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Investigating the Effects of a Hydrolytically Stable Hapten and a Th1 Adjuvant on Heroin Vaccine Performance

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Abstract

We challenged the performance of our previous heroin vaccine with a similar vaccine containing a more hydrolytically stable hapten analogue and a Th1 adjuvant (CpG ODN). Our results indicate that the elements of our previous vaccine are essential for its anti-heroin potency, i.e. a chemically labile hapten and an exclusively Th2 humoral response elicited by alum. Such design elements are critical for producing next generation heroin vaccines.

INTRODUCTION

Heroin addiction is a neuropsychiatric disorder characterized by compulsive administration of the powerful analgesic drug heroin despite negative physical, mental, legal and social consequences. Worldwide, heroin addiction causes significant detriment to society and facilitates the spread of blood-borne pathogens such as HIV, HBV and HCV.¹ Traditional pharmacotherapeutic treatment for heroin addiction consists of small molecule agonists/antagonists that blunt heroin cravings and/or withdrawal symptoms. Although these small molecule therapies are routinely employed to treat heroin addiction in the clinic, they have many disadvantages such as harmful side effects,² demand for specialized infrastructure and personnel,³ high cost⁴ and poor prevention of post-treatment drug relapse.^{5,6}

Immunopharmacotherapy (i.e. drugs of abuse vaccines) is a potentially more attractive option for the treatment of heroin addiction in comparison to pharmacotherapy. For the case at hand, a heroin vaccine will consist of a haptenic structure mirroring the opioid scaffold, which is chemically linked to an immunogenic carrier protein. When the hapten-protein conjugate is injected, the immunoconjugate elicits a humoral immune response against heroin. Polyclonal antibodies in the periphery bind the drug with a high degree of specificity precluding entry into the brain, thus blocking its psychological effects. Furthermore, formulation of the immunoconjugate with an immunostimulatory adjuvant enhances the humoral response against the target antigen. Therefore, a vaccine possessing an effective heroin-mimetic hapten and the appropriate adjuvant has great potential to effectively treat heroin addiction because in theory, it can ablate the pharmacodynamical impact of a heroin dose. Traditional treatments can only ameliorate symptoms caused by withdrawal or prevent

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ASSOCIATED CONTENT

Compound characterization spectra (¹H NMR, ¹³C NMR, LC-UV) and experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

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drug cravings, but do nothing to neutralize the drug once injected. Furthermore, drugs of abuse vaccines have a low side effect profile⁷ and can protect against drug relapse many months after immunization.⁸

In a previous study from our laboratory, we identified a heroin-KLH (keyhole limpet hemocyanin) hapten-protein immunoconjugate (**7a**, Figure 1) which, in formulation with the Th2 adjuvant alum (insoluble aluminum salts) generated high antibody titers specific for heroin and its deacetylated metabolites 6-acetylmorphine (6AM) and morphine. Additionally, heroin analgesia and self-administration studies in vaccinated rats validated the *in vivo* efficacy of our heroin vaccine.⁹ We hypothesized that the success of our vaccine is attributed to its dynamic nature: the hapten is able to mimic heroin metabolism, presenting epitopes similar to heroin, 6AM and morphine. In contrast, the deacetylated, more metabolically static version of **7a** (morphine-like hapten) elicited antibodies with poor affinity for heroin and 6AM and displayed reduced *in vivo* performance.⁹

Herein, we describe a more hydrolytically stable variation on our previous heroin hapten, in which the labile 3-acetate is replaced with a robust 3-acetamide isostere (**7b**, Figure 1). First, a vaccine embracing chemical epitope stability would test our hypothesis that a “dynamic” hapten may be essential for eliciting an anti-heroin humoral immune response. The 3-acetamide would resist hydrolysis, thus preventing the usual conversion to the deacetylated hapten. As a result, the antibody affinity profile for heroin and its metabolites may be altered drastically. Second, improved hapten stability may elevate anti-heroin antibody titers. Such was the case for a study aimed at generating a more potent anti-cocaine immune response: diester to diamide substitution led to a more potent antibody response to cocaine and showed impressive results in blunting the effects of the drug in animal models.¹⁰ Third, our previous hapten contained one critical liability involving its stability design. Under the conditions in which it is formulated (pH 7.4, PBS buffer, 25 °C) our heroin hapten in **7a** has an estimated 97 hour half-life.¹¹ As a consequence, freezing or injection of the immunoconjugate must occur immediately after preparation. Conversion of one or both of the labile esters in our hapten to amides (**7b,c,d**) would significantly increase vaccine half-life, potentially permitting long term storage.

We also sought to investigate the effect of a Th1 adjuvant (CpG ODN 1826) on heroin vaccine performance. CpG ODNs are well-studied adjuvants known to promote Th1 responses via stimulation of the innate immune receptor, TLR-9. Although Th1 immunity is associated with cell-mediated responses, it can concurrently manifest humoral responses. For example, K-type (aka B-type) CpG ODNs such as ODN 1826 induce robust B cell activation, antibody production, dendritic cell maturation and TNF- α /IL-6 secretion in cell culture.¹² In vaccine trials, CpG ODN 1826 in combination with alum (Th2 adjuvant) has shown an amazing ability to promote IgG antibody production against the target antigen (10–100 fold greater titer compared to alum alone) in both mice¹³ and humans.¹⁴ This improvement likely involves the ability of the adjuvants working in tandem to noncompetitively promote IgG isotypes associated with both Th1 and Th2 responses (IgG2a,b and IgG1 respectively).^{13a, 13c} Similarly, we applied this strategy to our heroin vaccine by formulating both CpG ODN and alum with our immunoconjugate. We hypothesize that activation of both Th1 and Th2 humoral responses may be beneficial for increasing heroin binding capacity over Th2 responses alone.

RESULTS AND DISCUSSION

To obtain the 3-acetamide analogue of our previous hapten in **7a**, we first synthesized the 3-acetamide analogue of heroin (Scheme 1). The 3-aminomorphine precursor has been reported previously,¹⁵ however, we were not able to reproduce the synthesis and therefore

developed a new, more efficient route to the 3-aza-analogue (Scheme 1). Morphine sulfate decahydrate was converted to **1** by selective triflation of the phenol with N-phenyltriflamide. Protection of the hindered C-6 alcohol was accomplished with the highly electrophilic reagent TBSOTf.¹⁶ The key step of the synthesis involved palladium-catalyzed Buchwald amination of aryl triflate **2** resulting in the replacement of the triflate with benzophenone, affording imine intermediate **3**.¹⁷ Subsequent hydrolysis of **3** with hydroxylamine HCl unveiled the aniline **4**.¹⁷ Finally, exposure of **4** to acetyl bromide accomplished concomitant desilylation and diacetylation of the 3-amino and 6-alkoxy substituents, furnishing **5**, the desired 3-aza-analog of heroin.¹⁸ Linker installment of **6** and subsequent conjugation to KLH via sulfhydryl-maleimide coupling were performed based on our previously established protocols (Experimental, Scheme 2).⁹

To compare the performance of the new hapten and adjuvant with our previous vaccine, mice were immunized with either hapten-protein conjugate **7b** or **7a** in formulation with alum or alum+CpG ODN 1826. Vaccine efficacy was measured via ELISA to assess anti-heroin antibody titers and competitive ELISA to assess antibody affinity and specificity for heroin and its metabolites.

Contrary to our hypothesis, ELISA results indicate a significant negative effect of CpG ODN 1826 on titer levels regardless of hapten structure (Table 1). Furthermore, the adjuvant seems to have a noticeable effect on antibody specificity and affinity for heroin and 6AM. ODN 1826 improved affinity for heroin and 6AM in the **7b** vaccine group, however, it caused marked reduction in 6AM affinity in the **7a** vaccine group.

We believe the CpG ODN 1826-mediated modulation of anti-heroin antibody responses involves its well documented ability to induce Th1 polarization.^{12–14} The ODN adjuvant possibly overpowered the Th2 inducing effects of alum. Given the 3–4 fold lower titer for ODN treated groups (Table 1), CpG ODN appears to reduce the overall humoral response against heroin potentially in favor of cell-mediated responses. In contrast to studies showing CpG ODN-mediated promotion of humoral responses against target proteins,^{12,13} our results indicate that this adjuvant and potentially all TLR-9 agonists are not well suited for vaccines against small molecules especially in the presence of alum. However, optimizing the dosage, vaccination schedule and molecular target of Th1 adjuvants may yield improvement in future studies.

An in depth analysis of Th1 vs. Th2 antibody isotypes revealed that CpG ODN shifted the ratio of IgG1/IgG2a titer to increase IgG2a (Th1) relative to IgG1 (Th2) in the **7a** groups (Table 1). A similar trend is observed for the **7b** groups. As expected, the ODN adjuvant favored the generation of Th1-associated antibodies although Th2-associated antibodies predominated. The shift in isotype ratio may be correlated with the shift in antibody specificities and affinities for heroin and 6AM (Table 1). In a broader sense, this result provides intriguing evidence that antibody isotype influences affinity and specificity for small molecules—a burgeoning theory that links the binding behavior of the antibody variable domain with the structure of the constant domain.¹⁹

In addition to adjuvant effects, hapten structure appears to greatly influence antibody character in accordance with our original hypothesis. Although both haptens appear equally immunogenic (similar titer levels, Table 1), 6AM affinity decreases significantly (~1000 fold) when the 3-acetamide is incorporated into the hapten. A likely explanation involves the fact that the acetamide cannot be hydrolyzed, precluding conversion to a 6AM- or morphine-like epitope, which both contain the C-3 alcohol. In contrast, 12-fold lower heroin affinities in the **7b** vaccine cannot be explained in the same manner since heroin is the parent compound in the metabolism pathway. The lower heroin affinities are likely a result

of the 3-acetamide hapten presenting a slightly altered epitope from the native heroin hapten (3-acetate) in **7a**. Although the absolute OD₅₀ values for heroin and 6AM for **7a** compare well with our previous study, morphine values do not, which may be attributable to differences between the host animals (mice herein vs. rats previously) and their carboxylesterase profiles.²⁰

Lastly, a mass spectrometric assay has confirmed the enhanced stability of our 3-acetamide hapten. In comparison to heroin, **5** has a noticeably longer half-life since the labile ester is replaced with an amide (Table 2). However, the 6-acetate of **5** is still prone to hydrolysis and therefore possesses a half-life similar to 6AM. Under basic conditions, 3-acetamido-6-acetylmorphine **5** directly converts to 3-acetamidomorphine **8** (Supporting Information). The results obtained from this study can be extrapolated to immunoconjugates **7a** and **7b**; the stability study indicates that we have in fact created a more stable heroin vaccine via 3-acetate to 3-acetamide substitution. We believe our half-life results in buffer present a suitable comparison of relative hapten stabilities which likely hold true in the various biological environments encountered by the vaccine *in vivo*. Additionally, hydrolysis of **7b** hapten directly to a 3-acetamidomorphine-like structure (dissimilar to both 6AM and morphine) explains the poor antibody affinity for 6AM, further demonstrating the need for chemical lability at the 3- position.

CONCLUSION

In this paper we have made significant progress in understanding the necessary design elements of a successful heroin vaccine. In testing the hydrolytically stable 3-acetamide immunoconjugate (**7b**) we have determined that the metabolically dynamic nature of our previous immunoconjugate (**7a**) appears to be essential for generating antibodies with high specificity and affinity for heroin and 6AM—the two key neutralization targets for blunting the addictive effects of heroin.^{9, 21} Our findings demonstrate proof of concept that blocking hapten metabolism through rational hapten design directly translates to antibody affinity for cognate, upstream drug metabolites. The overall outcome of our study indicates a clear direction for a next generation heroin vaccine.

Although our 3-acetamide vaccine displayed enhanced resistance to hydrolysis, its significantly reduced antibody affinity for 6AM limits its therapeutic potential as a heroin vaccine; however, development of a vaccine with the 6-acetamidoheroin hapten (**7c**, Figure 1) may be more successful. In contrast to **7b**, **7c** would present a labile heroin-like and a highly stable 6AM-like epitope to the immune system that may boost antibody affinity for 6AM. Synthesis of 6-acetamido-opioid analogues is well documented and consists of oxidation of the C-6 alcohol to the ketone followed by reductive amination and subsequent acetylation.²² Applying the same chemistry to **5** (Scheme 1) would result in diacetamidoheroin which can be used to create conjugate **7d**. This conjugate may be useful because it would possess maximum stability resulting in exclusive presentation of a heroin-like epitope *in vivo*.

To our knowledge, we are the first group to outfit a drugs of abuse vaccine with a Th1 CpG ODN adjuvant. Although the adjuvant reduced titer levels, it provides insight into the immunochemical mechanisms that underlie active immunization against small molecule drugs. Drugs of abuse vaccines are fundamentally different than vaccines against polyvalent bacteria or viral pathogens because they induce a robust humoral response that only serves to neutralize drug molecules. Cell-mediated responses to the drug are likely unnecessary, if not deleterious to vaccine performance. Our vaccine study suggests TLR-9 plays a role in the immunochemical mechanisms behind drugs of abuse vaccine action due the ability of CpG ODN 1826 to significantly modulate vaccine performance. CpG ODN 1826 activation

of TLR-9 may favor Th1 cell-mediated responses causing a reduction in alum-mediated Th2 humoral responses to heroin haptens. Also, CpG ODN-induced isotype switching of IgGs may alter antibody specificity and affinity profiles for heroin-like compounds; a potential example of IgG isotype switching influencing the small molecule binding profile of antibodies.

Our investigation of CpG ODN 1826 adjuvant on heroin vaccine efficacy highlights the importance of adjuvant formulation with drugs of abuse vaccines. Although haptenic variation has been heavily investigated to optimize therapeutic potency, thorough study of adjuvants in drugs of abuse vaccines has received less attention. The ability of adjuvants to polarize the immune response plays a critical role in fine tuning humoral responses to maximize drug molecule neutralization. In our case, TLR-9 stimulation appears to greatly downregulate anti-heroin humoral responses relative to alum, thus begging the question of how other TLRs influence immune response to drugs of abuse vaccines. Clearly, proper adjuvant selection and formulation is essential for the future success of drugs of abuse vaccines in combating drug addiction, and will be reported in due course.

EXPERIMENTAL SECTION

Synthetic preparation of haptens, ELISAs and mass spectrometric assay details are presented in the supporting information.

Hapten Conjugation to KLH

(Scheme 2) Trityl protected haptens **6a** and **6b** (4 mg, 5.3 Smol) were deprotected to thiols **6aSH** and **6bSH** by stirring at room temperature in a solution of DCM (0.5 mL), TIPS (100 SL) and TFA (100 SL) for 15 min. The solvent was blown off with nitrogen and the residue was placed under vacuum for 15 min. Triethylamine (50 μ L) and DCM (1 mL) were added to the residue, which was then purified by silica preparative TLC [15% MeOH/DCM to afford the thiol **6a/bSH** as a clear residue (2.0–2.2 mg, ~75%)]. To a pH 7.4 PBS solution of KLH (10 mg/mL, 350 μ L) was added sulfo-GMBS in conjugation buffer (CB) from Thermo Pierce (4.3 mM, 175 μ L). The solution was stirred for 1 h at room temperature and dialyzed at 4 °C [4 h X 5 (50 mL pH 7.4 PBS)]. Immediately following deprotection, **6a/bSH** (2.0–2.2 mg) was dissolved in a 20% DMSO/CB solution (250 μ L) and added to the maleimide-activated KLH solution. The solution was stirred for 3 h at room temperature and dialyzed at 4 °C [4 h X 3 (50 mL pH 7.4 PBS)]. Solutions of immunoconjugates **7a/b** were diluted 1:2 with glycerol and stored at –20 °C until injection. The final immunoconjugate concentrations of **7a** and **7b** were equal (1.5–1.8 mg/mL of protein in 1.8 mL buffer) as determined by both NanoDrop and BCA assay (Thermo Pierce). During the conjugation, the hapten concentration was monitored by an Ellman assay using a protocol from Thermo Pierce. Assay results reveal equal degrees of haptentation between both KLH immunoconjugates **7a** and **7b** (0.7–0.9 mmol hapten/mg KLH).

Lastly, BSA conjugates analogous to **7a** and **7b** were made in the same manner to perform MALDI-ToF mass spectrometric analysis and ELISAs. Mass spec results also show an equal degree of haptentation between the two BSA conjugates of five hapten copies per BSA protein (Supporting Information).

Immunization

Five groups of five female Swiss Webster mice (Taconic Farms; 6–8 weeks old; 25–30 g) were immunized with KLH (control), **7a**+alum, **7a**+alum+ODN, **7b**+alum or **7b**+alum+ODN. Immunoconjugates were administered i.p. in doses of 100 μ g per mouse on days 0, 14 and 28. All conjugates were formulated in a 1:1 v/v mixture of alum (Thermo Pierce)

immediately before injection. In the ODN treated groups, CpG ODN 1826 (Eurofins, 5'-TCCATGACGTTTCCTGACGTT-3') was added to the vaccine formulation (40 Sg per mouse per injection). Serum was collected at days 21 and 42. Titers were higher from the 42 day sera and used to obtain the reported data. No anti-heroin antibodies were detected in the sera of KLH control mice.

Statistics

Microsoft Excel 2007 was used for data management and for linear regression analysis of calibration curves for the mass spec stability assay. GraphPad Prism version 6 was used for calculating means $\pm$ SEMs, and for performing nonlinear regression analyses and t-tests for statistical significance. ELISA OD₅₀ values were determined by plotting log(dilution) vs. absorbance and then applying the 'one site-fit log IC₅₀' nonlinear regression. Half-life was determined by applying the 'one phase decay' exponential regression to a plot of time vs. %[5] relative to [5+8].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

KLH	keyhole limpet hemocyanin
6AM	6-acetylmorphine
ODN	oligodeoxynucleotide
GMBS	<i>N</i> -(γ -maleimidobutyryloxy) sulfosuccinimide ester)

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Figure 1.
Heroin Immunoconjugate Structures

Scheme 1.
Synthesis of 3-Acetamidoheroin

Scheme 2.
Conjugation of thiol heroin haptens to KLH

Table 1
Anti-heroin antibody titer, specificity and affinity of various immunoconjugate-adjuvant formulations

Vaccine	Ab Titer	IgG1 Titer	IgG2a Titer	1/2a Ratio	OD ₅₀ (μM)		
					Heroin	6AM	Morphine
7a +Alum	1:11,800 ± 2,000 ^a	21,800 ± 3,400	1,290 ± 40	16.9 ^g	3.44 ± 0.33 ^d	<0.391 ^{e,f}	>400
7a +Alum+ODN	1:3,840 ± 890 ^a	4,590 ± 70	480 ± 54	9.61 ^g	5.07 ± 1.48	42.9 ± 9.5 ^e	>400
7b +Alum	1:13,400 ± 1,700 ^b	14,600 ± 2,600	2,260 ± 180	6.46	42.9 ± 9.5 ^{e,d}	31.8 ± 17.1 ^f	>800
7b +Alum+ODN	1:4,750 ± 750 ^b	6,110 ± 680	1,180 ± 60	5.18	12.3 ± 3.9 ^e	2.22 ± 0.58	>800

All values are reported as means ± SEMs. Differences between values with matching letters are statistically significant (P<0.05).

Table 2

Half-lives (h) of opiates in buffer at 25 °C

pH	5	6AM ^a	Heroin ^a
7.4	stable *	stable *	96.8 ± 1.3
9.0	43.8 ± 0.4	45.9	5.9

* Half-life > 2 weeks.

^aHistorical data¹¹