	403.1975556		PBS/ααTTGA	8648974	336260	25.72109082	617.1241326
36	naïve	4953364	459006	10.79150164	238.2005491		naïve	1455558	86326	16.8611774	392.2532334
37	naïve	8551333	348876	24.51109563	586.4135947		naïve	1765775	281428	6.274340151	123.5517805
38	naïve	11239743	484800	23.18428837	552.7382834		naïve	1686129	293439	5.746097145	110.1445976
39	naïve	1071967	581287	1.844126911	11.10982009		naïve	242094	116438	2.079166595	17.07529429
40	naïve	6023459	525998	11.45148651	254.9514342		naïve	674843	383610	1.759190324	8.954069125
41	naïve	5524281	456106	12.11183585	271.7115697		naïve	8783614	307826	28.53434733	688.5265819
42	naïve	6670150	665324	10.02541619	218.756756		naïve	1071967	581287	1.844126911	11.10982009

(B)



Extended Figure 3: (A) Raw integration values for cocaine and cocaine-d_3 , ratio, and calculated cocaine concentration for brain and blood samples. (B) Standard curve used to calculate concentration with equation.



Extended Figure 4. End-point O.D. values for normal human anti-**1** subtyping read at 450 nm. Error bars represent SEM, n = 10.