Supporting Information

Bivalent conjugate vaccine induces dual immunogenic response that attenuates heroin and fentanyl effects in mice

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Supplementary Methods

Materials and reagents

The NHS-(PEG)$_2$-maleimide crosslinker [(SM-(PEG)$_2$], spin desalting columns (Zeba™, 7K MWCO), dialysis cassettes (Slide-A-Lyzer G2™, 10K MWCO), Pierce™ bicinchoninic acid (BCA) protein assay kit, and the bovine serum albumin (BSA) that was used for coupling reactions were purchased from Fisher Scientific (Rockford, IL). Tetanus toxoid (TT) was purchased from Mass Biologics (Mattapan, MA). Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) was purchased from Quality Biological Inc. (Gaithersburg, MD). Lipids used to prepare liposomal adjuvant, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), monophosphoryl lipid A (3D-PHAD®) (MPLA), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Alhydrogel® was purchased from Brenntag (Reading, PA). Mouse anti-Tetanus Toxoid monoclonal antibody was purchased from Abcam (Cambridge, MA). Peroxidase-linked sheep anti-mouse IgG (γ-chain specific) was purchased from The Binding Site (San Diego, CA). The 2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS) peroxidase substrate system was purchased from KPL, Inc. (Gaithersburg, MD). Mass spectrometry grade water and acetonitrile (ACN), methanol (MeOH), and rapid equilibrium dialysis (RED) plates (12 kDa MWCO) were purchased from Fisher Scientific (Rockford, IL). Sodium fluoride was purchased from Sigma-Aldrich (Milwaukee, WI). Mass spectrometry standards, fentanyl (99.9%) and fentanyl-*d$_5$* (99.7%) were from Cerilliant (Round Rock, TX). Mass spectrometry standards, heroin•HCl, heroin-*d$_5$*, 6-acetylmorphine•HCl, 6-acetylmorphine-*d$_3$*, morphine•HCl, and morphine-*d$_3$* were from Lipomed Inc. (Cambridge, MA). Naloxone,

methadone, buprenorphine, fentanyl•HCl and heroin•HCl that were used for animal challenge experiments were from Cayman Chemical (Ann Arbor, MI).

Parameters used for LC-MS/MS analysis

The parameters used in the LC-MS/MS analysis were based on previous works.[1-3] The column was maintained at 65 °C at a flow rate of 500 µL/min. The injection volume was 10 µL using a full-loop injection mode using the gradient shown in **Table S1**. To avoid carryover, the autosampler needle was rinsed with a weak wash (600 µL, 10 % MeOH in H_2O) and a strong wash (200 µL, 90 % ACN in H_2O) before each injection. All data were acquired using positive electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode. The electrospray and source settings were as follows: 0.7 kV (capillary voltage), 120 °C (source temperature), 500 °C (desolvation temperature), 900 L/h (desolvation gas flow, N_2), and 60 L/h (cone gas flow, N_2). The collision gas (Ar) flow in the collision cell was maintained at 0.3 mL/min. MRM transitions are provided in **Table S2**. Data were processed using external calibration with $1/X^2$ weighting in TargetLynx™ application of MassLynx™ version 4.2 software (Waters, Milford, MA).

Table S1. LC-MS/MS gradient

Time (min)	% A (10 mM NH$_4$HCOO with 0.1% HCOOH)	% B (MeOH with 0.1% HCOOH)
0	100	0
0.50	100	0
2.70	90	10
3.30	80	20
4.60	20	80
4.61	0	100
5.20	0	100
5.21	100	0
8.00	100	0

Table S2. MRM transitions, cone voltage, and collision energy settings

Analyte	Ret. Time (min)	MRM[a] transition (*m/z*)	Cone voltage (V)	Collision energy (V)	Dwell time (msec)
Fentanyl-*d$_5$*	4.74	342>105	40	35	328
Heroin-*d$_3$*	4.46	373>165	40	40	328
6-acetylmorphine-*d$_3$*	3.96	331>165	40	35	328
Morphine-*d$_3$*	2.06	289>165	40	40	328

[a]All ions were detected as [M+H]$^+$

Table S3. Half-maximal inhibitory concentration (IC_{50}) of fentanyl, heroin, 6-acetylmorphine, and morphine measured using competition ED-LC-MS/MS[a]

Drug	IC_{50} (nM)[b]
Fentanyl	2.20±0.14
Heroin	2.00±0.40
6-Acetylmorphine	2.35±0.33
Morphine	2.36±0.43

[a]Using pooled, post-immune (week 16) sera
[b]Mean ±SD of triplicate determinations

Figure S1. Effect of adding NaF in equilibrium dialysis buffer on suppressing heroin degradation. Sera from immunized mice has been shown to protect sequestered heroin from degradation.[4] Thus in this experiment, sera from unimmunized mice were used. Sera were incubated with 5 nM of isotopically labeled drugs and dialyzed against DPBS, pH 7.4 supplemented with 3 mg/mL NaF. Drug levels in the sample and buffer chambers were quantified after 24 h and fraction bound was calculated. Data shown are mean ± SEM. Statistical comparison was made using two-tailed, paired t-test, significance was defined at $p \leq 0.05$.
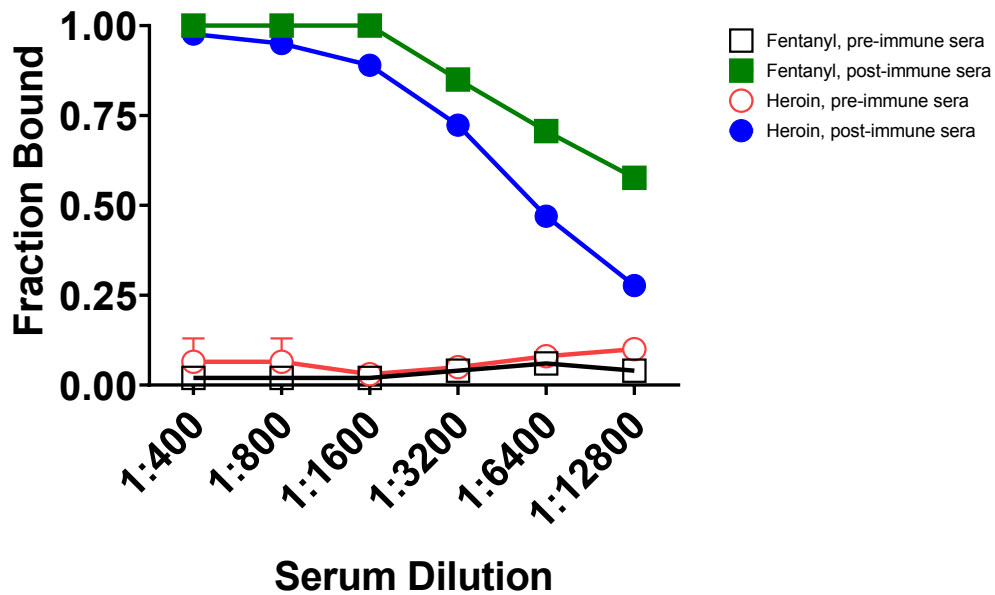
Figure S2. Serum sequestration of heroin + 9% (mol/mol) fentanyl *in vitro*. Pre-immune sera (week 0, red) and post-immune sera (week 16, blue) were diluted with a buffer that contained 5 nM of heroin spiked with 0.50 nM fentanyl (*i.e.* 9 mol% fentanyl in heroin) and dialyzed against buffer in an equilibrium dialysis plate. Drug concentrations in the sample and buffer chambers were determined after 24 h, and fraction bound was calculated. Data shown are mean ± SEM of triplicate determinations.
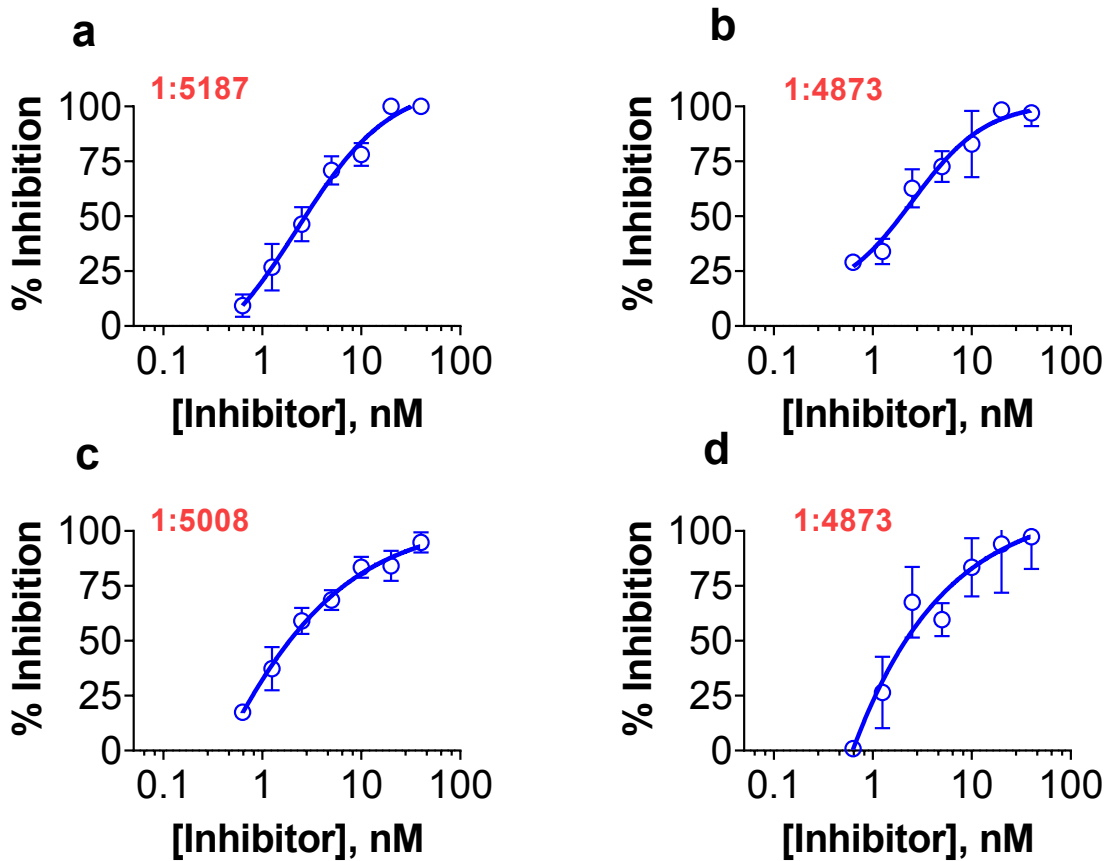
Figure S3. Competitive inhibition curves for the determination of IC_{50} and K_d. Post-immune sera (week 16, blue) were diluted with a buffer that contained 5 nM of isotopically labeled drugs at the indicated dilution that gave b = 0.4 to 0.7 value (indicated by in red font in each panel) and dialyzed against buffer containing 0 to 40 nM of their unlabeled versions in an equilibrium dialysis plate. Drug levels in the sample and buffer chambers were quantified after 24 h and fraction bound was calculated. a) fentanyl, b) heroin, c) 6-acetylmorphine, d) morphine. Data shown are mean ± SEM.

References

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