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Enhancing Efficacy and Stability of an Anti-Heroin Vaccine: Examination of Antinociception, Opioid Binding Profile, and Lethality

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KEYWORDS: heroin, opioids, vaccine, immunopharmacotherapy, adjuvants

Supporting Information Placeholder

ABSTRACT: In recent years, drug conjugate vaccines have shown promise as therapeutics for substance use disorder. As a means to improve the efficacy of a heroin conjugate vaccine, we systematically explored twenty vaccine formulations with varying combinations of carrier proteins and adjuvants. In regard to adjuvants, we explored a Toll-like receptor 9 (TLR9) agonist and a TLR3 agonist in the presence of alum. The TLR9 agonist was cytosine-guanine oligodeoxynucleotide 1826 (CpG ODN 1826), while the TLR3 agonist was virus-derived genomic doubled-stranded RNA (dsRNA). The vaccine formulations containing TLR3 or TLR9 agonist alone elicited strong anti-heroin antibody titers and blockade of heroin-induced antinociception when formulated with alum; however, a combination of TLR3 and 9 adjuvants did not result in improved efficacy. Investigation of month-long stability of the two lead formulations revealed that the TLR9 but not the TLR3 formulation was stable when stored as a lyophilized solid or as a liquid over 30 days. Furthermore, mice immunized with the TLR9 + alum heroin vaccine gained significant protection from lethal heroin doses, suggesting that this vaccine formulation is suitable for mitigating the harmful effects of heroin, even following month-long storage at room temperature.

Introduction

Heroin is a schedule I, highly addictive opioid drug and a significant public health concern. In the US, drug overdose deaths have nearly tripled between 1999 and 2014.[1] In 2015, 52,404 overdose deaths were reported, 63% of which involved opioids.[1] Recently, there has been a marked increase in prescriptions of synthetic opioid pain relievers (OPRs) for management of chronic pain.[2] Evidence suggests that misuse of OPRs is the strongest risk factor for initiating heroin abuse, and OPR users are 40 times more likely to abuse heroin.[3, 4] This phenomenon is driven by the relatively low cost of heroin and its wide availability.[3, 4] Current treatments for heroin addiction involve opioid replacement therapy e.g., methadone administration, to promote heroin detoxification.[5] Unfortunately, the addictive nature of heroin and other opioids, combined with the adverse effects of withdrawal and high cost of treatment, lead to a high incidence of drug relapse.[5 3, 4] In the face of increasing opioid abuse and overdose, the development of improved therapies that can attenuate the effects of opioids is crucial.

Vaccination is a promising strategy to promote cessation of heroin abuse and prevent relapse. Implementation of this strategy involves active immunization using a small molecule-protein conjugate, which elicits high-affinity, drug-specific antibodies. These polyclonal IgG antibodies sequester free drug in the blood and prevent access to the brain, subsequently reducing the drug compound's psychoactive effects. This approach has been pre-clinically validated for vaccines against nicotine,[6, 7] cocaine,[8, 9] and methamphetamine.[10, 11] For heroin specifically, vaccination efficacy has been repeatedly demonstrated in mice, rats, and non-human primates.[12-18]

In general, formulation of a vaccine with an adjuvant is an attractive approach to enhance the magnitude and length of vaccine immunity against the target antigen by stimulating antigen presenting cells, T-cells or B-cells. Historically, Alhydrogel (alum) has been the most commonly used adjuvant, but numerous alternatives have been pursued in recent years.[19] Adjuvants can act as pathogen-associated molecular patterns (PAMPs), which activate Toll-like receptors (TLRs) resulting in upregulation of an immune response. Specific PAMPs include lipopolysaccharides (LPS), double-stranded RNA (dsRNA) and unmethylated cytosine-guanine (CpG) motifs.[19] However, at this time only a limited number of adjuvants are approved for use in humans. By exploring new adjuvants or combinations of adjuvants, we can

rationally design vaccines with enhanced immunogenicity directed toward production of heroin-neutralizing antibodies.

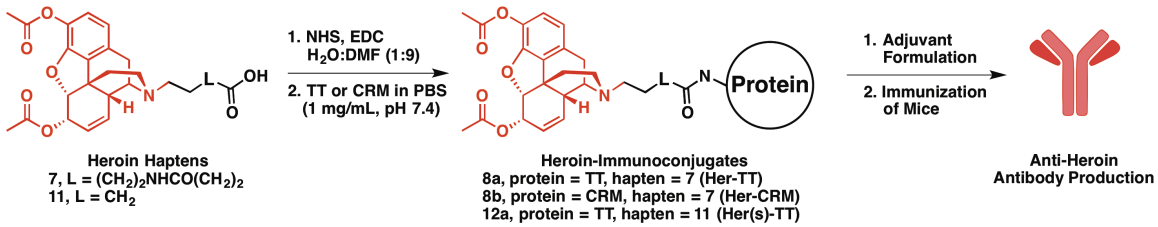


Figure 1. Structures of the heroin haptens, corresponding immunoconjugates and the general vaccine approach. The structure of heroin is highlighted in red.

CpG oligodeoxynucleotide (ODN) 1826 is a B-class ODN that stimulates B-cell responses though TLR9[20, 21] and was recently shown to elicit robust titers in anti-heroin vaccine studies.[13, 22] Natural or synthetic dsRNA, e.g., polyinosic:polycytidylicacid (poly I:C), is a molecular pattern associated with viral replication, which elicits an immune response via TLR3 and has been used as an effective adjuvant in several vaccine studies.[23-25] Given the potent immunostimulatory capacity of viral or bacterial PAMPs, we were interested in evaluating the efficacy of a yeast-derived viral dsRNA genome relative to CpG ODN, using a well-studied dsRNA virus of *Saccharomyces cerevisiae*, L-A.[26] To date, only the L-BC viral dsRNA genome generated from infected *S. cerevisiae* has been used as an adjuvant, where it increased immunogenicity of a prophylactic viral vaccine in mice.[27]

Here, we investigate L-A-derived dsRNA in combination with alum and/or CpG ODN in the context of our drug of abuse vaccine. Although alum is not necessary for TLR activation, it is one of the few adjuvants used in FDA-approved vaccines and has shown promising activity in anti-drug vaccines. In comparison to alum, we selected conjugatable adjuvant lipid vesicles (CALV) as an alternative vehicle for vaccine delivery.[28] CALVs are nanoparticulate liposomes designed to effectively deliver encapsulated antigens for immune uptake.

Our most successful anti-heroin vaccine to date involves a second generation heroin hapten adjuvanted with alum and CpG (Figure 1).[22] We used this formulation as a benchmark while investigating new adjuvant combinations and formulation conditions in an effort to find a lead vaccine candidate. We then measured the effects of adjuvant dosing on vaccine efficacy, and the vaccines were tested under various storage conditions for stability as a liquid or lyophilized solid after mixing with alum adjuvant and trehalose as a cryoprotectant. Our most successful formulation was then selected for an overdose challenge to see if protection was conferred against a lethal dose of heroin.

Experimental Section

Synthesis of heroin haptens and conjugation to carrier proteins

The synthesis and characterization of the heroin haptens and immunoconjugates are described in detail in the supplementary information (Figures S1-14), along with additional information on animals, formulation conditions, vaccine administration schedule, behavioral testing, ELISA and other experimental data. Our key hapten design element is a strategically placed linker on the nitrogen that ultimately presents an immune epitope with high structural congruence to heroin. (**7**, Figure 1 and Scheme S1).[22] We also prepared a heroin hapten with a truncated linker at this same position to probe the effect of linker length on vaccine efficacy (**11,** Figures 1 and S1). The haptens were activated and conjugated to carrier protein tetanus toxoid (TT) or a mutant diphtheria toxoid (CRM), using an EDC-mediated coupling reaction (Figure 1), followed by dialysis against pH 7.4 phosphate buffered saline (PBS). The degree of haptenation was determined by MALDI-ToF mass spectrometry, using a heroin-bovine serum albumin (Her-BSA) immunoconjugate as a surrogate for determining hapten density (Figures S9-14). Immunoconjugates were stored at -80 ˚C until the day of formulation and vaccination. More information on the synthesis and characterization data are described in the supplemental information.

Vaccine Formulation

After conjugation of the proteins, immunoconjugates were formulated with different adjuvants as described in Tables S1-5. The adjuvants were CpG ODN 1826, dsRNA, Alhydrogel (alum), and VesiVax® CALV.[28] CpG ODN 1826 is a phosphorothioated oligonucleotide with the following sequence (5' to 3'): TCCATGACGTTCCTGACGTT. The 4.6 kb viral dsRNA was derived from L-A infected *S. cerevisiae* (ATCC #22244). The viral dsRNA can be prepared according to literature procedure involving fermentation of killer yeast, *Saccharomyces cerevisiae* (ATCC 22244), containing the L-A virus grown in Difco YM media (Becton Dickson).[29-31] The VesiVax® CALV liposomes and dsRNA were obtained from Molecular Express, Inc. Each vaccine was prepared by shaking the mixture for twenty minutes prior to injection. The delivered dose of each component was 200 μg immunoconjugate, 50 μg of CpG ODN 1826 or dsRNA, and 1 mg of alum per animal for each injection, unless noted otherwise in Table 1 and Tables S1-5.

Chemical Stability Studies of Individual Vaccine Components

A systematic analysis of individual components under various storage conditions was performed to monitor potential unwanted degradation. Lack of chemical stability may represent potential causal factors for any change in potency that is observed over time. Samples were prepared according to standard vaccine formulation conditions and listed in Table S6. To perform these studies, we ran TBE-Urea gels for CpG, agarose gel, UV-Vis analysis and E-Gels for dsRNA, and SDS-PAGE gels for tetanus toxoid (TT) under various storage

conditions. Results from stability studies are in the supplemental information (Table S6 and Figures S22-28).

Animals and Vaccine Administration

All studies were performed in compliance with the Scripps Institutional Animal Care and Use Committee and all protocols adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals. Male Swiss Webster mice (Taconic Farms, Germantown, NY; 6-8 weeks old; 25-30 g) were immunized subcutaneously (s.c.) on days 0, 14, and 28, unless noted otherwise (Figure S15-17; Tables S1-5). Exact formulation parameters are given for each group in Tables S1-5. All animals in a given series were run at the same time, except for Series D and G. The bold lines separating the series indicate that the series were run in two sets, instead of simultaneously (Table S2 and S5). Mice were bled on day 38 using retro-orbital puncture in order to collect approximately 100-150 μL of whole blood, unless noted otherwise. Groups were composed of 4 to 6 mice. Mice were group-housed in an AAALAC-accredited vivarium containing temperature and humidity controlled rooms, and kept on a reverse light cycle (lights on: 9PM-9AM). Immunoconjugate **12a** with the shortened linker hapten was used in Group B4 and the hapten **11** was termed H(s) in Table 1 and S1.

Antinociception Assays

On week 6, antinociceptive responses under escalating heroin doses were evaluated to determine vaccine-mediated blockade of heroin psychoactivity.[32] A set of mice was tested for spinal (tail immersion) and supraspinal (hot plate) antinociceptive responses to thermal stimuli at 54 °C, according to our laboratory procedure.[33] Following administration of the drug, the analgesic effect (represented as maximal possible effect, % MPE) was measured for each test after every dose. The data were then fit using a non-linear regression in GraphPad PRISM to determine ED_{50} values. The ED_{50} is calculated from plotting the %MPE with respect to heroin dose. Since the effect is based on %MPE, the ED_{50} describes when 50% of the animals within a group experienced the maximum effect of heroin-induced antinociception.

ELISAs

Bleeds were taken on weeks 6 and 10 for Series B, and maximum titer levels occurred at week 6 (Figure S18). Therefore, we opted to perform bleeds on day 38 for Series C-G, and perform antinociception assays around week 6. Since heroin is rapidly metabolized to 6-AM before entering the brain,[34, 35] an ELISA using heroin or 6-monoacetylmorphine (6-AM) as coating antigens was performed for Series E to characterize antigen specificity of the antibody response. The equivalent titer response to coating antigen may suggest that the heroin immunoconjugate hydrolyzes to 6-AM before or during antigen presentation (Figure S19). Additional information on ELISAs is described in the supplemental information.

Analyzing Cross-Reactivity of Polyclonal Anti-Heroin Antibodies by Surface Plasmon Resonance

The binding IC_{50} for mouse serum IgGs from Group G6 and 6-AM was determined by competitive binding assay via surface plasmon resonance (SPR) using a Biacore 3000 instrument (GE Healthcare) equipped with a research-grade CM5 sensor chip according to literature methods.[36] Diluted mouse serum from day 38 was incubated with serial dilutions of heroin, 6-AM, methadone, oxycodone, naloxone, buprenorphine, norbuprenorphine, naltrexone and morphine and injected into a Biacore 3000 containing a Her-BSA-loaded sensor chip. The heroin-BSA conjugate, was immobilized on the sensor chip using a NHS, EDC-mediated coupling reaction. The conjugate was resuspended in 10 mM sodium acetate (pH 4.0) was immobilized at a density of 5,000 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). The pooled mouse sera was diluted in running buffer (HBS-EP + buffer) and titrated on both coated flow cells, so as to give a response of ~100 RU within 3 minutes of injection and 2.5 minute dissociation at a flow rate of 30 μL/min. The chip surface was regenerated by injection of 10 mM Gly-HCl (pH 1.5) for 30 seconds before the next round of assays. Signal produced by antibody binding to the SPR chip without drug present was used as a reference for 100% binding. Rapid hydrolysis of heroin interfered with collecting sufficient binding data.

Statistical analysis

Tests for homogeneity of variance and normal distribution were performed on behavioral observation test scores. If conditions were met, analyses of variance (ANOVAs) were performed. Results were analyzed via one-way ANOVA with Dunnett's *post hoc* comparisons for titers and Tukey's *post hoc* test for analgesia. Pearson correlation coefficient was used to test the linear relationship between anti-heroin midpoint titers to analgesia results for all animals tested (hot plate, P = 0.002, R^2 = 0.093, Figure S21A and C; tail immersion, P = 0.009, R^2 = 0.047, Figure S21B and D). However, there is no meaningful correlation between titers and antinociception data; titer data is reflective of antibody binding to hapten and not necessarily to free drug.

Results and Discussion

Series A-C: Preliminary evaluation of dsRNA as an adjuvant

To evaluate the series of heroin vaccine formulations, mice (n = 4-6/group) were vaccinated subcutaneously (s.c.) with the specific formulations listed in Tables S1-5. Series A through C were designed to broadly explore the scope of vaccine conditions with the new dsRNA adjuvant in multiple contexts and to compare the adjuvant to our most successful heroin vaccine: Her-TT adjuvanted with 50 ug CpG adjuvanted and 1 mg of alum (Group A6). We used our previously reported second-generation heroin hapten[17] in the majority of our formulations, (**7**, Figure 1) although a truncated heroin hapten (**11**, Figure 1), was compared to **7** and showed no difference in behavioral efficacy (Group B4, Table 1). Moreover, ELISA results revealed that antibody titers for both hapten **7** and **11** vaccination groups were similar regardless of coating antigens (**8c** and **12b**), suggesting that the hapten linker does not noticeably affect immunogenicity or antibody-hapten binding (Figure S19A and B). In moving forward with

hapten **7**, optimization of vaccine formulation conditions for the dsRNA included varying the carrier protein, the delivery system (i.e., CALV liposomal delivery or alum as a depot), and combining CpG and dsRNA. Findings from the first three series (highlighted in red in Table 1) were used to guide successive series of refinement. The subsequent Series D and E were designed to focus on a specific TLR agonist and observe its response to dose ranging with alum. After establishing an optimal dose with each TLR agonist, the integrity of the vaccine was tested under various storage conditions (Series F and G).

Following behavioral assays and titer measurements of all the series, a one-way ANOVA was performed on the resulting data (Table 1). The ANOVA confirmed a significant effect of formulation conditions in the hot plate assay [$F(37, 135) = 5.851$; $p < 0.001$]. A similar result was observed for the ANOVA in the tail flick assay [$F(37,135) = 22.92$; $p < 0.001$]. A Dunnett or Tukey *post hoc* test was then used to confirm significance among the groups. In Series A-C, we observed several interesting trends pertaining to (1) RNA vs. DNA-based adjuvants, (2) carrier protein, (3) delivery vehicle, and (4) preliminary vaccine stability (Figure 2).

Comparison of CpG and dsRNA as adjuvants revealed equipotency in the context of TT as the carrier protein co-administered with alum (Figure 2A). Intriguingly, the addition of CpG to the dsRNA/TT/alum formulation did not improve efficacy (Group B2, Figure 2A), indicating that the adjuvants do not act synergistically and possibly interfere with each other's adjuvant effects.

When a non-toxic mutant of diphtheria toxin, CRM, was employed as a carrier in eliciting an immunogenic response, we found that CRM adjuvanted with dsRNA was superior in both antinociception assays, as compared to TT ($p < 0.001$, Figure 2B). However despite this increased efficacy, we opted to perform the rest of the vaccine studies with TT due to the fact that the CRM conjugate had an unfortunate tendency to precipitate upon storage.

In comparing CALV liposomes and alum, anti-heroin antibody titers were higher in alum formulations than liposomal formulations (Groups D6 and C2 found in Figures 2C and S20F, respectively). Moreover, CALV formulations (Groups A4, C1-C3 Figure 2, Table 1) were not as effective as vaccines containing alum in protecting mice from heroin-induced antinociception (Figure 2C, Table 1). A notable difference in efficacy was observed when CRM was adjuvanted with alum versus CALV liposomes, although this trend was not observed for TT. It is possible that the large disparity between the two delivery conditions may be due to the marked aggregation of Her-CRM during conjugation, which would impede subsequent encapsulation by liposomes. On the other hand, Her-TT's solubility would theoretically permit encapsulation by CALV liposomes, possibly explaining the fact that CALV Her-TT liposomes gave the same magnitude of protection against heroin compared to Her-TT adjuvanted with a low dose of alum (0.2 mg/dose). Based on the finding that CALV was moderately effective as a Her-TT adjuvant, but never superior to alum, we did not move forward with CALV in our DNA and RNA dose-ranging studies.

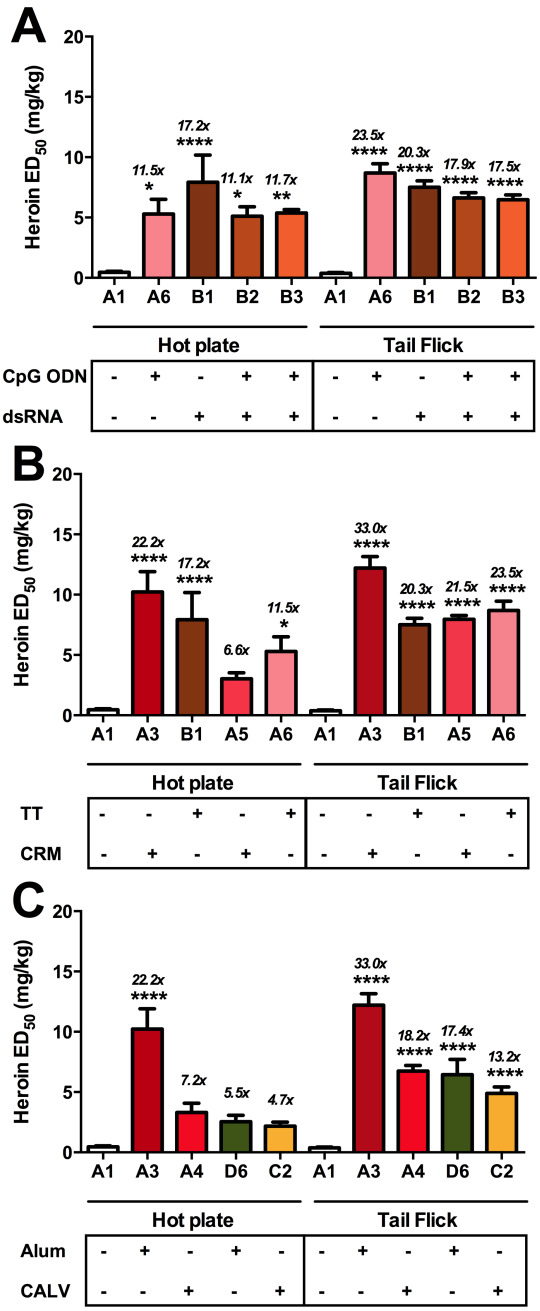


Figure 2. Effects of adjuvants and carrier proteins on heroin vaccine efficacy in antinociception assays. Differences in formulation are shown below the x-axis in each panel. **A1** is a vehicle control. Panel A shows the effects of RNA vs. DNA. All vaccines contain 50 μg Her-TT and 1 mg of alum. Only **B3** used trehalose as a cryoprotectant for lyophilization treatment, all other vaccines contained glycerol. Panel B shows the effect of carrier protein. All vaccines contain 50 μg of immunoconjugate, 1 mg of alum and glycerol. **A3** and **B1** contain 50 μg of dsRNA, and **A5** and **A6** contain 50 μg of CpG. Panel C displays the effect of alum versus CALV as delivery vehicles. All vaccines contain 50 μg dsRNA. Groups **A3** and

A4 contain 50 μg of Her-CRM, and groups **D6** and **C2** contain 50 μg of Her-TT. Italicized numbers above the bars represent the ED_{50} ratio vs. nonvaccinated control animals from control A1. A one-way ANOVA was performed for each antinociception assay, followed by a Dunnett's *post hoc* comparison test, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus control A1.

Series D and E: RNA and DNA Adjuvant Dose-Ranging with Alum

In any vaccine, the beneficial immunopotentiation of adjuvants needs to be balanced against the risk of adverse side effects. Unfortunately, potent adjuvant action is often correlated with increased toxicity, presenting as inflammation at the site of immunization. Even adjuvants used in FDA-approved vaccines like alum are known to produce inflammation at the injection site.[37,38] Preliminary assessment of toxicities in Series A-C showed occasional injection site redness and swelling, particularly in formulations containing dsRNA. Although injection site reactions are typical with alum-containing vaccines, we hypothesized that refining adjuvant dosing parameters might reduce the incidence and severity of these reactions.

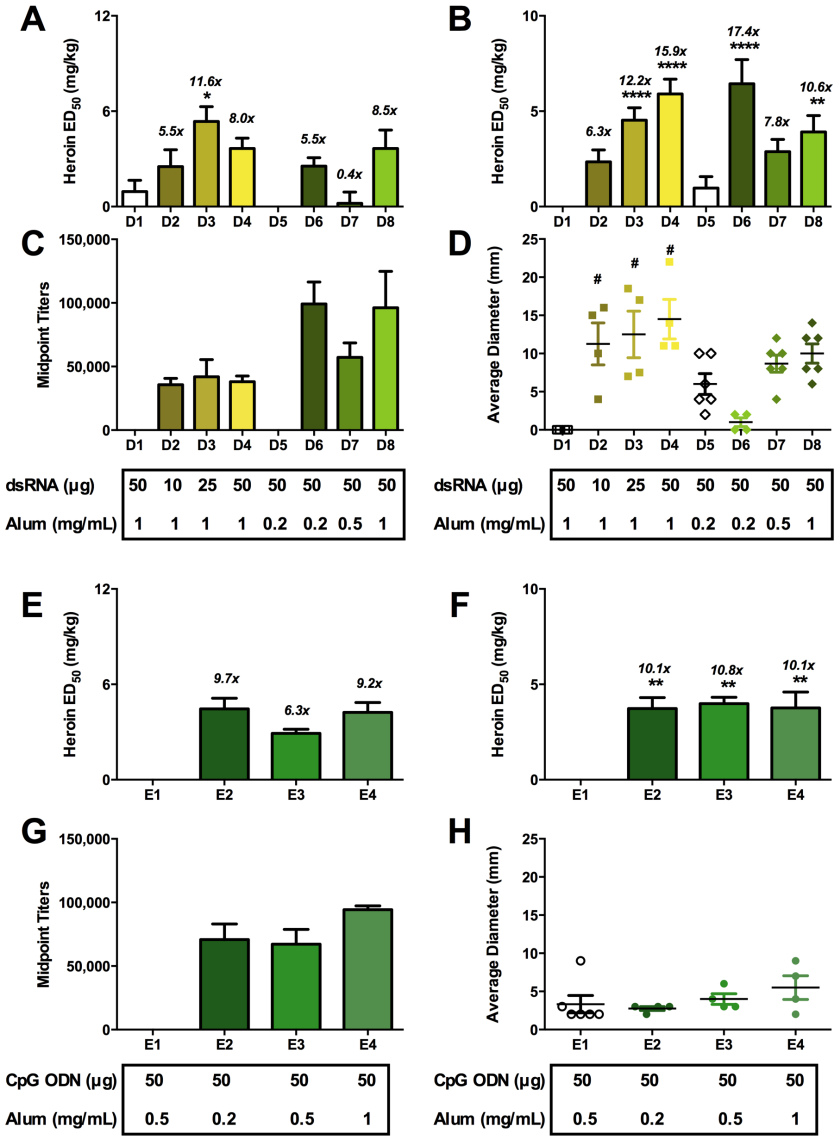


Figure 3. Dose-ranging effects of dsRNA or CpG with alum on vaccine efficacy. Differences in vaccine formulation between the groups are shown below the x-axis. All vaccines in the dsRNA series (Panels A-D) contained 50 μg of Her-TT or KLH (for controls) and glycerol. All vaccines in the CpG series (Panels E-H) contained 50 μg of Her-TT or KLH (for controls) and 50 μg CpG. Panels A and E are hot plate antinociceptive tests, Panels B and F are tail immersion tests, Panels C and G are anti-heroin midpoint titers and D and H are injection site reactions measured on the day of antinociception. Italicized numbers above the bars represent the ED_{50} ratio vs. nonvaccinated control animals from control A1. A one-way ANOVA was performed for each antinociception assay and the titer data, followed by a Dunnett's or Tukey's *post hoc* comparison test, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus control A1. $^{\#}P < 0.0001$ versus control C1.

Initial screenings of candidate formulations suggested that the preparations containing both dsRNA and alum yielded superb antibody and antinociceptive responses (Table 1). We specifically investigated different dsRNA to alum ratios in the mouse antinociception models to further refine the vaccine formulation. We hypothesized that at lower doses of alum and/or dsRNA, we might be able to lessen the severity of the injection site reactions without an appreciable loss of immunogenicity. Increasing the amount of dsRNA in vaccine formulations with 1 mg of alum (Groups D2-D4, Table 1) increased the size and/or incidence of injection site reactions. The increased inflammatory effect was also reflected in an increase in vaccine efficacy in the tail immersion response, but not in hot plate antinociception test (Figure 3A and B). However, we found that lower doses of alum (0.2 mg) dramatically reduced the injection site reactions without compromising the efficacy of the vaccine for the dsRNA series (Figure 3A, B and D). In terms of the CpG series, we found that decreasing the alum had no effect on efficacy and that CpG formulations with the lowest alum dose were still adequately efficacious (Figure 3E-F). CpG dosing was previously reported and demonstrated a positive correlation between vaccine efficacy and CpG dose with no increase in adverse reactions.[22]

Series F and G: Potency Time-Course Studies of Vaccines Under Various Storage Conditions and Time Periods

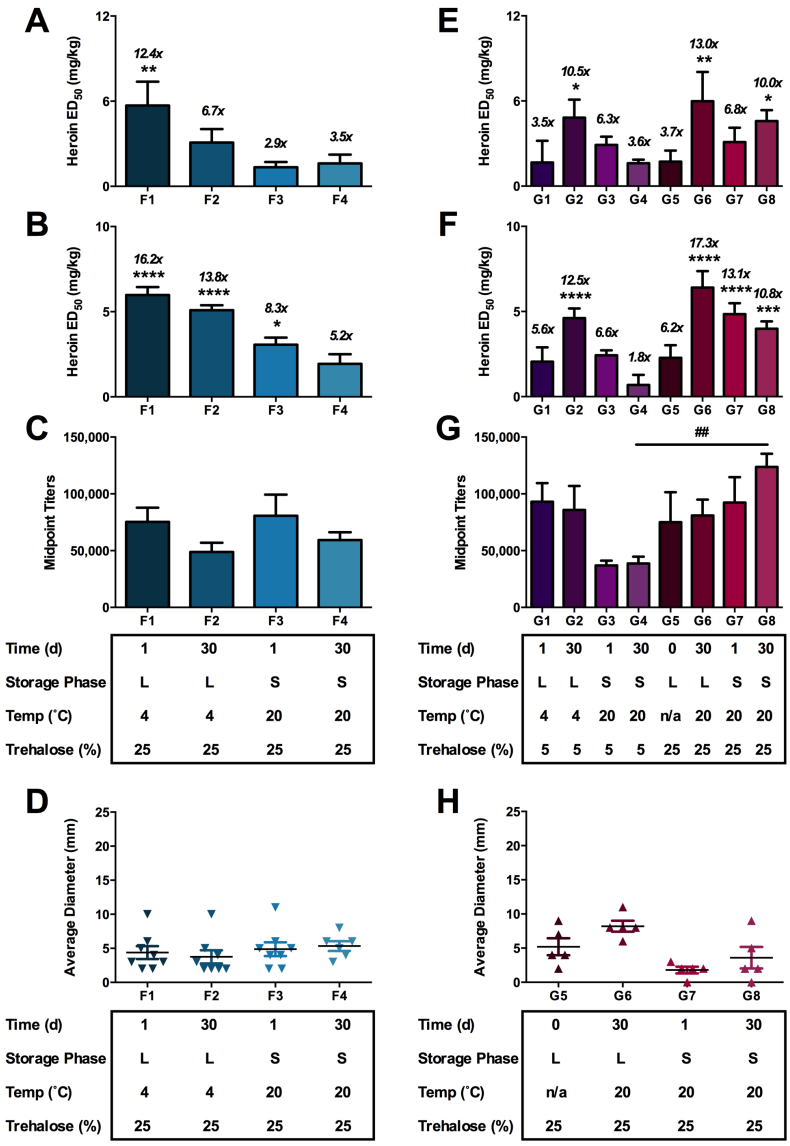


Figure 4. The stability of dsRNA + alum (A-D) and CpG + alum (E-H) vaccines under liquid and solid storage conditions over time. Differences in formulation parameters are shown below the x-axis. Vaccines in the dsRNA stability series contained 50 μg Her-TT, 0.2 mg alum, 50 μg dsRNA and 25% trehalose. Vaccines in the CpG series contained 50 μg of Her-TT, 1 mg alum, 50 μg CpG and either 5 or 25% trehalose. Panels A and E are hot plate antinociceptive tests, Panels B and F are tail immersion tests, Panels C and G are anti-heroin midpoint titers and D and H are injection site reactions measured the day of antinociception. Italicized numbers above the bars represent the ED_{50} ratio vs. nonvaccinated control animals from control A1. In the legend, L and S stand for liquid or solid,

respectively. A one-way ANOVA was performed for each antinociception assay and the titer data, followed by a Dunnett's or Tukey's *post hoc* comparison test, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$ versus control A1. $^{\#\#}P < 0.01$ Tukey's comparison test for titer between G4 and G8, which differed only in percentage of trehalose added.

Another important goal in vaccine design is achieving long-term shelf stability without loss in efficacy, typically via lyophilization. Recently, it was suggested that our hapten was unstable due to the presence of labile ester groups and subsequently was expected to exhibit a "limited shelf-life" due to undesired degradation during storage.[16] In order to examine the clinical viability of our heroin vaccine, we initiated potency studies over various time points and storage conditions to test its efficacy against heroin over time. Consequently, protection of the vaccine components against damage during the freezing and drying process is essential.[39] Trehalose can be used as an effective cryoprotectant to prevent alum aggregation during lyophilization,[40, 41] therefore we investigated the stability and efficacy of our heroin vaccines under various storage conditions in the presence of trehalose.

In a preliminary study, we tested a lyophilized vaccine formulation containing 15% w/v trehalose as a cryoprotectant (Group B3, Table 1). When immunized with the reconstituted vaccine, this group demonstrated similar efficacy to the non-lyophilized vaccine Group B2 in antinociceptive assays (Figure 2A, Table 1). This initial result prompted us to explore a broader range of conditions for each nucleotide-based adjuvant and their relative shelf stability over time. In addition, before initiating full 30-day potency time-course studies, we tested a range of trehalose concentrations with three concentrations of alum and qualitatively assessed undesired alum aggregation (Supplemental Figure S27). Chemical stability studies were also conducted on each individual and combined vaccine component to awssess for degradation under conditions, such as freezing in liquid nitrogen or lyophilization. The results of the studies are in Figure S22-28, using dosing parameters of the most promising vaccine conditions for each series. It was determined that each component was found to be relatively stable over time and under different storage conditions.

We also noted that alum was found to bind antigen in phosphate buffered saline (Figure S22-24, S26 and S28), which was not expected considering that negligible binding was observed both *Haemophilus influenza* type b and meningococcal group C conjugate vaccines in phosphate buffered saline with alum.[42] However, in the case of our heroin conjugate vaccine, alum retains its ability to bind antigen even after lyophilization and resuspension.

For both the dsRNA and CpG series, Her-TT immunoconjugate was formulated with trehalose and dsRNA or CpG, samples were initially stored in the -80 ˚C freezer, defrosted, mixed with alum and then subjected to the following storage conditions (Figure S16): (1) formulated with alum one day before injection and stored as a liquid at 4 ˚C (Groups F1 and G1); (2) formulated with alum thirty days before injection and stored as a liquid at 4 ˚C (Groups F2 and G2) or stored at room temperature (RT, Group G6); (3) formulated with alum one day before injection, lyophilized, and stored at RT (Groups F3, G3, and G7); (5) formulated with alum thirty days before injection, lyophilized, and stored at RT (Groups F4, G4, and G8, Table 1). As a negative control in the CpG series, Groups G1-G4, Table 1 were spiked with a lower amount of trehalose (>5%) to measure its effect on protection from lyophilization. On the day of injection, all lyophilized samples were resuspended in water *via* twenty minutes of vortex mixing, then administered to mice.

In interpreting the dsRNA series results, lyophilized vaccines (Groups F3 and F4) were not as effective in tail immersion and hot plate thermal nociception as compared to liquid storage for one day (Group F1, Figure 4A and B, Table 1). Samples stored for thirty days also showed modestly lower titer levels (Groups F2 and F4, Figure 4C, Table 1). These results could be explained by the possible instability of the dsRNA genome at room temperature, as cold storage (-20 to -80 ˚C) is optimal for most extracted DNA samples.[43] On the other hand, extended incubation and storage apparently enhanced efficacy for the CpG series (G Series, Figure 4E-H), possibly due to the formation of immunologically active antigen-alum aggregates during storage.[44] In assessing the effects of the cryoprotectant, liquid samples with CpG were effective regardless of the presence of trehalose over time (Group G2); however, lyophilized samples without at least 15% trehalose do not survive under storage conditions after thirty days as evidenced by reduced *in vivo* efficacy (Group G4, Figure 3E-G, Table 1). When a sufficient amount of trehalose was used in the vaccine formulations, lyophilized vaccines performed better at both one and thirty-day time points in thermal nociception assays and titer (G3 vs. G7 for one day, G4 vs. G8 for thirty day lyophilized, Figure 4E-G, Table 1). Promisingly, the efficacy of the vaccine was retained after 30 days as a liquid (G2 and G6) or when lyophilized (G8), and there was no significant difference between the samples that were lyophilized thirty days or one day prior to injection (G8 and G7, respectively, Table 1).

Lethality Challenge with Series G

Upon demonstrating that our vaccine could block substantial doses of heroin in the antinociception assay, we examined the ability of our vaccine to mitigate heroin-induced lethality. Based on the antinociceptive data for the stability studies, we defined an efficacious vaccine as a vaccine group having an $ED_{50} \geq 4.5$ mg/kg in at least one measure of thermal nociception. Using this criterion, the CpG series with 25% cryoprotectant were the most successful. Thus, vaccinated mice (n = 17) from the CpG stability studies and nonvaccinated mice (n = 5) were administered a 160 mg/kg dose of heroin and survival was measured (Figure 5A). The survival rate for the pooled efficacious vaccine group was 77% (10 of 13 mice survived), as compared to 20% survival for the nonvaccinated (1 of 5 mice survived, Figure 5B). Taken together, these results

clearly indicate that the heroin vaccine is highly effective in diminishing the effects of a lethal heroin challenge in rodents.

Table 1. Summary of vaccine formulations and results. Red section indicates the adjuvant selection studies, the blue section indicates the adjuvant and alum dosing; the green section indicates the stability studies.

Group	Vaccine	Immunoconjugate (μg/dose)[a]	Alum (mg/dose)[a]	Adjuvant (mg/dose)[a]	Cryoprotectant (w/v or v/v)[b]	Mice (/group)	Antinociception Assay[c] Hot Plate (ED_{50})	Antinociception Assay[c] Tail Flick (ED_{50})	Midpoint Titers[d] ($x10^3$)
A1	vehicle	–	1	–	glycerol	6	0.5 ± 0.1[e]	0.4 ± 0.1[e]	n.d.[f]
A2	H-CRM-RNA	50 μg Her-CRM	–	50 μg dsRNA	glycerol	4	0.6 ± 0.5	6.8 ± 0.5	6 ± 1
A3	H-CRM-Alum-RNA	50 μg Her-CRM	1	50 μg dsRNA	glycerol	4	10.2 ± 1.7	12.2 ± 0.9	21 ± 5
A4	H-CRM-CALV-RNA	50 μg Her-CRM	–	2.5 mg CALV + 50 μg dsRNA	glycerol	4	3.3 ± 0.8	6.7 ± 0.5	4 ± 2
A5	H-CRM-Alum-CpG	50 μg Her-CRM	1	50 μg CpG	glycerol	4	3.0 ± 0.5	8.0 ± 0.3	19 ± 3
A6	H-TT-Alum-CpG	50 μg Her-TT	1	50 μg CpG	glycerol	4	5.3 ± 1.2	8.7 ± 0.8	18 ± 10
B1	H-TT-Alum-RNA	50 μg Her-TT	1	50 μg dsRNA	glycerol	4	7.9 ± 2.3	7.5 ± 0.5	28 ± 3
B2	H-TT-Alum-CpG+RNA	50 μg Her-TT	1	50 μg CpG + 50 μg dsRNA	glycerol	4	5.1 ± 0.8	6.6 ± 0.4	55 ± 8
B3	H-TT-Alum-CpG+RNA-Lyo	50 μg Her-TT	1	50 μg CpG + 50 μg dsRNA	15% trehalose	6	5.4 ± 0.3	6.5 ± 0.4	46 ± 4
B4	H(s)-TT-Alum-CpG	50 μg Her(s)-TT	1	50 μg CpG	glycerol	4	5.7 ± 0.7	9.6 ± 0.4	44 ± 2
B5	(IP) H-TT-Alum-CpG	50 μg Her-TT	1	50 μg CpG	glycerol	4	9.6 ± 1.5	13.4 ± 1.2	103 ± 30
C1	H-TT-Alum-RNA-CALV	50 μg Her-TT	0.2	2.5 mg CALV + 50 μg dsRNA	glycerol	5	2.9 ± 0.8	3.2 ± 0.6	68 ± 9
C2	H-TT-RNA-CALV	50 μg Her-TT	–	2.5 mg CALV + 50 μg dsRNA	glycerol	5	2.2 ± 0.3	4.9 ± 0.5	28 ± 4
C3	H-TT-CALV	50 μg Her-TT	–	2.5 mg CALV	glycerol	5	1.0 ± 1.5	2.5 ± 0.6	15 ± 5
D1	KLH (vehicle)	50 μg KLH	1	50 μg dsRNA	glycerol	4	0.9 ± 0.7[e]	0.0 ± 0.0[e]	n.d.[f]
D2	H-TT-Alum-RNA(L)	50 μg Her-TT	1	10 μg dsRNA	glycerol	4	2.5 ± 1.1	2.3 ± 0.6	36 ± 5
D3	H-TT-Alum-RNA(M)	50 μg Her-TT	1	25 μg dsRNA	glycerol	4	5.4 ± 0.9	4.5 ± 0.7	42 ± 13
D4	H-TT-Alum-RNA(H)	50 μg Her-TT	1	50 μg dsRNA	glycerol	4	3.7 ± 0.7	5.9 ± 0.8	38 ± 4
D5	KLH (vehicle)	50 μg KLH	0.2	50 μg dsRNA	glycerol	4	0.0 ± 0.0[e]	1.0 ± 0.6[e]	n.d.[f]
D6	H-TT-Alum(L)-RNA	50 μg Her-TT	0.2	50 μg dsRNA	glycerol	4	2.6 ± 0.5	6.4 ± 1.3	99 ± 17
D7	H-TT-Alum(M)-RNA	50 μg Her-TT	0.5	50 μg dsRNA	glycerol	4	0.2 ± 0.7	2.9 ± 0.6	57 ± 11
D8	H-TT-Alum(H)-RNA	50 μg Her-TT	1	50 μg dsRNA	glycerol	4	3.7 ± 1.2	3.9 ± 0.9	96 ± 29
E1	KLH (vehicle)	50 μg KLH	0.5	50 μg CpG	–	4	0.0 ± 0.0[e]	0.0 ± 0.0[e]	n.d.[f]
E2	H-TT-Alum(L)-CpG	50 μg Her-TT	0.2	50 μg CpG	–	4	4.5 ± 0.7	3.7 ± 0.6	71 ± 12
E3	H-TT-Alum(M)-CpG	50 μg Her-TT	0.5	50 μg CpG	–	4	2.9 ± 0.3	4.0 ± 0.3	67 ± 12
E4	H-TT-Alum(H)-CpG	50 μg Her-TT	1	50 μg CpG	–	4	4.2 ± 0.6	3.8 ± 0.8	94 ± 3
F1	H-TT-Alum-RNA (1 d)	50 μg Her-TT	0.2	50 μg dsRNA	25% trehalose	5	5.7 ± 1.7	6.0 ± 0.5	75 ± 13
F2	H-TT-Alum-RNA (30 d)	50 μg Her-TT	0.2	50 μg dsRNA	25% trehalose	5	3.1 ± 0.9	5.1 ± 0.3	49 ± 8
F3	H-TT-Alum-RNA-Lyo	50 μg Her-TT	0.2	50 μg dsRNA	25% trehalose	5	1.3 ± 0.4	3.1 ± 0.4	81 ± 19
F4	H-TT-Alum-RNA-Lyo (30 d)	50 μg Her-TT	0.2	50 μg dsRNA	25% trehalose	5	1.6 ± 0.6	1.9 ± 0.6	59 ± 7
G1	H-TT-Alum-CpG (1 d, 4°C)	50 μg Her-TT	1	50 μg CpG	>5% trehalose	5	1.7 ± 1.5	2.1 ± 0.8	93 ± 16
G2	H-TT-Alum-CpG (30 d, 4 °C)	50 μg Her-TT	1	50 μg CpG	>5% trehalose	5	4.8 ± 1.3	4.6 ± 0.6	86 ± 21
G3	H-TT-Alum-CpG-Lyo	50 μg Her-TT	1	50 μg CpG	>5% trehalose	5	2.9 ± 0.6	2.4 ± 0.3	37 ± 4
G4	H-TT-Alum-CpG-Lyo (30 d)	50 μg Her-TT	1	50 μg CpG	>5% trehalose	5	1.6 ± 0.3	0.7 ± 0.6	39 ± 6
G5	H-TT-Alum-CpG (0 d)	50 μg Her-TT	1	50 μg CpG	25% trehalose	5	1.7 ± 0.8	2.3 ± 0.7	75 ± 26
G6	H-TT-Alum-CpG (30 d, RT)	50 μg Her-TT	1	50 μg CpG	25% trehalose	5	6.0 ± 2.1	6.4 ± 1.0	81 ± 14
G7	H-TT-Alum-CpG-Lyo	50 μg Her-TT	1	50 μg CpG	25% trehalose	5	3.1 ± 1.0	4.9 ± 0.6	92 ± 22
G8	H-TT-Alum-CpG-Lyo (30 d)	50 μg Her-TT	1	50 μg CpG	25% trehalose	5	4.6 ± 0.8	4.0 ± 0.4	124 ± 12

[a] The amount of each vaccine component is given as the concentration per dose per mouse, respectively.

[b] Cryoprotectant amounts are given for trehalose as w/v percentages for the total vaccine volume. In vaccines where glycerol was employed, the immunoconjugates were diluted 50% (v/v) with glycerol before being stored at -80 °C. Therefore glycerol content in total vaccine volumes ranged from 12 to 25% (v/v).

[c] Behavioral assay results are reported as the mean heroin ED_{50} (mg/kg) ± SEM for each vaccine

[d] Midpoint titers are reported as the mean anti-heroin midpoint titers ± SEM for each vaccine.

[e] The heroin ED_{50} (mg/kg) for vehicles did not exhibit antinociceptive protection. However, some controls are noted as zero due to the lack of convergence of the data through PRISM. An appropriate ED_{50} for these controls can be designated ≥ 1.0 mg/kg.

[f] Stands for not detected. Anti-heroin antibody titers were not detected in control serum.

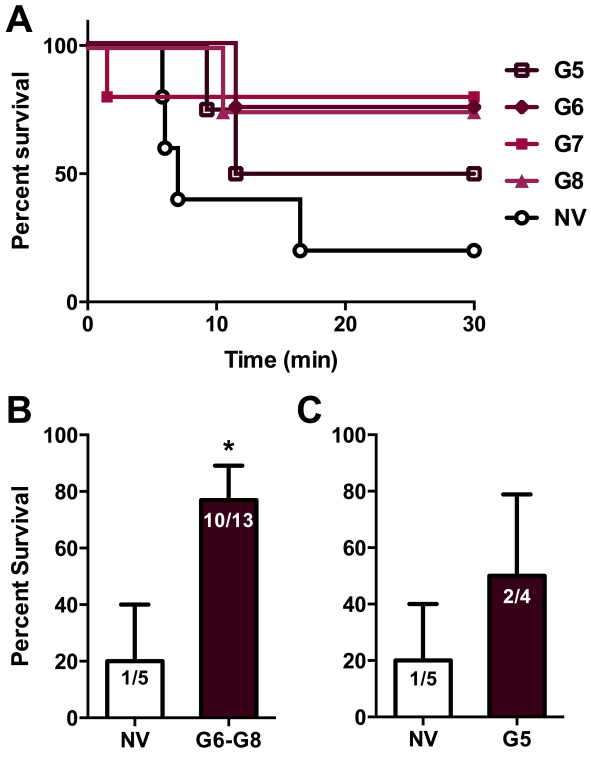
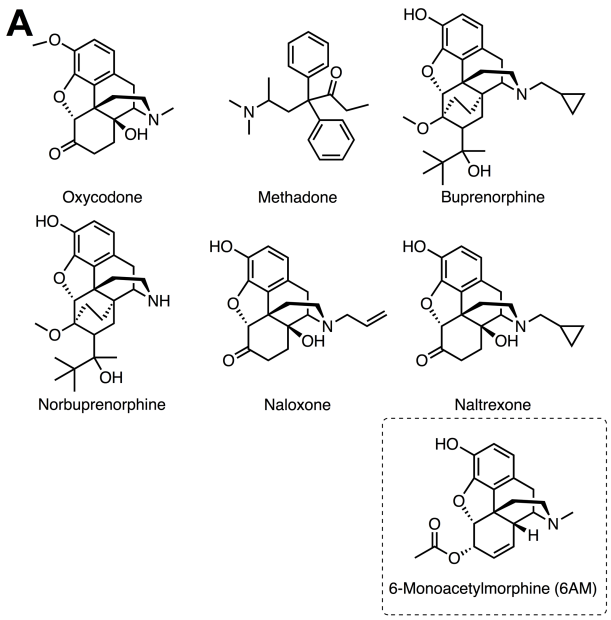


Figure 5. Efficacy of heroin vaccine against a lethal heroin challenge. All vaccines contain 50 μg Her-TT, 50 μg CpG, 1 mg alum and 25% trehalose. Panel A shows the survival curve of each vaccinated treatment group and nonvaccinated (NV, n = 5) mice challenged with a 160 mg/kg dose (i.p.) and observed for thirty minutes. Panel B shows the vaccinated mice (n = 13) from the groups that demonstrated efficacious vaccine potency in comparison to the control (n = 5). Panel C shows the vaccinated mice that did not meet our efficacy cut-off criterion (n = 4, G5) versus the nonvaccinated mice (n = 5). Nonvaccinated mice were given a 2 mg/kg dose of heroin the same day the vaccinated mice underwent antinociception assays. The lethal challenge was performed the following week. A nonparametric, unpaired Mann-Whitney U test was performed and revealed survival between the two groups were statistically significant ($P < 0.05$). Bars represent mean survival percentage ± SEM.

Cross-Reactivity of Antibodies from Group G6

A major benefit of vaccination over traditional pharmacotherapies stems from the increased duration of action of circulating antibodies and decreased side effects. The advancement of a heroin vaccine may benefit from a combination therapy with existing drugs, such as methadone or burprenorphine, to mitigate opioid cravings during cessation therapy. To test whether combination therapy was feasible with our heroin vaccine, we selected Group G6 (Table 1) from the stability series and characterized the polyclonal antibody response by SPR. Sera from Group G6 were pooled to measure the binding affinities of polyclonal antibodies in vaccinated mouse serum G6 for heroin, 6-AM, and morphine using a Biacore 3000 equipped with a Her-BSA-coated chip. Diluted mouse sera was then preincubated with serial dilutions of FDA-approved therapeutic opioids (Figure 6A) to test for potential cross-reactivity that might interfere with combination therapy.



B

Compound	Cross-Reactivity (%)
Oxycodone	0.08
Methadone	0.07
Buprenorphine	0.08
Norbuprenorphine	0.08
Naloxone	0.10
Naltrexone	0.10

Figure 6. Cross-reactivity of anti-heroin polyclonal antibodies from Group G6 to other therapeutic opioids as determined by surface plasmon resonance (SPR) binding assay. Panel A contains the structures of the relevant opioids. Panel B shows the cross-reactivity of therapeutic opioids (10 μM) compared to 6-AM on a Her-BSA-loaded sensor chip incubated with diluted mouse sera from G6. Surface plasmon resonance revealed the IC_{50} value of 6-AM for Group G6 was ~100 nM. The IC_{50} value of heroin could not be determined by SPR due to the rapid hydrolysis of heroin to 6-AM during experimental runs at 37 C for 15 minute runs per dose.

Using the SPR competition assay, it was determined that the polyclonal antibodies from G6 had a binding affinity for 6-AM corresponding to ~100 nM. As previously reported,[12, 22]

this hapten is "dynamic" making antibodies against multiple active species, including 6-AM, which is the primary mediator of heroin's psychoactivity.[45]

It can also be inferred that the formulation parameters for G6 storage as a liquid at room temperature, presents minimal 6-AM hydrolysis over 30 days in phosphate buffered saline (pH 7.4) with trehalose (25% w/v) suggesting that alum adsorption may inhibit hydrolysis. In addition, it was demonstrated that affinities for FDA-approved opioids were >1,000 times lower compared to 6-AM (Figure 6B), indicating minimal cross-reactivity to therapeutic opioids. These data suggest that Her-TT vaccinated subjects may use pharmacotherapies in tandem with vaccination.

We have examined adjuvant formulation and carrier protein in the context of our heroin vaccine in order to improve vaccine efficacy. Substituting CRM197 for TT as a carrier protein gave similar efficacy in heroin antinociception tests. Evaluation of an RNA-based adjuvant similar to TLR3 agonist poly(I:C), showed an increase in vaccine efficacy versus our previously used TLR9 adjuvant, CpG, while a combination of the two was not as effective. Furthermore, formulation of the RNA adjuvant without alum or with a liposome (CALV) showed poor vaccine efficacy. Dosing of the adjuvants with alum and dsRNA or CpG was optimized to reduce injection site reactions while maintaining vaccine efficacy. The RNA-based adjuvant in combination with a lower dose of alum was promising, while CpG was unaffected by alum dosing, so both RNA and DNA adjuvant vaccines were further explored in stability studies.

In the dsRNA stability studies, it was determined that vaccines containing dsRNA perform the best one day after formulation. Liquid dsRNA and CpG samples stored for thirty days at 4 ˚C were comparable, but the CpG vaccine stored as a liquid at RT surpassed both adjuvant samples in the measures of vaccine efficacy. In terms of lyophilized treatment, trehalose is essential for lyophilized vaccine performance. Both lyophilized CpG samples with 25% trehalose (w/v) achieved much higher ED_{50}'s than the lyophilized dsRNA samples. Therefore for our lethality challenge, we tested the CpG stability series and found that the vaccine conferred protection against a lethal dose of heroin. Based on the results of this systematic formulation assessment for vaccines against heroin abuse, the CpG + alum Her-TT formulation has demonstrated the most promise to move beyond preclinical development.

ASSOCIATED CONTENT

Supporting Information. Detailed hapten synthetic procedures and characterization data including, ^{1}H and ^{13}C NMR spectra are included in the supplemental information. Additional data include immunoconjugate characterization by MALDI-ToF, titer data, and detailed vaccination tables and corresponding schedules. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

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Notes

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ABBREVIATIONS

OPR, opioid pain reliever; 6-AM, 6-monoacetylmorphine; PAMPS, pathogen-associated molecular patterns; LPS, lipopolysaccharides; CpG ODN, cytosine-phosphodiester-guanine oligodeoxynucleotide; dsRNA, double-stranded RNA; TLR, Toll-like receptor; TT, tetanus toxoid; CRM, nontoxic mutant of diphtheria toxin; KLH, keyhole limpet hemocyanin; PBS, phosphate buffered saline; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; MALDI-ToF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; CALV, conjugatable adjuvant lipid vesicles; s.c., subcutaneous; SPR, surface plasmon resonance; Lyo, lyophilized.

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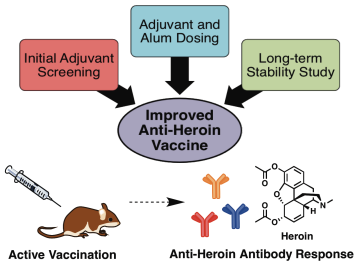
Adjuvant and Alum Dosing
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Long-term Stability Study
Improved Anti-Heroin Vaccine
Active Vaccination
Anti-Heroin Antibody Response
Heroin