

## **Monoclonal antibodies counteract opioid-induced behavioral and toxic effects in mice and rats.**

<sup>1,2</sup>Carly Baehr, <sup>1,3</sup>April Huseby Kelcher, <sup>1</sup>Aaron Khaimraj, <sup>4</sup>Dana E. Reed, <sup>4</sup>Sujata G. Pandit, <sup>4</sup>David AuCoin, <sup>5</sup>Saadyah Averick, and <sup>1,6</sup>Marco Pravetoni

<sup>1</sup>University of Minnesota Medical School Department of Pharmacology, <sup>2</sup>Department of Veterinary Population Medicine, <sup>3</sup>Department of Psychiatry and Behavioral Sciences; <sup>4</sup>University of Nevada, Reno School of Medicine, <sup>5</sup>Allegheny Health Network, Pittsburgh, PA, and <sup>6</sup>University of Minnesota Center for Immunology.

**Running title:** Development of mAb to counteract opioids

Corresponding author: Marco Pravetoni, [prave001@umn.edu](mailto:prave001@umn.edu)

Address: University of Minnesota Department of Pharmacology, Nils Hasselmo Hall, 312 Church St SE,  
Minneapolis, MN 55455

Phone: 612-625-6243 Fax: 612-625-8408

Text pages: 16

Number of tables: 0

Number of figures: 5

References: 46

Abstract: 189 words

Introduction: 749 words

Discussion: 830 words

Abbreviations: mAb, monoclonal antibody; OUD, opioid use disorder; BSA, bovine serum albumin;

OVA, ovalbumin; PE, phycoerythrin; sKLH, subunit keyhole limpet hemocyanin; 6-AM, 6-monoacetyl  
morphine

Section assignment: Drug Discovery and Translational Medicine or Neuropharmacology

## ABSTRACT

Monoclonal antibodies (mAb) and vaccines have been proposed as medical countermeasures to treat opioid use disorder (OUD) and prevent opioid overdose. In contrast to current pharmacotherapies (e.g., methadone, buprenorphine, naltrexone, and naloxone) for OUD and overdose, which target brain opioid receptors, mAb and vaccine-generated polyclonal antibodies sequester the target opioid in the serum and reduce drug distribution to the brain. Further, mAb offer several potential clinical benefits over approved medications, such as longer serum half-life, high selectivity, reduced side effects, and low abuse liability. Using magnetic enrichment to isolate opioid-specific B cell lymphocytes prior to fusion with myeloma partners, this study identified a series of murine hybridoma cell lines expressing mAb with high affinity for opioids of clinical interest, including oxycodone, heroin and its active metabolites, and fentanyl. In mice, passive immunization with lead mAb against oxycodone, heroin, and fentanyl reduced drug-induced antinociception and the distribution of the target opioid to the brain. In mice and rats, mAb pre-treatment reduced fentanyl-induced respiratory depression and bradycardia, two risk factors for opioid-related overdose fatality. Overall, these results support use of mAb to counteract toxic effects of opioids and other chemical threats.

### **Significance Statement.**

The incidence of fatal overdoses due to the widespread access to heroin, prescription opioids, and fentanyl suggests that current FDA-approved countermeasures are not sufficient to mitigate the opioid epidemic. Monoclonal antibodies (mAb) may provide acute protection from overdose by binding to circulating opioids in serum. Use of mAb prophylactically, or post-exposure in combination with naloxone, may reduce hospitalization and increase survival.

## INTRODUCTION

An estimated 2.5 million people in the United States are living with an opioid use disorder (OUD), and 67,000 fatal drug overdoses occurred in the US in 2018, of which 70% involved opioids (Centers for Disease Control and Prevention, 2020). Current interventions for OUD consist of pharmacological agonists (methadone), partial agonists (buprenorphine), and antagonists (naloxone and naltrexone) targeting the opioid receptors in the brain to exert therapeutic effects. Although opioid pharmacotherapy has substantial clinical utility in medication-assisted treatment for OUD, and naloxone is a critical emergency medication for reversing opioid overdose, these medications have been insufficient to curb the prevalence of OUD and incidence of overdose (Sharma *et al.*, 2017; Han *et al.*, 2019). Limitations of current medications include undesirable side effects, abuse liability or diversion of agonists, the need for detoxification prior to initiation of antagonist treatment to avoid symptoms of precipitated withdrawal, and the requirement for frequent dosing, which presents a high burden of compliance. Consequently, complementary or alternative therapies are needed to supplement current medications.

Immunotherapeutics, consisting of monoclonal antibodies (mAb) and vaccines, offer a promising strategy to treat OUD and reduce the incidence of overdose (reviewed in (Bremer and Janda, 2017; Pravetoni and Comer, 2019)). Monoclonal antibodies and vaccine-induced polyclonal antibodies selectively alter the pharmacokinetics of the target drug through binding and sequestration of drug molecules in serum, preventing distribution to the brain without directly affecting receptor signaling. Both mAb and vaccines may offer several advantages over opioid antagonists, including fewer side effects; additionally, pharmacotherapy may require controlled detoxification in order to prevent precipitated withdrawal (Jarvis *et al.*, 2018; Rzasz Lynn and Galinkin, 2018), whereas mAb and vaccines are not expected to alter endogenous opioid signaling, nor require detoxification (Raleigh *et al.*, 2020). Additionally, antibodies typically exhibit high specificity for their target with little cross-reactivity for structurally distinct opioids or opioid antagonists (Raleigh *et al.*, 2017). Therefore, mAb and vaccines can be considered both as an alternative and as a supplement to existing small molecule therapies for OUD.

Anti-opioid vaccines have demonstrated pre-clinical efficacy and selectivity in reducing opioid brain distribution, opioid-induced respiratory depression and antinociception, intravenous self-administration, and lethality in rodent and non-human primate models (Pravetoni *et al.*, 2013; Bremer *et al.*, 2017; Raleigh *et al.*, 2017; Nguyen *et al.*, 2018; Sulima *et al.*, 2018; Tenney *et al.*, 2019). However, efficacy of anti-drug vaccines is dependent on generation of high concentrations of polyclonal antibodies, which may require multiple immunizations over weeks or months. Further, active immunization may only achieve sufficient antibody concentrations in a subset of individuals (Cornuz *et al.*, 2008; Martell *et al.*, 2009; Kosten *et al.*, 2014). In contrast, direct administration of high-affinity drug-specific mAb would provide almost immediate protection against the target drug, and allow for greater control over serum antibody concentration. Drug-targeting mAb have demonstrated pre-clinical efficacy against cocaine, methamphetamines, nicotine, and opioids (Fox *et al.*, 1996; Keyler *et al.*, 2005; Kashanian *et al.*, 2015; Pravetoni, 2016; Kvello *et al.*, 2019; Marckel *et al.*, 2019; Smith *et al.*, 2019). Additionally, favorable safety and pharmacokinetic profiles for a chimeric mAb against methamphetamine support the clinical translation of mAb for OUD (Stevens *et al.*, 2014).

Decades after its invention, hybridoma technology remains an effective method for generation of novel mAb. However, fusion of Ab-expressing cells with myeloma fusion partner cells is a stochastic event, and isolation of desired clones stably expressing mAb against the antigen of interest often requires screening of hundreds of clones. To streamline the generation of hybridomas, it has been reported that antigen-based magnetic enrichment can be used to pre-select target-specific B cells prior to hybridoma fusion (Spanier *et al.*, 2016). Magnetic enrichment or “baiting” is frequently employed to increase a desired cell population for flow cytometry analysis (Boonyaratankornkit and Taylor, 2019), and single-cell sorting has been utilized for isolation antigen-specific cells for development of recombinant mAb against a variety of targets and in multiple species (Smith *et al.*, 2009; Ho *et al.*, 2016; Starkie *et al.*, 2016; Lei *et al.*, 2019). Using an antigen-based enrichment platform previously validated for flow cytometry analysis of opioid-specific B cell populations (Taylor *et al.*, 2014; Laudénbach *et al.*, 2015), hybridomas were isolated from mice vaccinated against three commonly misused opioids: oxycodone,

heroin, and fentanyl. The mAb isolated using this method demonstrated binding to their target drug *in vitro*, as well as *in vivo* efficacy in reducing opioid distribution and behavioral effects when administered in rodent models, supporting further pre-clinical development of opioid-targeting mAb as a therapy to treat OUD and prevent overdose.

## MATERIALS AND METHODS

**Animals.** All procedures were approved by the Institutional Animal Care and Use Committees of the University of Minnesota and Hennepin Healthcare Research Institute, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition, National Academies Press). Male and female Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were 7 weeks on arrival, and male Sprague Dawley rats (Envigo, Indianapolis, IN) were 8-10 weeks on arrival. All animals were housed under standard conditions with a 12/12 hour light/dark cycle and given food and water *ad libitum*.

**Haptens and conjugates.** The oxycodone (OXY), morphine (M), and fentanyl (F) haptens containing a tetraglycine [(Gly)<sub>4</sub>] linker were synthesized as previously described (Pravetoni *et al.*, 2012; Raleigh *et al.*, 2013, 2019), and then conjugated via carbodiimide chemistry (Baruffaldi *et al.*, 2018) to subunit keyhole limpet hemocyanin (sKLH) carrier protein for immunogens, phycoerythrin (PE) for magnetic enrichment, and either ovalbumin (OVA) or bovine serum albumin (BSA) for screening assays. An additional fentanyl-based hapten (Li *et al.*, 2017) containing a biotin moiety was used for antibody characterization by biolayer interferometry (BLI, Suppl. Fig 6).

**Active immunization and hybridoma fusion.** Male Balb/c mice (n=4 per group) were immunized on days 0 and 28 (vaccine formulations detailed in Suppl. Table 1), and pooled lymph nodes and spleens were collected 4 days after the second immunization. The antigen-based magnetic enrichment procedure to isolate opioid-specific B cells was performed as described in (Robinson *et al.*, 2019). Briefly, tissues were processed to a single-cell suspension, and cells were pelleted at 1600 rpm for 5 min. Pellets were resuspended in DMEM, the opioid-based hapten conjugated to PE was added to a final concentration of 6.7 nM, and the mixture was incubated 25 min at room temperature. Cells were washed with 10 mL DMEM, and pellet was resuspended in 125  $\mu$ L DMEM with 25  $\mu$ L anti-PE microbeads (Miltenyi Biotec, Auburn, CA). Cells and beads were incubated for 15 min at room temperature, and antigen-specific cells collected using magnetic separator columns (Miltenyi). Columns were washed with DMEM and antigen-specific cells were eluted with 5 mL ClonaCell-HY Medium A (StemCell Technologies, Cambridge,

MA). The enriched antigen-specific cells were counted, washed with serum-free media, and fused with Sp2/0Ag14 (ATCC® CRL1581™, American Type Culture Collection, Manassas, VA) mouse myeloma cells at a 1:5 ratio using the ClonaCell-HY Hybridoma kit (StemCell) according to manufacturer's recommended protocol. Fused hybridoma cells were grown with HAT selection for 10-14 days at 37°C and 5% CO<sub>2</sub>, and visible colonies were transferred to 96-well plates containing 100 µL culture medium per well and incubated for 2-4 days prior to screening.

**Hybridoma screening.** To screen for antigen-positive colonies by ELISA, 96-well plates were coated with 5 ng/well OXY-OVA, M-BSA or F-BSA, blocked with 1% gelatin, and incubated with 50 µL conditioned medium diluted 1:1 with PBS-T for 2 hours. Plates were washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Jackson) overnight, and HRP activity was measured using SigmaFast OPD substrate (Millipore Sigma) with absorbance read at 492 nm on Tecan Infinite M1000 microplate reader (Tecan, Männedorf, Switzerland).

**Determination of relative antibody affinity.** For competitive ELISA, 96-well plates were coated with 5 ng/well cognate antigen overnight and blocked with 1% gelatin. Plates were incubated with purified antibody, 0.04-0.06 µg/mL, for 2 hours in the presence of free opioid as competitor in a range of concentrations (1 mM - 1 pM). Plates were washed and incubated overnight with HRP-conjugated goat anti-mouse secondary antibody, and HRP activity was measured using SigmaFast OPD substrate. BLI was performed using ForteBio BLItz system (Molecular Devices, San Jose, CA) with streptavidin biosensors. Biosensors were loaded with 2 µM fentanyl-biotin (see Supplemental Material for synthesis) for 60 sec, binding was measured with 100 nM mAb for 2 min, and dissociation was measured in PBS for 2 min.

**Antibody scale up and purification.** Hybridomas were adapted to DMEM (Corning Inc, Corning, NY) supplemented with 10% fetal bovine serum, hypoxanthine/thymidine (Sigma), and 2-mercaptoethanol and inoculated into Integra Celline 1000 bioreactors (Wheaton, Millville, NJ). Supernatant containing secreted mAb was purified by affinity chromatography with recombinant Protein A Sepharose (GE Healthcare,

Chicago, IL). Antibody was sterilized by 0.2  $\mu$ m filtration, aliquoted in preservative-free PBS, pH 7.2, and stored at 4°C. Purified mAb was analyzed by SDS-PAGE and dynamic light scattering (see Supplemental Material for additional methods).

**Drug challenges and pharmacokinetics.** Mice were passively immunized with control antibody (Ab, Gammagard, Baxalta Inc) or anti-opioid mAb in sterile PBS, 40-80 mg/kg as indicated in figure legends. To determine bioavailability and serum stability of mAb, approximately 50  $\mu$ L of blood was collected by facial vein sampling at least 1 hour prior to drug challenge. Mice were injected s.c. with 5.0 mg/kg oxycodone, 1.0 mg/kg heroin, or 0.1 or 0.5 mg/kg fentanyl, and antinociception was evaluated by latency to respond on a hot plate set to 54°C (Columbus Instruments, Columbus, OH) at 30 min post-injection. Antinociception was reported as percent maximum possible effect (%MPE), and was calculated as (latency post injection – baseline latency)/(60 – baseline latency) x 100. At 31 min post-injection, mice were euthanized by CO<sub>2</sub> inhalation and decapitated, and brain and blood were collected for drug concentration analysis. Oxycodone concentration was measured by GC-MS as described (Pravetoni *et al.*, 2013), fentanyl concentration was measured by LC-MS, and concentration of heroin, 6-acetyl morphine (6-AM), and morphine were analyzed by LC-MS (Raleigh *et al.*, 2013). For fentanyl, mice were evaluated for heart rate and breath rate using a MouseOx Plus pulse oximeter (Starr Life Sciences, Oakmont, PA). Rats were passively immunized with 60 mg/kg  $\alpha$ -fentanyl mAb i.p. and 24 hours later challenged with 0.1 mg/kg fentanyl s.c. Antinociception, heart rate, and respiratory behavior were measured every 15 min post-injection.

**Data analysis.** Statistical analysis was performed using Prism (GraphPad, La Jolla, CA). Mean %MPE, drug concentrations, oxygen saturation (%SaO<sub>2</sub>), heart rate (beats per minute, bpm) and breath rate (breaths per minute, brpm) were analyzed by ordinary one-way ANOVA followed by post-hoc analysis by Tukey's multiple comparisons test, or by two-way ANOVA followed by Sidak's multiple comparisons test when appropriate for measurements over time.

## RESULTS

**Antigen-based magnetic enrichment provides a high-throughput platform for generation of hybridomas expressing anti-opioid mAb.** Murine hybridomas expressing anti-opioid ( $\alpha$ -opioid) antibodies were generated after active immunization with lead  $\alpha$ -opioid conjugate vaccines (see Supp. Table S1). To isolate  $\alpha$ -oxycodone mAb, a vaccine consisting of an oxycodone-based hapten (OXY, Fig 1B) conjugated to the sKLH carrier protein (OXY-sKLH) was chosen for immunization. The OXY-sKLH vaccine has previously been shown to elicit protective titers in rats (Raleigh *et al.*, 2018), and OXY-specific B cells in mice (Laudenbach *et al.*, 2015). A magnetic enrichment strategy was used to isolate opioid-specific B cells prior to hybridoma fusion (see scheme, Fig 1A). To minimize co-isolation of carrier-specific splenocytes, OXY was conjugated to PE and used with anti-PE magnetic microbeads for enrichment of OXY-specific cells. Enrichment reduced the number of cells to be fused from approximately  $3 \times 10^8$  total splenocytes to  $3.8 \times 10^7$  enriched cells, and fusion of the OXY-specific enriched cell pool with Sp2/0 cells resulted in isolation of approximately 30 OXY-positive clones from a screening of 294 colonies (>10%).

Because previous research indicates that depletion of interleukin-4 (IL-4) with neutralizing antibodies enhances vaccine efficacy (Laudenbach *et al.*, 2018), a second cohort of mice was immunized with OXY-sKLH in conjunction with a neutralizing murine  $\alpha$ -IL-4 mAb (clone 11B11). An additional 12 hybridomas expressing  $\alpha$ -oxycodone mAb were isolated from this fusion, and were evaluated by ELISA to determine the IgG subclasses of expressed mAb (Supp. Fig. S1). Whereas all clones isolated from immunization with aluminum adjuvant alone expressed IgG<sub>1</sub>  $\alpha$ -oxycodone mAb, immunization with aluminum in combination with  $\alpha$ -IL-4 allowed isolation of several clones expressing IgG<sub>2a</sub>  $\alpha$ -oxycodone mAb, suggesting that choice of adjuvant impacts the profile of mAb generated from hybridomas. In addition, these data further support use of molecular adjuvants or immunomodulators such as the  $\alpha$ -IL-4 mAb to improve the quality of the polyclonal antibody responses and the likelihood of isolating mAb of a desired IgG subclass.

For generation of  $\alpha$ -morphine and  $\alpha$ -fentanyl mAb, mice were immunized with conjugate vaccines containing either a morphine-based hapten (M, Fig 1C) or a fentanyl-based hapten (F, Fig 1D) conjugated to sKLH. Active immunization with M-sKLH has been previously shown to generate polyclonal antibody response targeting heroin and its active metabolites 6-AM and morphine in mice and rats (Raleigh *et al.*, 2013). Active immunization with F-sKLH has been shown to protect against fentanyl-induced respiratory depression and bradycardia in rats (Raleigh *et al.*, 2019). A similar magnetic enrichment strategy as that for  $\alpha$ -oxycodone mAb was used to isolate hybridomas expressing  $\alpha$ -morphine and  $\alpha$ -fentanyl mAb, resulting in a reduction in splenocytes available for fusion to approximately  $2 \times 10^7$  and  $2.5 \times 10^7$  enriched cells respectively, and yielding approximately 5% of hybridoma clones producing mAb specific for the immunizing antigen.

**Initial biophysical characterization and scalability.** Hybridomas expressing  $\alpha$ -opioid mAb were initially cultured in 10 cm dishes using ClonaCell-HY medium, and mAb purified from supernatant at a 10 mg scale for initial *in vitro* characterization. Selected hybridoma clones were then transferred across laboratory sites and adapted to growth in Integra CL 1000 bioreactors, which yielded up to 250 mg. Lead mAb were purified and characterized by dynamic light scattering and SDS-PAGE to evaluate aggregation state and molecular weight (Supp. Fig S2). These data support scalability of the lead mAb generated in this study, and preliminary feasibility of technology transfer to a contract research organization with the goal of scaling up the mAb production to support late-stage characterization and *in vivo* studies in large animal models.

***In vivo* efficacy of  $\alpha$ -oxycodone mAb.** Selected  $\alpha$ -oxycodone mAb were purified from hybridoma supernatant, and relative affinity was determined by competitive ELISA (Fig 2A). Anti-oxycodone mAb exhibited  $IC_{50}$  within the 10 nM – 1  $\mu$ M range. Several clones were selected for further scale up and characterization; while clone HY1-3E3 exhibited the highest *in vitro* affinity, the isolated mAb exhibited

poor *in vivo* efficacy in initial tests (Supp. Fig. S3). Therefore, two clones with robust mAb expression were selected as leads, including one IgG<sub>1</sub> clone (HY1-3G8), and one IgG<sub>2a</sub> clone (HY2-A12).

To evaluate whether  $\alpha$ -oxycodone mAb is able to reduce the effects of oxycodone *in vivo*, mice were passively immunized with purified mAb 24 hours before a 5.0 mg/kg oxycodone challenge. Doses of either 40 or 80 mg/kg HY1-3G8 significantly reduced antinociception in mice compared to a control Ab (Fig 2B), and 40 mg/kg HY2-A12 reduced antinociception ( $p=0.065$ ). Thirty minutes after administration of drug, mice were euthanized and the concentration of oxycodone in the brain and serum were measured by GC-MS. Passive immunization with 40 mg/kg of either IgG<sub>1</sub> or IgG<sub>2a</sub>  $\alpha$ -oxycodone mAb reduced brain distribution of drug by approximately 49% (Fig 2D), whereas 80 mg/kg of IgG<sub>1</sub>  $\alpha$ -oxycodone mAb reduced brain distribution by 65%. These data suggest that IgG subclass may be not be a major contributor to antibody efficacy *in vivo*, and that mAb efficacy is dose-dependent.

***In vivo* efficacy of  $\alpha$ -heroin mAb.** For  $\alpha$ -heroin mAb, relative affinity of purified mAb was evaluated by competitive ELISA using plates coated with M-BSA and free morphine as competitor. Three clones exhibited  $IC_{50} < 2$  nM (Fig 3A), and HY4-1F9 was chosen for scale up and *in vivo* characterization. Mice were passively immunized with purified  $\alpha$ -heroin mAb, and given a 1 mg/kg heroin challenge 24 hours after passive immunization. Treatment with mAb reduced heroin-induced antinociception (Fig 3B), but the effect was not statistically significant ( $p=0.086$  for 40 mg/kg;  $p=0.127$  for 80 mg/kg). Because heroin is rapidly metabolized *in vivo* to active metabolites morphine and 6-AM, the concentrations of these metabolites in the brain and serum 30 minutes post-challenge were measured by LC-MS as a correlate of drug distribution. Measured levels of heroin were near or below the lower limit of quantitation, and excluded from analysis. At a dose of 1 mg/kg heroin, pre-treatment with 40 mg/kg of  $\alpha$ -heroin mAb reduced brain distribution of heroin metabolites by 35%, and 80 mg/kg mAb reduced distribution by 57% of control.

To investigate the distribution of  $\alpha$ -opioid mAb after passive immunization, 40 mg/kg of the lead  $\alpha$ -heroin mAb HY4-1F9 was administered to mice either s.c. or i.p., and blood was sampled at intervals

following administration to determine concentration of HY4-1F9 in serum (Supp. Fig S4B). Importantly, the resulting serum mAb concentrations were equivalent between these routes, suggesting that both s.c. and i.p. are viable for delivery of mAb and supporting use of the more convenient s.c. delivery for  $\alpha$ -opioid prophylaxis in potential clinical applications.

***In vivo efficacy of  $\alpha$ -fentanyl mAb.*** Relative affinities of  $\alpha$ -fentanyl mAb were measured by BLI, with the highest affinity mAb HY6-F9 exhibiting a dissociation constant of  $\sim 0.5$  nM. The two lead mAb selected for scale up included HY6-B5 and HY6-F9, which were IgG<sub>1</sub> and IgG<sub>2a</sub> subtypes respectively (Supp. Fig. S1). To evaluate the efficacy of these mAb, male and female mice were passively immunized with 40 mg/kg of either HY6-B5 or HY6-F9. Because fentanyl-induced respiratory depression is a major contributor to overdose fatalities (Fox *et al.*, 2018), the effects of fentanyl on respiratory behavior were measured 30 minutes after administration of 0.1 mg/kg fentanyl. Passive immunization with either HY6-B5 or HY6-F9 reduced fentanyl antinociception (Fig 4B), and HY6-F9 prevented fentanyl-induced suppression of respiration and heart rate (Fig 4C-D) compared to pre-drug baseline values. Female mice treated with control Ab showed slightly greater fentanyl-induced antinociception than male mice treated with control Ab, but no other statistically significant differences between male and female mice in the same treatment group were observed, and HY6-F9 significantly reduced fentanyl-induced antinociception and bradycardia in both male and female mice (Suppl. Fig 5).

To evaluate the effect of passive immunization at higher doses of fentanyl, separate cohorts of male and female mice were passively immunized with 40 mg/kg HY6-F9, and challenged with 0.5 mg/kg fentanyl. At this dose of fentanyl, passive immunization with HY6-F9 increased oxygen saturation and reduced brain concentration of fentanyl 85% as compared to control Ab (Fig 4E-F), but did not reduce the effect of fentanyl on antinociception or bradycardia (*data not shown*).

Because HY6-F9 was effective in reducing the effects of fentanyl in mice, *in vivo* efficacy of this mAb was also evaluated in rats. Rats were passively immunized with 60 mg/kg mAb and challenged with 0.1 mg/kg fentanyl, and antinociception and respiratory behavior were measured every 15 minutes for one

hour. Anti-fentanyl mAb was effective at reducing antinociception (Fig 5A) and preventing loss of oxygen saturation and heart rate (Fig 5B-C) after administration fentanyl.

## DISCUSSION

Monoclonal antibodies and vaccines against drugs or other chemical threats offer a unique tool with potential applications for treatment of OUD, and prevention of overdose or toxicity. Here, we describe the development of mAb against three opioid targets (oxycodone, heroin, and fentanyl), and demonstrate their efficacy *in vivo*. The magnetic enrichment strategy used to develop the mAb generated in this study reduced the number of total hybridoma clones 10-fold, simplifying the screening process. Similar methods have been successfully applied to generation of hybridomas against protein and peptide targets, including MHC-peptide complexes (Spanier *et al.*, 2016). However, this is the first report to our knowledge to apply magnetic enrichment of splenocytes prior to hybridoma fusion to the generation of mAb against a chemical target.

Selected mAb isolated using this approach exhibited both *in vitro* binding capability to their target drug and *in vivo* efficacy in reducing behavioral and physiological effects of opioids. In comparison to active vaccination, which may require weeks to months with multiple boosts to mount a protective immune response, passive immunization with  $\alpha$ -opioid mAb offers the benefit of greater control over the dose, affinity, and peak timing of protective Ab. However, because efficacy of mAb and vaccines against opioids depends on binding and sequestration of the drug of interest in serum, favorable stoichiometric ratios of Ab binding sites to drug in serum are required to achieve the reduction in brain distribution necessary to block physiological effects. Typically, doses of 30-120 mg/kg of an  $\alpha$ -opioid mAb are required for passive immunization (Smith *et al.*, 2019), and efficacy is dose-dependent with larger mAb doses offering greater protection against opioid intoxication (Kvello *et al.*, 2016, 2019). The doses of 40-80 mg/kg shown here result in mAb serum concentrations comparable to levels of polyclonal Ab achieved with their corresponding vaccinating antigens OXY-sKLH and M-sKLH (Suppl. Fig S3), and similar *in vivo* efficacy (Raleigh *et al.*, 2014, 2017). Though the relatively high manufacturing cost of mAb production can be a significant limitation to clinical translation of mAb-based therapies for OUD due to high dosing requirements, advances in manufacturing technologies for biologics (Buyel *et al.*, 2017; Diamos *et al.*, 2020) are expected to facilitate cost-effective mAb production in the future.

For both the  $\alpha$ -oxycodone mAb (Fig 2D) and the  $\alpha$ -heroin mAb (Fig 3D), a dose of 80 mg/kg produced a greater reduction in brain distribution in comparison to a lower dose of mAb, and a corresponding increase in the concentration of drug sequestered in the serum compartment (Fig 2C, 3C). Notably,  $\alpha$ -heroin mAb appeared less effective than  $\alpha$ -oxycodone mAb in terms of reducing brain concentration of drug, despite a 5-fold lower drug dose and a higher apparent affinity for the  $\alpha$ -heroin mAb. The finding that Ab against oxycodone are more efficacious than those against morphine is consistent with trends observed following active immunization with OXY-sKLH and M-sKLH; and efficacy depends on the route of heroin or oxycodone administration (Raleigh *et al.*, 2018). Because mAb can theoretically only interact with circulating drug in the serum compartment, whereas circulating unbound drug is subject to metabolism and tissue distribution effects, it can be difficult to predict mAb doses required for efficacy against various opioids. For example, a dose of 5 mg/kg oxycodone is in 30-fold excess of available binding sites when considering the total dose ratio, whereas measured serum concentrations of oxycodone and mAb estimate that the amount of drug in the serum compartment is ~50-60% that of available binding sites. Regardless, 40 mg/kg mAb was sufficient to significantly impact physiological response to oxycodone (Fig 2B) and reduce brain concentration ~50%, whereas brain concentration of heroin after 1 mg/kg challenge (corresponding to a 5-fold heroin:mAb binding sites total dose ratio) was reduced ~35% (Fig 3D).

Monoclonal antibodies against fentanyl and fentanyl analogs are of particular interest to public health due to their high potency. Because fentanyl-induced respiratory depression and bradycardia are implicated in overdose fatalities, heart rate and respiration were used as primary measures of  $\alpha$ -fentanyl mAb efficacy. Passive vaccination was effective at reducing toxic effects of fentanyl in both mice and rats (Fig 4-5), supporting the potential of mAb as a therapeutic to protect against fentanyl toxicity. However, at the higher fentanyl dose (0.5 mg/kg) in mice, mAb reduced respiratory depression compared to control Ab, but was less protective against fentanyl-induced antinociception and bradycardia despite an 85% reduction in brain distribution in mAb-treated mice. While further study with larger doses of  $\alpha$ -fentanyl mAb will be required to establish whether mAb can protect or rescue from high concentrations fentanyl

that may be encountered in an overdose scenario, other studies have successfully demonstrated mAb-based protection from potentially lethal fentanyl doses (Smith *et al.*, 2019). In this context, mAb could act as either a standalone treatment or as a supplement to opioid antagonists (e.g. naloxone) for overdose treatment and prevention. Preclinical efficacy of these opioid-targeting biologics has been demonstrated in multiple models and with various dose ranges and routes of administration, supporting further clinical development of this therapeutic approach.

## **ACKNOWLEDGEMENTS**

The authors thank Jennifer Vigliaturo and Michael Raleigh for technical support and discussions.

### **Authorship Contributions.**

Participated in research design: Baehr, Pravetoni and AuCoin.

Contributed novel reagents: Averick.

Performed experiments: Baehr, Huseby Kelcher, Khaimraj, Reed and Pandit.

Wrote the manuscript: Baehr and Pravetoni.

## References.

- Baruffaldi F, Kelcher AH, Laudénbach M, Gradinati V, Limkar A, Roslawski M, Birnbaum A, Lees A, Hassler C, Runyon S, and Pravetoni M (2018) Preclinical Efficacy and Characterization of Candidate Vaccines for Treatment of Opioid Use Disorders Using Clinically Viable Carrier Proteins. *Molecular Pharmaceutics* **15**.
- Boonyaratanakornkit J, and Taylor JJ (2019) Techniques to Study Antigen-Specific B Cell Responses. *Frontiers in immunology* **10**:1694, Frontiers Media S.A.
- Bremer PT, and Janda KD (2017) Conjugate Vaccine Immunotherapy for Substance Use Disorder. *Pharmacological Reviews* **69**:298.
- Bremer PT, Schlosburg JE, Banks ML, Steele FF, Zhou B, Poklis JL, and Janda KD (2017) Development of a Clinically Viable Heroin Vaccine. *Journal of the American Chemical Society*, doi: 10.1021/jacs.7b03334.
- Buyel JF, Twyman RM, and Fischer R (2017) Very-large-scale production of antibodies in plants: The biologization of manufacturing. *Biotechnology Advances* **35**:458–465.
- Centers for Disease Control and Prevention (2020) Opioid Overdose.
- Cornuz J, Zwahlen S, Jungi WF, Osterwalder J, Klingler K, van Melle G, Bangala Y, Guessous I, Müller P, Willers J, Maurer P, Bachmann MF, and Cerny T (2008) A vaccine against nicotine for smoking cessation: a randomized controlled trial. *PloS one* **3**:e2547–e2547, Public Library of Science.
- Diamos AG, Hunter JGL, Pardhe MD, Rosenthal SH, Sun H, Foster BC, DiPalma MP, Chen Q, and Mason HS (2020) High Level Production of Monoclonal Antibodies Using an Optimized Plant Expression System. *Frontiers in Bioengineering and Biotechnology* **7**:472.

- Fox BS, Kantak KM, Edwards MA, Black KM, Bollinger BK, Botka AJ, French TL, Thompson TL, Schad VC, Greenstein JL, Geftter ML, Exley MA, Swain PA, and Briner TJ (1996) Efficacy of a therapeutic cocaine vaccine in rodent models. *Nature Medicine* **2**:1129–1132.
- Fox LM, Hoffman RS, Vlahov D, and Manini AF (2018) Risk factors for severe respiratory depression from prescription opioid overdose. *Addiction (Abingdon, England)* **113**:59–66.
- Han Y, Yan W, Zheng Y, Khan MZ, Yuan K, and Lu L (2019) The rising crisis of illicit fentanyl use, overdose, and potential therapeutic strategies. *Translational psychiatry* **9**:282, Nature Publishing Group UK.
- Ho IY, Bunker JJ, Erickson SA, Neu KE, Huang M, Cortese M, Pulendran B, and Wilson PC (2016) Refined protocol for generating monoclonal antibodies from single human and murine B cells. *Journal of immunological methods* **438**:67–70.
- Jarvis BP, Holtyn AF, Subramaniam S, Tompkins DA, Oga EA, Bigelow GE, and Silverman K (2018) Extended-release injectable naltrexone for opioid use disorder: a systematic review. *Addiction (Abingdon, England)* **113**:1188–1209.
- Kashanian S, Shams A, Ghahremani H, and Paknejad M (2015) Preparation and Characterization of a Monoclonal Antibody Against Morphine. *Monoclonal Antibodies in Immunodiagnosis and Immunotherapy* **34**:270–274, Mary Ann Liebert Inc.
- Keyler DE, Roiko SA, Benlhabib E, LeSage MG, st. Peter J v, Stewart S, Fuller S, Le CT, and Pentel PR (2005) Monoclonal Nicotine-specific Antibodies Reduce Nicotine Distribution to Brain in Rats: Dose- and Affinity-response Relationships. *Drug Metabolism and Disposition* **33**:1056.
- Kosten TR, Domingo CB, Shorter D, Orson F, Green C, Somoza E, Sekerka R, Levin FR, Mariani JJ, Stitzer M, Tompkins DA, Rotrosen J, Thakkar V, Smoak B, and Kampman K (2014) Vaccine for

cocaine dependence: a randomized double-blind placebo-controlled efficacy trial. *Drug and alcohol dependence* **140**:42–47.

Kvello AMS, Andersen JM, Øiestad EL, Mørland J, and Bogen IL (2016) Pharmacological effects of a monoclonal antibody against 6-monoacetylmorphine upon heroin-induced locomotor activity and pharmacokinetics in mice. *Journal of Pharmacology and Experimental Therapeutics* **358**:181–189, American Society for Pharmacology and Experimental Therapy.

Kvello AMS, Andersen JM, Øiestad EL, Steinsland S, Aase A, Mørland J, and Bogen IL (2019) A monoclonal antibody against 6-acetylmorphine protects female mice offspring from adverse behavioral effects induced by prenatal heroin exposure. *Journal of Pharmacology and Experimental Therapeutics* **368**:106–115, American Society for Pharmacology and Experimental Therapy.

Laudenbach M, Baruffaldi F, Robinson C, Carter P, Seelig D, Baehr C, and Pravetoni M (2018) Blocking interleukin-4 enhances efficacy of vaccines for treatment of opioid abuse and prevention of opioid overdose. *Scientific Reports* **8**.

Laudenbach M, Baruffaldi F, Vervacke JS, Distefano MD, Titcombe PJ, Mueller DL, Tubo NJ, Griffith TS, and Pravetoni M (2015) The frequency of naive and early-activated hapten-specific B cell subsets dictates the efficacy of a therapeutic vaccine against prescription opioid abuse. *Journal of immunology (Baltimore, Md : 1950)* **194**:5926–5936.

Lei L, Tran K, Wang Y, Steinhardt JJ, Xiao Y, Chiang C-I, Wyatt RT, and Li Y (2019) Antigen-Specific Single B Cell Sorting and Monoclonal Antibody Cloning in Guinea Pigs. *Frontiers in Microbiology* **10**:672.

Li S, Cohen-Karni D, Kovaliov M, Tomycz N, Cheng B, Whiting D, and Averick S (2017) Synthesis and biological evaluation of fentanyl acrylic derivatives. *RSC Advances*, doi: 10.1039/C7RA01346A.

- Marckel JA, Wetzel HN, Amlal S, Amlal H, and Norman AB (2019) A Recombinant Humanized Anticocaine Monoclonal Antibody Alters the Urinary Clearance of Cocaine and Its Metabolites in Rats. *Drug metabolism and disposition: the biological fate of chemicals* **47**:184–188, The American Society for Pharmacology and Experimental Therapeutics.
- Martell BA, Orson FM, Poling J, Mitchell E, Rossen RD, Gardner T, and Kosten TR (2009) Cocaine vaccine for the treatment of cocaine dependence in methadone-maintained patients: a randomized, double-blind, placebo-controlled efficacy trial. *Archives of general psychiatry* **66**:1116–1123.
- Nguyen JD, Hwang CS, Grant Y, Janda KD, and Taffe MA (2018) Prophylactic vaccination protects against the development of oxycodone self-administration. *Neuropharmacology* **138**:292–303.
- Pravetoni M (2016) Biologics to treat substance use disorders: Current status and new directions. *Human vaccines & immunotherapeutics* **12**:3005–3019, Taylor & Francis.
- Pravetoni M, and Comer SD (2019) Development of vaccines to treat opioid use disorders and reduce incidence of overdose.
- Pravetoni M, le Naour M, Harmon TM, Tucker AM, Portoghese PS, and Pentel PR (2012) An oxycodone conjugate vaccine elicits drug-specific antibodies that reduce oxycodone distribution to brain and hot-plate analgesia. *The Journal of pharmacology and experimental therapeutics* **341**:225–232, The American Society for Pharmacology and Experimental Therapeutics.
- Pravetoni M, le Naour M, Tucker AM, Harmon TM, Hawley TM, Portoghese PS, and Pentel PR (2013) Reduced antinociception of opioids in rats and mice by vaccination with immunogens containing oxycodone and hydrocodone haptens. *Journal of Medicinal Chemistry*, doi: 10.1021/jm3013745.
- Raleigh MD, Accetturo C, and Pravetoni M (2020) Combining a candidate vaccine for opioid use disorders with extended-release naltrexone increases protection against oxycodone-induced

behavioral effects and toxicity. *Journal of Pharmacology and Experimental Therapeutics* JPET-AR-2020-000014.

Raleigh MD, Baruffaldi F, Peterson SJ, le Naour M, Harmon TM, Vigliaturo JR, Pentel PR, and Pravetoni M (2019) A Fentanyl Vaccine Alters Fentanyl Distribution and Protects against Fentanyl-Induced Effects in Mice and Rats. *Journal of Pharmacology and Experimental Therapeutics* **368**.

Raleigh MD, Laudénbach M, Baruffaldi F, Peterson SJ, Roslawski MJ, Birnbaum AK, Carroll FI, Runyon SP, Winston S, Pentel PR, and Pravetoni M (2018) Opioid Dose- and Route-Dependent Efficacy of Oxycodone and Heroin Vaccines in Rats. *Journal of Pharmacology and Experimental Therapeutics* **365**.

Raleigh MD, Pentel PR, and Lesage MG (2014) Pharmacokinetic correlates of the effects of a heroin vaccine on heroin self-Administration in rats. *PLoS ONE*, doi: 10.1371/journal.pone.0115696.

Raleigh MD, Peterson SJ, Laudénbach M, Baruffaldi F, Carroll FI, Comer SD, Navarro HA, Langston TL, Runyon SP, Winston S, Pravetoni M, and Pentel PR (2017) Safety and efficacy of an oxycodone vaccine: Addressing some of the unique considerations posed by opioid abuse. *PLoS ONE* **12**.

Raleigh MD, Pravetoni M, Harris AC, Birnbaum AK, and Pentel PR (2013) Selective effects of a morphine conjugate vaccine on heroin and metabolite distribution and heroin-induced behaviors in rats. *Journal of Pharmacology and Experimental Therapeutics*, doi: 10.1124/jpet.112.201194.

Robinson C, Baehr C, Schmiel SE, Accetturo C, Mueller DL, and Pravetoni M (2019) Alum adjuvant is more effective than MF59 at prompting early germinal center formation in response to peptide-protein conjugates and enhancing efficacy of a vaccine against opioid use disorders. *Human Vaccines and Immunotherapeutics*, doi: 10.1080/21645515.2018.1558697.

- Rzasa Lynn R, and Galinkin JL (2018) Naloxone dosage for opioid reversal: current evidence and clinical implications. *Therapeutic advances in drug safety* **9**:63–88, SAGE Publications.
- Sharma A, Kelly SM, Mitchell SG, Gryczynski J, O’Grady KE, and Schwartz RP (2017) Update on Barriers to Pharmacotherapy for Opioid Use Disorders. *Current Psychiatry Reports* **19**:35.
- Smith K, Garman L, Wrammert J, Zheng N-Y, Capra JD, Ahmed R, and Wilson PC (2009) Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. *Nature Protocols* **4**:372–384.
- Smith LC, Bremer PT, Hwang CS, Zhou B, Ellis B, Hixon MS, and Janda KD (2019) Monoclonal Antibodies for Combating Synthetic Opioid Intoxication. *Journal of the American Chemical Society*, doi: 10.1021/jacs.9b04872.
- Spanier JA, Frederick DR, Taylor JJ, Heffernan JR, Kotov DI, Martinov T, Osum KC, Ruggiero JL, Rust BJ, Landry SJ, Jenkins MK, McLachlan JB, and Fife BT (2016) Efficient generation of monoclonal antibodies against peptide in the context of MHCII using magnetic enrichment. *Nature communications* **7**:11804, Nature Publishing Group.
- Starkie DO, Compson JE, Rapecki S, and Lightwood DJ (2016) Generation of Recombinant Monoclonal Antibodies from Immunised Mice and Rabbits via Flow Cytometry and Sorting of Antigen-Specific IgG+ Memory B Cells. *PloS one* **11**:e0152282–e0152282, Public Library of Science.
- Stevens MW, Henry RL, Owens SM, Schutz R, and Gentry WB (2014) First human study of a chimeric anti-methamphetamine monoclonal antibody in healthy volunteers. *mAbs* **6**:1649–1656, Taylor & Francis.
- Sulima A, Jalah R, Antoline JFG, Torres OB, Imler GH, Deschamps JR, Beck Z, Alving CR, Jacobson AE, Rice KC, and Matyas GR (2018) A Stable Heroin Analogue That Can Serve as a Vaccine Hapten to Induce Antibodies That Block the Effects of Heroin and Its Metabolites in Rodents and

That Cross-React Immunologically with Related Drugs of Abuse. *Journal of Medicinal Chemistry*, doi: 10.1021/acs.jmedchem.7b01427.

Taylor JJ, Laudenbach M, Tucker AM, Jenkins MK, and Pravetoni M (2014) Hapten-specific naïve B cells are biomarkers of vaccine efficacy against drugs of abuse. *Journal of immunological methods* **405**:74–86.

Tenney RD, Blake S, Bremer PT, Zhou B, Hwang CS, Poklis JL, Janda KD, and Banks ML (2019) Vaccine blunts fentanyl potency in male rhesus monkeys. *Neuropharmacology*, doi: 10.1016/j.neuropharm.2019.107730.

## **Footnotes.**

Financial Support. This work was supported by the National Institutes of Health under CounterACT Administrative Supplement [U01 DA038876] (MP), [T32 DA007097] (CB), and [T32 DA037183] (AHK). Dynamic Light Scattering analysis was conducted in the Minnesota Nano Center, which is supported by the National Science Foundation through the National Nano Coordinated Infrastructure Network (NNCI) [Award Number ECCS-1542202].

Disclosures of interest. MP and CB are inventors of provisional patent application 62/857,020 “Anti-opioid compounds and methods of making and using same,” filed June 4, 2019. All other authors have no conflicts of interest.

## FIGURE LEGENDS

### **Figure 1. B cell-based platform for generating $\alpha$ -opioid hybridomas via magnetic enrichment.**

(A) Workflow for hybridoma generation. Antigen-specific cells from immunized mice are selected by magnetic enrichment for fusion with Sp2/0 myeloma cells; HAT-resistant colonies are transferred to plates and screened for expression of anti-opioid antibodies by ELISA. Structures of haptens used for immunization for (B)  $\alpha$ -oxycodone, (C)  $\alpha$ -morphine, and (D)  $\alpha$ -fentanyl hybridomas.

### **Figure 2. Characterization and efficacy of $\alpha$ -oxycodone mAb.**

(A) Dissociation constants of  $\alpha$ -oxycodone mAb were determined by competitive ELISA. (B-D) Passive immunization with  $\alpha$ -oxycodone mAb reduces oxycodone distribution to the brain. Mice (n=5/group) were passively immunized with 40 mg/kg or 80 mg/kg  $\alpha$ -oxycodone mAb i.p. and challenged with 5.0 mg/kg oxycodone s.c. (B) Antinociception was evaluated by latency to respond on a hot plate; oxycodone levels in (C) serum and (D) brain were determined by GC-MS. Mean  $\pm$  SD; \*p<0.05; \*\*\*\*p<0.0001.

### **Figure 3. Characterization and efficacy of $\alpha$ -heroin mAb.**

(A) Relative affinities of  $\alpha$ -heroin mAb were determined by competitive ELISA. (B-D) Passive immunization with  $\alpha$ -heroin mAb reduces heroin distribution to the brain. Mice (n=6/group) were passively immunized with 40 mg/kg or 80 mg/kg  $\alpha$ -heroin mAb i.p. and challenged with 1.0 mg/kg heroin s.c. (B) Antinociception was evaluated by latency to respond on a hot plate 30 min post-injection; heroin metabolites 6-AM and morphine in (C) serum, and (D) brain were determined by LC-MS. Mean  $\pm$  SD; \*\*p<0.01; \*\*\*\*p<0.0001.

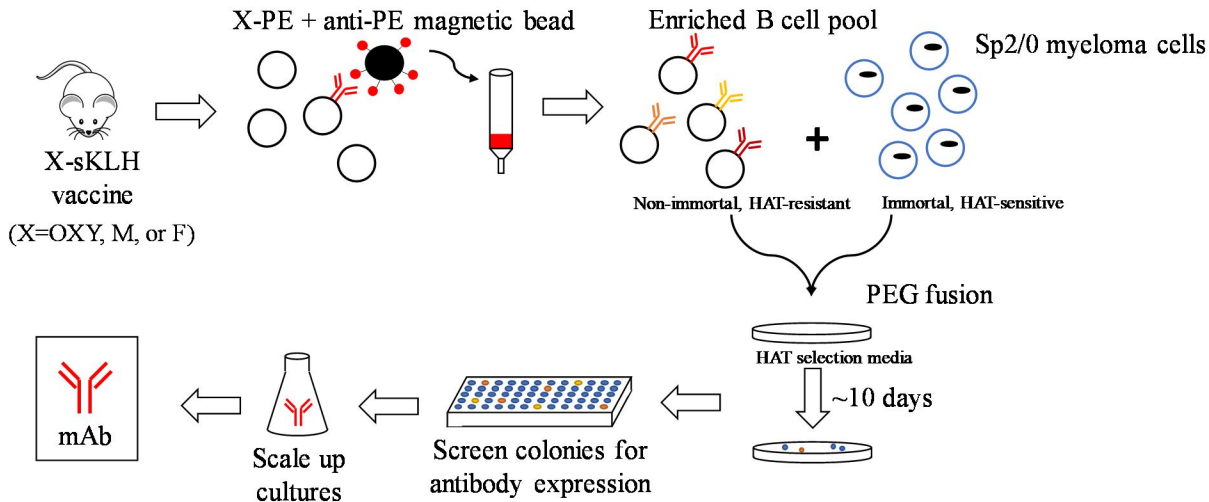
### **Figure 4. Characterization and efficacy of $\alpha$ -fentanyl mAb.**

(A) Dissociation constants of  $\alpha$ -fentanyl mAb were determined by biolayer interferometry. (B-D) Passive immunization with  $\alpha$ -fentanyl mAb reduces fentanyl-induced antinociception, respiratory depression and bradycardia. Mice (n=3 male and 3 female per group) were passively immunized with 40 mg/kg anti-fentanyl mAb i.p. and challenged with 0.1 mg/kg fentanyl s.c. (B) Antinociception was evaluated by latency to respond on a hot plate; (C) breath rate and (D) heart rate were measured by oximetry at 30 min post-injection. (E-F) In a separate

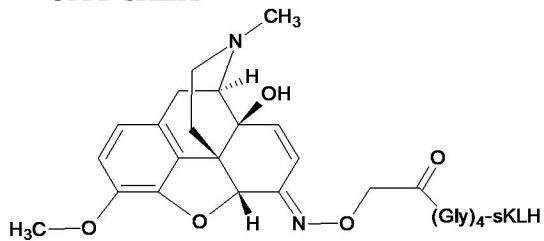
experiment, mice (n=4 male and 4 female per group) were passively immunized with 40 mg/kg  $\alpha$ -fentanyl mAb and challenged with 0.5 mg/kg fentanyl. (E) oxygen saturation was measured by oximetry and (F) brain fentanyl concentration was measured by LC-MS at 30 min post-injection. Mean  $\pm$  SD; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001.

**Figure 5. Efficacy of  $\alpha$ -fentanyl mAb in rats.** Rats (n=4/group) were passively immunized with 60 mg/kg  $\alpha$ -fentanyl mAb i.p. and challenged with 0.1 mg/kg fentanyl s.c. (A) Antinociception by latency to respond on a hot plate, (B) oxygen saturation, and (C) heart rate were evaluated every 15 min up to 1 hour post-injection. Mean  $\pm$  SD; \*p<0.05; \*\*p<0.01; \*\*\*p<0.001.

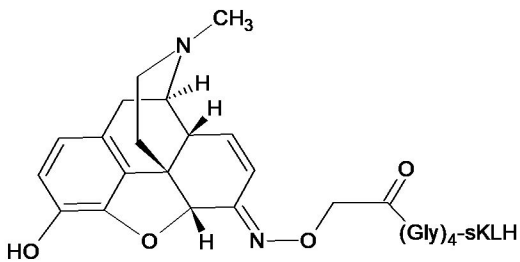
A



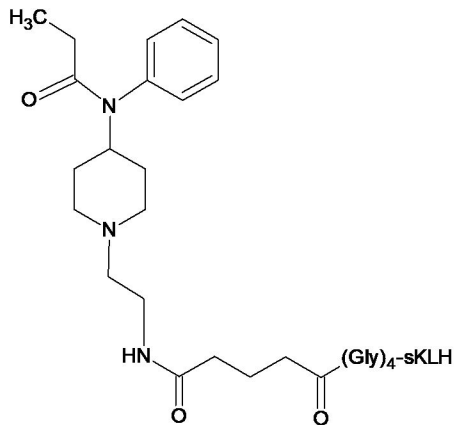
**B** OXY-sKLH

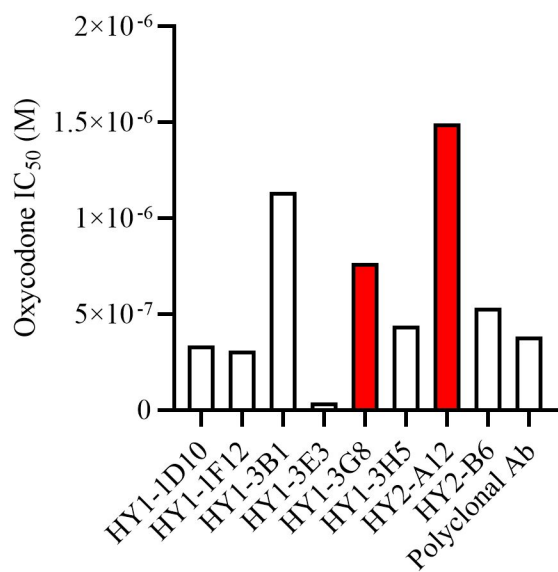
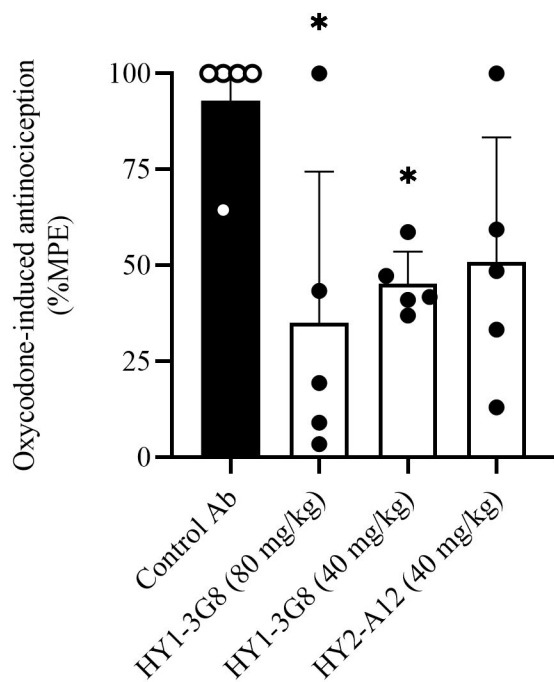
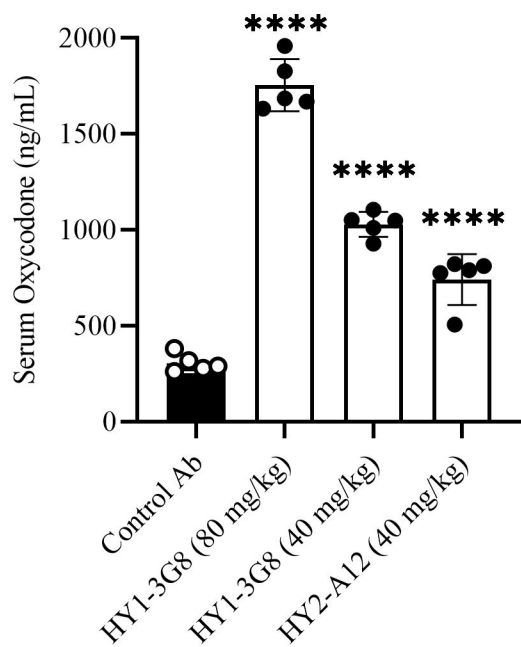
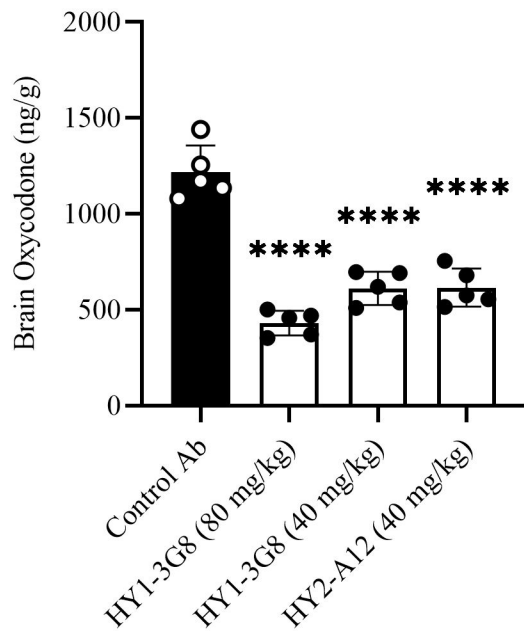


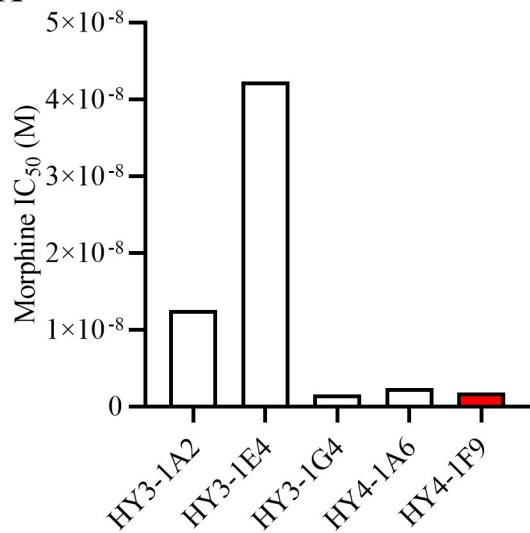
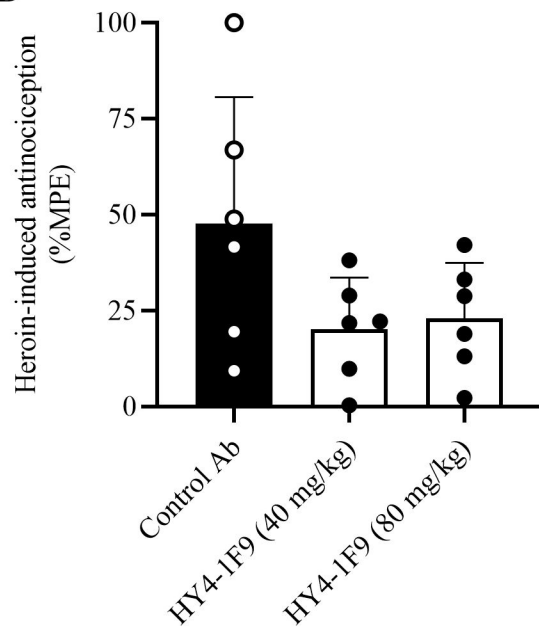
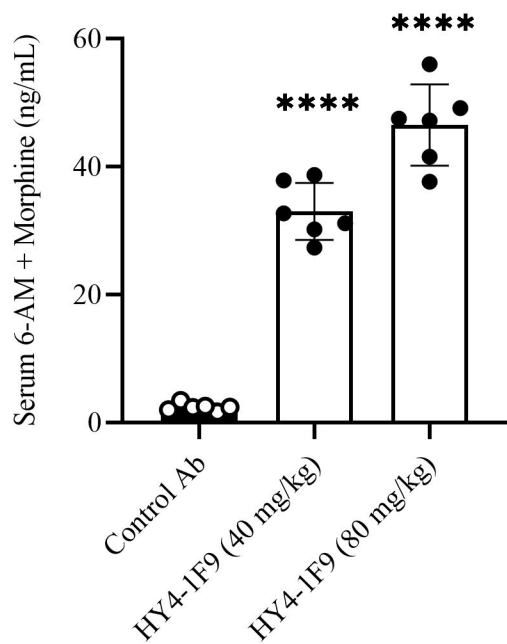
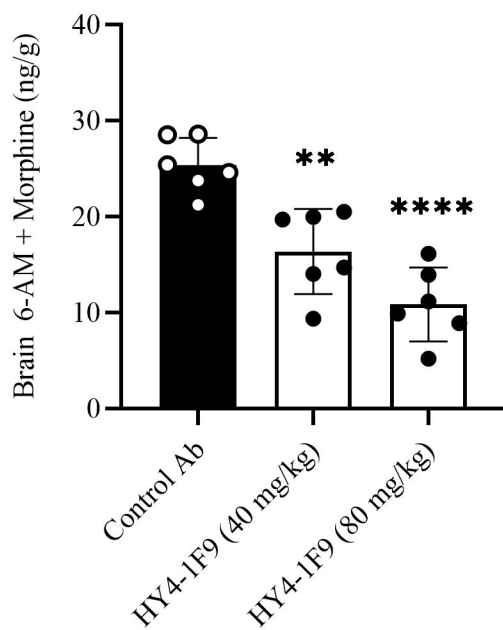
C M-sKLH

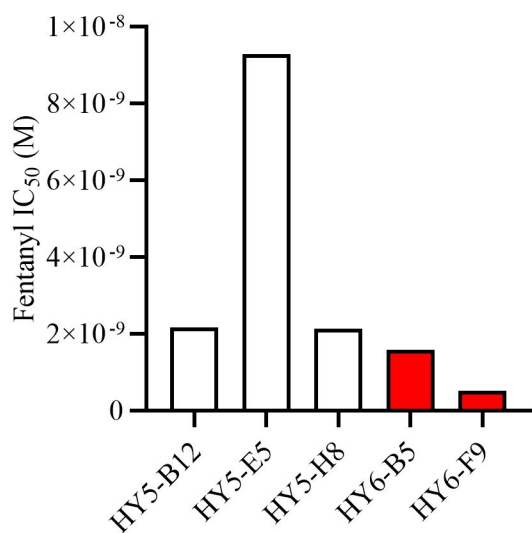
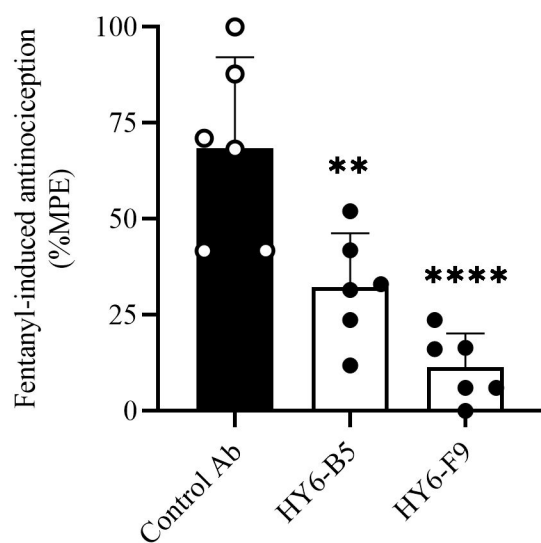
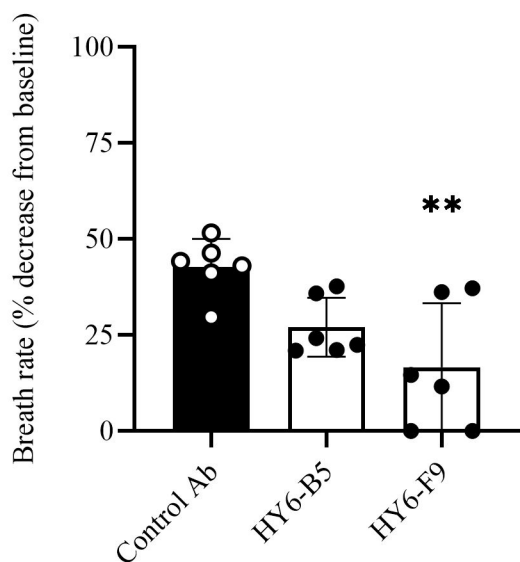
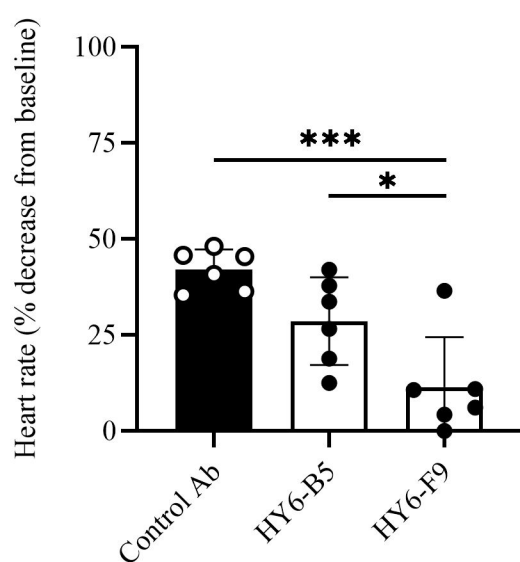
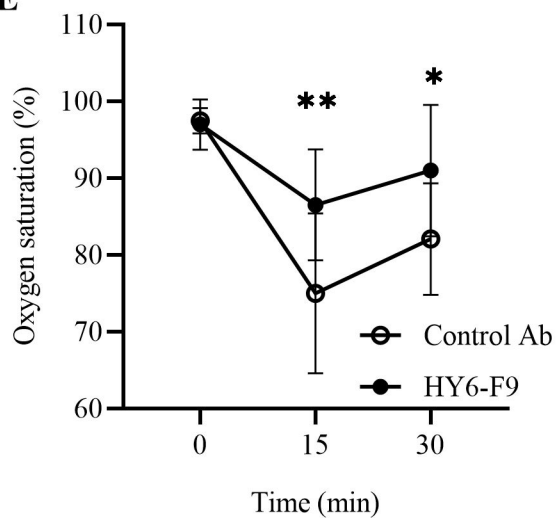


**D** F-sKLH



**A****B****C****D**

**A****B****C****D**

**A****B****C****D****E****F**