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Brief Article

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Injection Route and TLR9 Agonist Addition Significantly Impact Heroin Vaccine Efficacy

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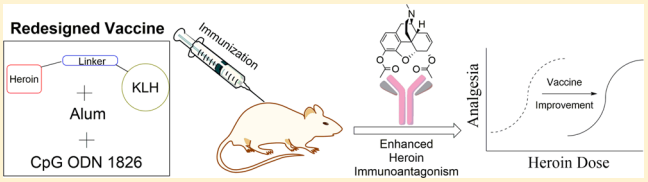
Supporting Information

ABSTRACT: Active immunization is an effective means of blocking the pharmacodynamic effects of drugs and holds promise as a treatment for heroin addiction. Previously, we demonstrated the efficacy of our first-generation vaccine in blocking heroin self-administration in rats, however, many vaccine components can be modified to further improve performance. Herein we examine the effects of varying heroin vaccine injection route and adjuvant formulation. Mice immunized via subcutaneous (sc) injection exhibited inferior anti-heroin titers compared to intraperitoneal (ip) and sc/ip coadministration injection routes. Addition of TLR9 agonist cytosine-guanine oligodeoxynucleotide 1826 (CpG ODN 1826) to the original alum adjuvant elicited superior antibody titers and opioid affinities compared to alum alone. To thoroughly assess vaccine efficacy, full dose−response curves were generated for heroin-induced analgesia in both hot plate and tail immersion tests. Mice treated with CpG ODN 1826 exhibited greatly shifted dose−response curves (10−13-fold vs unvaccinated controls) while non-CpG ODN vaccine groups did not exhibit the same robust effect (2−7-fold shift for ip and combo, 2−3-fold shift for sc). Our results suggest that CpG ODN 1826 is a highly potent adjuvant, and injection routes should be considered for development of small molecule−protein conjugate vaccines. Lastly, this study has established a new standard for assessing drugs of abuse vaccines, wherein a full dose−response curve should be performed in an appropriate behavioral task.



KEYWORDS: *vaccine, heroin, analgesia, dose−response, antibody, route of administration, adjuvant, CpG ODN*

INTRODUCTION

Heroin is a highly addictive semisynthetic opioid derived from the diacetylation of morphine. Indeed, heroin abuse is a significant problem that incurs large social and economic costs worldwide.[1,2] Alarmingly, the number of heroin users in the U.S. has grown by 50% from 2002 to 2010.[3] Furthermore, since 2007 the number of deaths due to heroin overdose has also increased.[4] Due to the severity of heroin addiction, there is a dire need for anti-heroin addiction treatments that are more effective than currently available pharmacological agents (e.g., methadone, buprenorphine, and naltrexone). The first report of a working heroin vaccine was disclosed in 1974,[5] however, further research on heroin immunopharmacotherapy has only been conducted in the past decade.[6,7] Previously, we reported the design of a heroin−KLH (keyhole limpet hemocyanin) immunoconjugate (Figure 1) that showed ample promise in combination with alum adjuvant as a vaccine against heroin addiction.[8] As a testament to the vaccine's efficacy, immunization of heroin-dependent rats prevented relapse to compulsive intake of heroin in self-administration models.[9] Herein, we sought to further improve our vaccine's performance through injection route and adjuvant exploration.

Vaccine administration route may influence immune response, due to a difference in physiological environments of the subcutaneous tissue versus the peritoneal cavity. Interestingly, no study to date has investigated injection route of small molecule conjugate vaccines. In addition to administration route, adjuvants can also impact vaccine performance. In this capacity, TLR agonists could play an important role in vaccine design with their ability to modulate immune responses. For example, CpG ODN acts as a pathogen associated molecular pattern (PAMP) to stimulate the innate immune receptor TLR9.[10] A specific CpG ODN, CpG 1826 (Figure 1), is a member of "B-class" ODNs which activate B-cell immune responses.[11] Impressively, vaccine trials engaging a specific CpG ODN sequence (#1826) in combination with alum have demonstrated its ability to safely enhance IgG antibody titers against the target antigen in mice.[12−15] Furthermore, many phase I and II clinical trials have investigated B-type CpG ODNs as vaccine adjuvants against hepatitis B,[16] malaria,[17] pneumonia,[18] melanoma,[19] and lymphoma.[20] Results from most clinical studies demonstrate CpG ODN efficacy in enhancing immunity to the target antigen while displaying a favorable safety profile. Additionally, this adjuvant is inexpensive, stable, readily obtainable and can be easily manipulated for vaccine formulation. CpG ODN 1826 therefore holds

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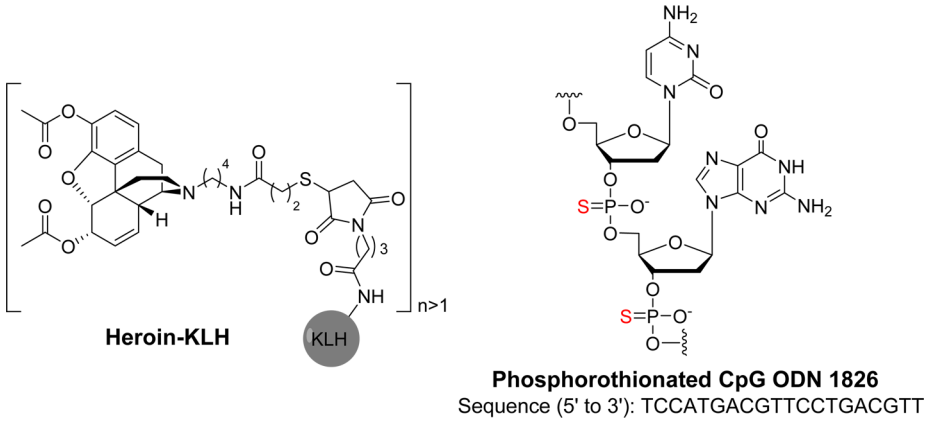

Figure 1. Heroin–KLH and CpG ODN 1826 structures.

promise as an adjunct to our heroin vaccine "cocktail" as a means of further potentiating opioid-neutralizing antibodies.

In the research we disclose herein, both injection route and TLR9 agonist CpG ODN 1826 significantly affected antibody titer levels and opiate affinity, translating to marked differences in mitigation of heroin-induced analgesia. Full heroin dose–response curves were generated in both hot-plate and tail flick tests to clearly demonstrate differences in vaccine efficacy. Our results have wide-reaching applicability for both refining and evaluating drugs of abuse vaccines.

MATERIALS AND METHODS

Animals and Vaccinations. All studies were performed in compliance with the Scripps Insitutional Animal Care and Use Committee and were in concordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 6–8 week old male Swiss Webster mice (Taconic) were immunized ip, sc, or ip/sc with 50 μg of heroin–KLH conjugate on days 0, 14, and 28. The mice were bled on days 21 and 42. Since titers were much higher on day 42 than 21, day 42 sera were used for the reported immunochemical assays. Heroin–KLH (Figure 1) was prepared via thiol–maleimide coupling. A heroin–BSA conjugate was prepared as a surrogate to quantify hapten loading by MALDI-ToF MS (4 copies per BSA, Supporting Information) as performed previously.[21] A single batch of the conjugate was used for all immunizations. Each vaccine was prepared by shaking a 1:1 v/v mixture of Her–KLH and Imject Alum (Thermo Pierce) for 30 min, and the final volume injected was 150 μL per mouse. In the oligonucleotide adjuvant group, phosphorothioated CpG ODN 1826 (Figure 1, Eurofins MWG Operon) was adsorbed to alum alongside Her–KLH and each mouse received 30 μg of the ODN per injection (ip).

ELISA. For all ELISAs, plates were coated with 250 ng/mL heroin–BSA conjugate at 0 °C overnight. A pH 6.4 PBS buffer was used throughout the assay. Titers are reported at OD_{50}. Overall titers were determined with a IgG/IgM/IgA secondary (SouthernBiotech) while IgG titers were determined with secondaries specific for IgG1 or IgG2a (Invitrogen). For competitive ELISAs the limiting dilution was found by normalizing titer curves for each treatment group (pooled sera) and choosing the dilution that produced 80% of the maximum absorbance. Competition with heroin or its primary metabolites, 6-acetylmorphine (6AM) or morphine (Cambridge Isotopes), was run in triplicate for each group. DMSO was used to solubilize the opioids with a final assay DMSO concentration of 0.5%. Generally, competitive ELISA IC_{50} values underestimate actual K_D by a factor of 10^3. Radioimmunoassay for determining K_D was not possible because of the instability of heroin.

Hot Plate and Tail Immersion Antinociceptive Testing. At day 46, mice were tested for spinal (tail immersion) and supraspinal (hot plate) antinociceptive responses,[22] both set to 54 °C. The hot plate test was measured by placing the mouse in an acrylic cylinder (14 cm diameter × 22 cm) and timing latency to perform one of the following nociceptive responses: licking of hindpaw, shaking of hindpaw, or jumping. Mice were removed from the surface immediately following response, with typical baseline latency between 8 and 15 s, and a 35 s cutoff to prevent tissue damage. The tail immersion test was administered by lightly restraining mice in a small pouch constructed from absorbent laboratory underpads and dipping 1 cm of the tip of the tail into a heated water bath, with the time to withdrawal timed. Typical baseline response was 1–2 s, and a cutoff of 10 s was imposed to prevent tissue damage. Baselines for hot plate followed by tail flick were performed, and then drug was immediately administered. Following a set time (10 min for heroin, 15 min for morphine/oxycodone) the tests were repeated, and if no full analgesic response was noted, the animals continued to receive further cumulative drug injections and repeated testing until cutoff times were reached. Drugs were tested in a counterbalanced manner with at least 3 days between drug cumulative-dosing regimens.

Computational Analysis. Computational and statistical analysis was performed in GraphPad PRISM. All values are reported as means ± SEM. Titers were determined by applying the one site fit logIC50 nonlinear regression. Normalization was performed by fitting titer curves with the one site fit logIC50 regression and then by expressing values as a percentage of the maximum absorbance. Competition curves generated at the 80% limiting dilution were first normalized and then fitted with the log(inhibitor) vs normalized response – variable slope regression to calculate IC_{50} values. Titers were compared via one-way ANOVA with Fisher's LSD post hoc comparisons.

Antinociceptive data was transformed from time to % maximum possible effect (% MPE), which is calculated as % MPE = (test − baseline)/(cutoff − baseline) × 100. This data was then fit using a log(agonist) vs normalized response nonlinear regression. These produced ED_{50} values for each drug under each pain test for the individual treatment groups, allowing calculation of potency ratios. Differences in potency

Table 1. Anti-Heroin Antibody Titers and Opioid Affinities

vaccine	anti-heroin titer	IgG1[a]	IgG2a[a]	1/2a ratio	competitive OD_{50} heroin (μM)	6AM (nM)	morphine (mM)
ip Her−KLH + alum	18,900	29,300 ± 6,000$^{\#\#}$	2,000 ± 700	14.4	11.9 ± 2.7	283 ± 67	7.30 ± 1.21
sc Her−KLH + alum	5,900	5,250 ± 1,400	620 ± 300	8.5	2.56 ± 0.34	185 ± 32	3.71 ± 0.47
ip + sc Her−KLH + alum	23,700	27,100 ± 4,000$^{\#\#}$	2,600 ± 600$^{\#}$	10.4	2.71 ± 0.34	490 ± 70	2.23 ± 0.38
ip Her−KLH + alum + CpG ODN	139,000	111,200 ± 33,900$^{*,\#\#}$	70,200 ± 32,000*	1.6	0.436 ± 0.070	30 ± 12	0.619 ± 0.090

[a]Fisher's test: *$p < 0.05$ versus non-CpG groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus sc group.

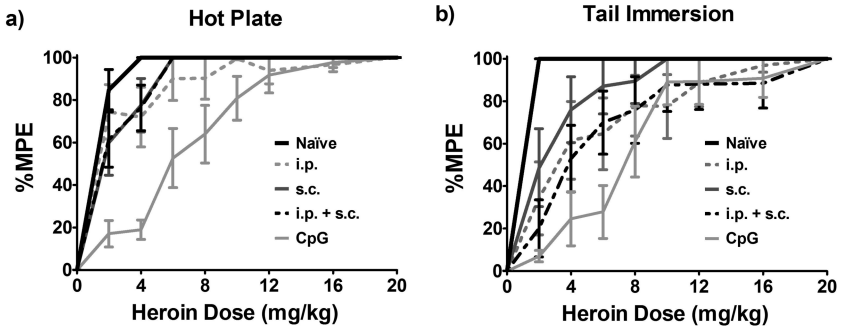

Figure 2. Cumulative heroin analgesic response in the (a) hot plate and (b) tail immersion (54 °C) antinociceptive tests. Data are expressed as % maximum possible effect, with heroin administered in 2 mg/kg cumulative intervals. Curve-fitting of data used to determine potency values in Table 2.

Table 2. Estimated ED_{50} (mg/kg; 95% Confidence Intervals) for Heroin Antinociceptive Activity in Hot Plate and Tail Immersion Tests

treatment group	hot plate (54 °C) ED_{50} (95% CI)	hot plate potency ratio[a]	tail immersion (54 °C) ED_{50} (95% CI)	tail immersion potency ratio[a]
naive	0.46 (0.33−0.64)	1.0	0.37 (0.27−0.50)	1.0
ip Her−KLH + alum	0.90 (0.55−1.45)	2.0	2.57 (1.60−4.13)	6.9$^{**,\#}$
sc Her−KLH + alum	0.87 (0.57−1.33)	1.9	0.92 (0.58−1.46)	2.5*
ip + sc Her−KLH + alum	0.86 (0.59−1.25)	1.9	3.71 (2.43−5.67)	10.0$^{***,\#}$
ip Her−KLH + alum + CpG ODN	4.36 (3.14−6.06)	9.5$^{***,\$}$	5.15 (3.51−7.57)	13.9$^{***,\#}$

[a]Tukey's test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus naive control group, $^{\$}p < 0.05$ versus all other vaccine groups, $^{\#}p < 0.05$ versus sc treatment.

were determined by one-way ANOVA followed by Tukey's post hoc test.

RESULTS

Antibody Titers and Opioid Affinities. Mice were immunized with our first generation heroin vaccine (heroin−KLH conjugate formulated with alum adjuvant), and antibody titers were measured by ELISA against heroin−BSA conjugate. A significant effect of the vaccines on antibody titers was observed [IgG1, $F_{2,24} = 7.2$, $p < 0.01$; IgG2a, $F_{2,23} = 4.8$, $p < 0.01$]. Ip and ip/sc administration of the heroin vaccine gave nearly identical titers, which were 5-fold higher compared to sc injection (Table 1). On top of a significant effect of ip administration routes compared to sc, the addition of CpG ODN 1826 further increased titers 6-fold compared to alum alone ($p < 0.05$, Fisher's tests).

Based upon competitive ELISA data, the ip group exhibited the poorest overall opioid affinities, although the ip/sc group 6AM affinity was weakest of all the groups (Table 1). In contrast, affinities for heroin, 6AM, and morphine were 2−4-fold better in the sc group. When CpG ODN 1826 was added to our existing formulation, affinities improved 27-fold for heroin, 9-fold for 6AM, and 12-fold for morphine. Statistical comparisons were not performed because affinities were determined from pooled sera.

To assess Th1 and Th2-associated humoral responses, we compared titers of anti-heroin IgG2a and IgG1, respectively. As shown previously, our heroin vaccine exhibits an exclusively Th2 immune response.[21] However, addition of CpG ODN in the current study elicited a robust Th1 humoral response as indicated by the 27-fold increase in IgG2a titers (Table 1; $p < 0.05$). Furthermore, CpG ODN enhanced the Th2-associated humoral response by boosting IgG1 titers 4-fold over alum alone ($p < 0.05$).

Antinociceptive Testing. To assess vaccine performance in blocking the analgesic effects of heroin, vaccinated animals were subjected to two antinociceptive tests, hot plate and tail immersion. Full dose−response curves and corresponding ED_{50} values were generated to thoroughly test vaccine efficacy in a wide range of heroin doses, then repeated for selectivity against morphine and oxycodone. Results in the behavioral tests generally paralleled ELISA results. The vaccines showed varying degrees of shifting the heroin antinociceptive dose−response to the right, with tail immersion being more responsive to the vaccines (Figure 2). A significant effect of vaccination was observed based on the derived estimates of heroin's

antinociceptive ED_{50} in both tests [hot plate, $F_{4,310} = 31.1$, $p < 0.001$; tail immersion, $F_{4,310} = 25.5$, $p < 0.001$]. Post hoc comparison of heroin ED_{50} values for the sc group were over 2-fold lower ($p < 0.05$) in the tail immersion assay compared to either ip or ip/sc coadministration groups (Table 2; Tukey's test). Ip and ip/sc performances were fairly similar to one another and gave ED_{50} values that were 7−10-fold better than those of naive animals ($p < 0.01$). Addition of CpG ODN 1826 increased ED_{50} values by 5-fold in hot plate ($p < 0.01$) and 2−5-fold in tail immersion ($p < 0.01$) over alum alone.

Similar antinociceptive testing was conducted for morphine as that for heroin, and a significant vaccination ANOVA was determined [hot plate, $F_{4,115} = 6.4$, $p < 0.001$; tail immersion, $F_{4,135} = 10.9$, $p < 0.001$]. Similar to previous findings, our original vaccine formulation is primarily more effective on heroin than morphine. No significant shift of morphine potency was found compared to naive, regardless of route of administration (Table S1 in the Supporting Information). However, the addition of the CpG ODN 1826 provided sufficient boosts in titer and affinity to generate a significant reduction in morphine potency, though only in the tail immersion test ($p < 0.001$). As expected, and in confirmation of the selectivity of the vaccine for heroin and its primary metabolites, no heroin vaccine treatment provided protection against oxycodone, a structurally similar opioid (Table S2 in the Supporting Information).

DISCUSSION

The current study highlights the impact of varying the injection route of our first generation heroin vaccine, along with enhancing immunogenicity by addition of the CpG ODN adjuvant. Few studies have directly compared injection routes for drugs of abuse vaccines with the exception of one study that found intradermal (id) nicotine vaccine delivery to be effective compared to intramuscular (im).[23] However, id delivery is generally not compatible with alum adjuvant: a necessary component of our heroin vaccine. Although the im route was not investigated in the current study, previous studies have shown that im is comparable to sc in terms of vaccine immunogenicity.[24]

Alum has been shown to stimulate dendritic cells (DCs) both intraperitoneally and subcutaneously through the NALP3 inflammasome, enhancing Th2 immune responses to the antigen.[25] Although a Th2 response was observed for both ip and sc administration routes, titers were 6-fold higher for ip. The difference in physiological environments between the subcutaneous tissue and peritoneal cavity may contribute to the observed discrepancy in ip versus sc immunization. The heroin−KLH immunogen in the peritoneal fluid can drain directly to lymph nodes for immune recognition,[26] however, the large particle size of the immunogen when formulated with alum prevents immediate drainage out of subcutaneous tissue.[27] Consequently, the immunogen is taken up by DCs and transported to lymph nodes to evoke an adaptive immune response.[28] According to our study, immunogenicity of our heroin−KLH immunogen is best achieved via intraperitoneal administration possibly due to more immediate drainage to lymph nodes. In sum, these findings stress the need for testing vaccination route in studying drugs of abuse vaccines, which could be especially important at the clinical level.

Previously, we reported reduced immunogenicity of our heroin vaccine in the presence of CpG ODN;[21] however, these results were derived from an oligonucleotide with native phosphodiester linkages. In the current study we employed nuclease-resistant phosphorothioate linkages between base pairs to enhance *in vivo* stability. As a result, we obtained drastically increased titers and opioid affinities similar to previous studies that showed that CpG ODN bolsters antinicotine vaccine efficacy.[23,29] Interestingly, we observed a marked increase not only in IgG2a titers but also in IgG1. In a previous study, only Th1 associated antibodies were boosted by CpG ODN 1826.[12,30] The observed dually enhanced Th1 and Th2 humoral responses highlight a unique compatibility between this vaccine "cocktail": heroin−KLH conjugate, alum and CpG ODN 1826.

Comparing titers, opioid affinities, and pain curves in the current study reveals that immunochemical assay results do not always present a clear indication of vaccine efficacy (especially vaccines against heroin that target three different opioids). While the sc immunized group exhibited higher opioid affinities and lower titers, analgesia testing demonstrated that the efficacy of the sc group was inferior to all other groups. Although the non-CpG groups appeared different in terms of titers and affinities, the hot plate test revealed no differences between the groups. The lack of efficacy in the hot plate test compared to tail immersion suggests a differential ability to block spinal reflexive analgesia, largely mediated by spinal μ-opioid receptors, versus more complex pain-related behavior of hot plate that involves peripheral, spinal, and supraspinal processing. The hot plate test has also been found to be less sensitive compared to tail flick in reducing morphine-induced analgesic properties in μ-opioid receptor deficient mice.[31] It should also be noted that, compared with previous titer and hot plate evaluation in rats using the same vaccination procedure, mice exhibit lower titer responses and less substantial shifts in heroin hot plate analgesia.[9]

The large enhancement in vaccine performance from CpG ODN suggests that, when assessing a vaccine, >10-fold greater antidrug titers and affinities must be achieved to also present improved efficacy at the behavioral level. Moreover, previous evidence demonstrates that weak blockade by drug-directed vaccines tends to actually promote increased drug seeking,[32,33] often leading to subjects adjusting dosing in order to supersede the vaccine. This necessitates rigorous behavioral testing over a wide range of doses to provide an accurate indication of vaccine efficacy in reducing drug psychoactivity.

We have shown that the enhanced antidrug titers and opioid affinities from CpG ODN translate directly to reduced pharmacodynamics of the drug; in our case heroin-induced latency to nociception (analgesia) was greatly diminished in hot plate and tail immersion tests. Previous studies have shown that when CpG ODN is used as an adjuvant in nicotine vaccines, a greater degree of drug immunoantagonism is achieved; antibodies in vaccinated animals bind a greater amount of nicotine in the blood leading to a lower concentration of nicotine in the brain.[23,29] However, it is not clear to what degree this result would manifest behaviorally. Analgesia testing is a robust method of measuring to what degree heroin is generating psychoactive effects in an animal and is therefore highly relevant to screening vaccines for immunotherapeutic antagonism.

We demonstrated previously that blunted analgesic responses to heroin in mice vaccinated with our first generation heroin vaccine corresponded to a blockade of compulsive heroin intake in rats.[8,9] The over 2-fold greater ED_{50} for heroin analgesia in the CpG ODN vaccine group over the first

generation vaccine group (alum alone) implies that CpG ODN addition to the vaccine lends a significant increase in protection from heroin addiction. In extrapolating the tail immersion test results to humans, an 80 kg adult when immunized with the standard vaccine would have to administer 207 mg heroin to experience an effect compared to 30 mg in an unvaccinated adult. When CpG ODN is added to the vaccine, an adult would have to administer 417 mg heroin to experience an equivalent effect. At this large a dose required for psychoactivity, maintaining a heroin addiction would likely become impractical both behaviorally and economically. Overall, the findings from our study are expected to facilitate the development of vaccines not only against opioids but also against other drugs of abuse.

■ ASSOCIATED CONTENT

Supporting Information

Tables S1 and S2, tabulating the antinociceptive potency of morphine and oxycodone, respectively, for the various vaccinated treatments. MALDI-ToF spectra of heroin−BSA conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

KLH, keyhole limpet hemocyanin; 6AM, 6-acetylmorphine; CpG ODN, cytosine-guanine oligodeoxynucleotide

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