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

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Ⓢ *Supporting Information*

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 cost,[4] 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 consists of a haptenic structure mirroring the opioid scaffold, which is chemically linked to an immunogenic carrier protein. When the hapten–protein conjugate is injected, it 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 drug cravings but do nothing to neutralize the drug once injected. Furthermore, drugs of abuse vaccines have a low side effect profile[7] 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
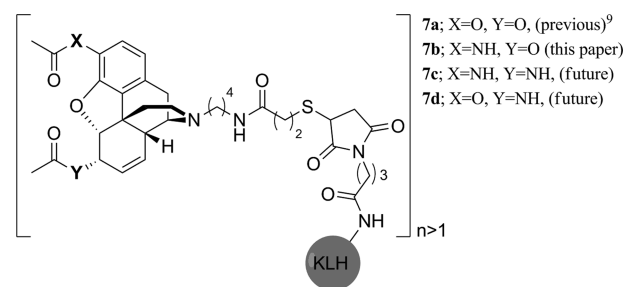

7a; X=O, Y=O, (previous)[9]
7b; X=NH, Y=O (this paper)
7c; X=NH, Y=NH, (future)
7d; X=O, Y=NH, (future)

**Figure 1.** Heroin immunoconjugate structures.

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

Scheme 1. Synthesis of 3-Acetamidoheroin
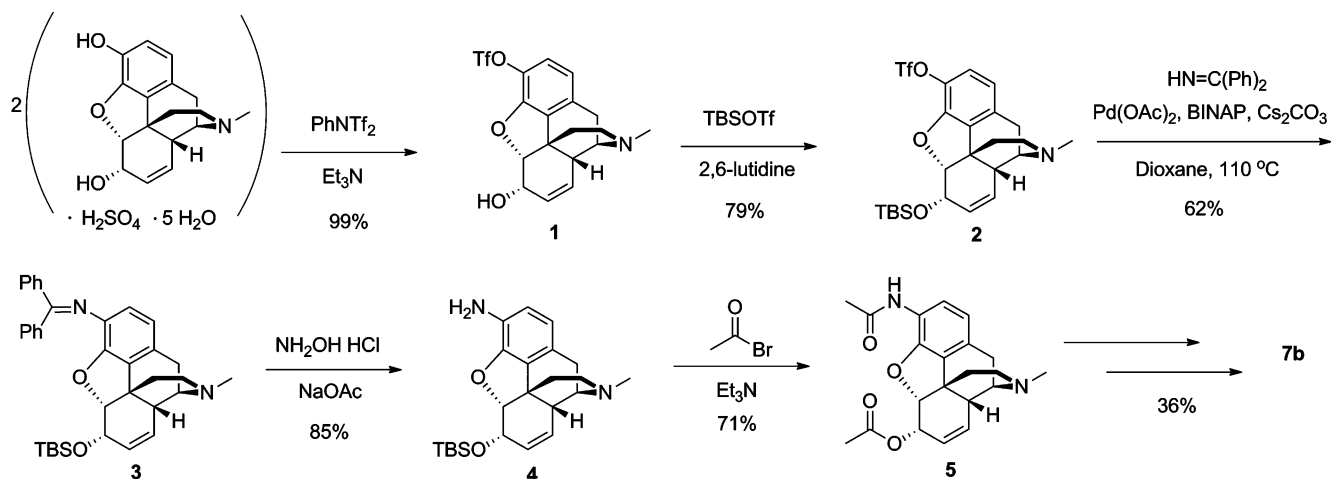
the effects of the drug in animal models.[10] Third, our previous hapten contained one critical liability involving its stability design. Under the conditions in which it is formulated (pH 7.4, PBS buffer, 25 °C), our heroin hapten in **7a** has an estimated 97 h half-life.[11] As a consequence, freezing 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 mice[13] 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,c] 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 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 to afford the 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 bisacetylation of the 3-amino and 6-alkoxy substituents, furnishing **5**, the desired 3-aza-analogue of heroin.[18] Linker installment of **5** to hapten **6** and subsequent conjugation to KLH via sulfhydryl−maleimide coupling were performed based on our previously established protocols (Supporting Information and Scheme 2).[9]

Scheme 2. Conjugation of Thiol Heroin Haptens to KLH
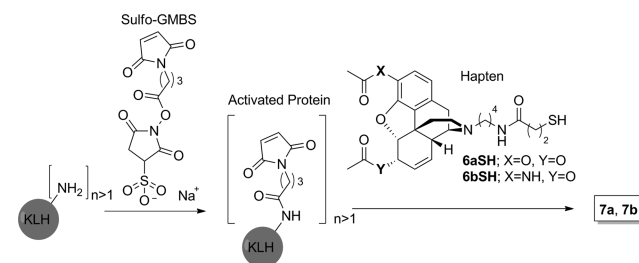
To compare the performance of the new hapten and adjuvant with our previous vaccine, mice were immunized with either hapten−protein conjugate **7b** or **7a** in formulation with alum or alum + CpG ODN 1826. Vaccine efficacy was measured by ELISA to assess anti-heroin antibody titers and competitive 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 the heroin immunoconjugate potentially in favor of cell-mediated responses. In contrast to studies showing CpG ODN-

Table 1. Anti-Heroin Antibody Titer, Specificity, and Affinity of Various Immunoconjugate-Adjuvant Formulations[a]

vaccine	IgG titer	IgG1 titer	IgG2a titer	1/2a ratio	OD$_{50}$ (μM)		
					heroin	6AM	morphine
7a + alum	11800 ± 2000 a	21800 ± 3400	1290 ± 40	16.9 g	3.44 ± 0.33 d	<0.391 e,f	>400
7a + alum + ODN	3840 ± 890 a	4590 ± 70	480 ± 54	9.61 g	5.07 ± 1.48	42.9 ± 9.5 e	>400
7b + alum	13400 ± 1700 b	14600 ± 2600	2260 ± 180	6.46	42.9 ± 9.5 c,d	31.8 ± 17.1 f	>800
7b + alum + ODN	4750 ± 750 b	6110 ± 680	1180 ± 60	5.18	12.3 ± 3.9 c	2.22 ± 0.58	>800

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

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,[19] 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.[20]

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 because 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 OD$_{50}$ 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.[21]

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 because 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.[9,22] 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.[23] 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 CpG ODN adjuvant.[24] 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

Table 2. Half-Lives (h) of Opiates in Buffer at 25 °C

pH	5	6AM[b]	heroin[b]
7.4	stable[a]	stable[a]	96.8 ± 1.3
9.0	43.8 ± 0.4	45.9	5.9

[a]Half-life >2 weeks. [b]Literature data.[11]

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 to 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 μmol) were deprotected to thiols **6aSH** and **6bSH** by stirring at room temperature in a solution of DCM (0.5 mL), TIPS (100 μL), and TFA (100 μL) for 15 min. The solvent was blown off with nitrogen, and the residue was placed under vacuum for 15 min. Triethylamine (50 μL) 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 μL) was added sulfo-GMBS in conjugation buffer (CB) from Thermo Pierce (4.3 mM, 175 μL). The solution was stirred for 1 h at room temperature and dialyzed at 4 °C [4 h × 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 μL) 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 × 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 haptenation 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 spectrometric results also show an equal degree of haptenation 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 ip in doses of 100 μg 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′-TCCATGACGTTCCTGACGTT-3′) was added to the vaccine formulation (40 μg per mouse per injection). Serum was collected at days 21 and 42. Titers were higher in the 42 day sera which was 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 ± SEMs and for performing nonlinear regression analyses and t tests for statistical significance. ELISA OD_{50} values were determined by plotting log(dilution) vs absorbance and then applying the "one site-fit log IC_{50}" 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**].

ASSOCIATED CONTENT

$\mathbf{S}$ Supporting Information

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

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

KLH, keyhole limpet hemocyanin; 6AM, 6-acetylmorphine; ODN, oligodeoxynucleotide; GMBS, N-(γ-maleimidobutyryloxy) sulfosuccinimide ester)

REFERENCES

(1) (a) Degenhardt, L.; Hall, W. Extent of illicit drug use and dependence, and their contribution to the global burden of disease. *Lancet* **2012**, *379* (9810), 55−70. (b) Garten, R. J.; Lai, S.; Zhang, J.; Liu, W.; Chen, J.; Vlahov, D.; Yu, X. F. Rapid transmission of hepatitis C virus among young injecting heroin users in Southern China. *Int. J. Epidemiol.* **2004**, *33* (1), 182−188. (c) Hien, N. T.; Giang, L. T.; Binh, P. N.; Deville, W.; van Ameijden, E. J.; Wolffers, I. Risk factors of HIV infection and needle sharing among injecting drug users in Ho Chi Minh City, Vietnam. *J. Subst. Abuse* **2001**, *13* (1−2), 45−58. (d) Samuel, M. C.; Doherty, P. M.; Bulterys, M.; Jenison, S. A. Association between heroin use, needle sharing and tattoos received in prison with hepatitis B and C positivity among street-recruited injecting drug users in New Mexico, USA. *Epidemiol. Infect.* **2001**, *127* (3), 475−84. (e) Nutt, D. J.; King, L. A.; Phillips, L. D. Drug harms in the UK: a multicriteria decision analysis. *Lancet* **2010**, *376* (9752), 1558−1565.

(2) (a) Benyamin, R.; Trescot, A. M.; Datta, S.; Buenaventura, R.; Adlaka, R.; Sehgal, N.; Glaser, S. E.; Vallejo, R. Opioid complications and side effects. *Pain Physician* **2008**, *11* (Suppl 2), S105−S120. (b) van Dorp, E. L.; Yassen, A.; Dahan, A. Naloxone treatment in

opioid addiction: the risks and benefits. *Expert Opin. Drug Saf.* **2007**, *6* (2), 125−132.

(3) Maxwell, S.; Shinderman, M. S. Optimizing long-term response to methadone maintenance treatment: a 152-week follow-up using higher-dose methadone. *J. Addictive Dis.* **2002**, *21* (3), 1−12.

(4) Mark, T. L.; Woody, G. E.; Juday, T.; Kleber, H. D. The economic costs of heroin addiction in the United States. *Drug Alcohol Depend.* **2001**, *61* (2), 195−206.

(5) Dekimpe, M. G.; Gucht, L. M. V. D.; Hanssens, D. M.; Powers, K. I. Long-Run Abstinence after Narcotics Abuse: What Are the Odds? *Manage. Sci.* **1998**, *44* (11), 1478−1492.

(6) Gossop, M.; Stewart, D.; Browne, N.; Marsden, J. Factors associated with abstinence, lapse or relapse to heroin use after residential treatment: protective effect of coping responses. *Addiction* **2002**, *97* (10), 1259−1267.

(7) Kosten, T. R.; Rosen, M.; Bond, J.; Settles, M.; Roberts, J. S. C.; Shields, J.; Jack, L.; Fox, B. Human therapeutic cocaine vaccine: safety and immunogenicity. *Vaccine* **2002**, *20* (7−8), 1196−1204.

(8) Martell, B. A.; Mitchell, E.; Poling, J.; Gonsai, K.; Kosten, T. R. Vaccine Pharmacotherapy for the Treatment of Cocaine Dependence. *Biol. Psychiatry* **2005**, *58* (2), 158−164.

(9) Stowe, G. N.; Vendruscolo, L. F.; Edwards, S.; Schlosburg, J. E.; Misra, K. K.; Schulteis, G.; Mayorov, A. V.; Zakhari, J. S.; Koob, G. F.; Janda, K. D. A vaccine strategy that induces protevtice immunity against heroin. *J. Med. Chem.* **2011**, *54*, 5195−5204.

(10) Carrera, M. R.; Ashley, J. A.; Wirsching, P.; Koob, G. F.; Janda, K. D. A second-generation vaccine protects against the psychoactive effects of cocaine. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (4), 1988−1992.

(11) Barrett, D. A.; Dyssegaard, A. L.; Shaw, P. N. The effect of temperature and pH on the deacetylation of diamorphine in aqueous solution and in human plasma. *J. Pharm. Pharmacol.* **1992**, *44* (7), 606−608.

(12) (a) Verthelyi, D.; Ishii, K. J.; Gursel, M.; Takeshita, F.; Klinman, D. M. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J. Immunol.* **2001**, *166* (4), 2372−2377. (b) Krug, A.; Rothenfusser, S.; Hornung, V.; Jahrsdorfer, B.; Blackwell, S.; Ballas, Z. K.; Endres, S.; Krieg, A. M.; Hartmann, G. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur. J. Immunol.* **2001**, *31* (7), 2154−2163. (c) Hartmann, G.; Krieg, A. M. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* **2000**, *164* (2), 944−953.

(13) (a) Brazolot Millan, C. L.; Weeratna, R.; Krieg, A. M.; Siegrist, C. A.; Davis, H. L. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95* (26), 15553−15558. (b) Weeratna, R.; Comanita, L.; Davis, H. L. CPG ODN allows lower dose of antigen against hepatitis B surface antigen in BALB//c mice. *Immunol. Cell Biol.* **2003**, *81* (1), 59−62. (c) Zakhartchouk, A. N.; Sharon, C.; Satkunarajah, M.; Auperin, T.; Viswanathan, S.; Mutwiri, G.; Petric, M.; See, R. H.; Brunham, R. C.; Finlay, B. B.; Cameron, C.; Kelvin, D. J.; Cochrane, A.; Rini, J. M.; Babiuk, L. A. Immunogenicity of a receptor-binding domain of SARS coronavirus spike protein in mice: implications for a subunit vaccine. *Vaccine* **2007**, *25* (1), 136−143.

(14) Vajdy, M.; Selby, M.; Medina-Selby, A.; Coit, D.; Hall, J.; Tandeske, L.; Chien, D.; Hu, C.; Rosa, D.; Singh, M.; Kazzaz, J.; Nguyen, S.; Coates, S.; Ng, P.; Abrignani, S.; Lin, Y. L.; Houghton, M.; O'Hagan, D. T. Hepatitis C virus polyprotein vaccine formulations capable of inducing broad antibody and cellular immune responses. *J. Gen. Virol.* **2006**, *87* (Pt 8), 2253−2262.

(15) Wentland, M. P.; Duan, W.; Cohen, D. J.; Bidlack, J. M. Selective protection and functionalization of morphine: synthesis and opioid receptor binding properties of 3-amino-3-desoxymorphine derivatives. *J. Med. Chem.* **2000**, *43* (19), 3558−3565.

(16) Corey, E. J.; Cho, H.; Rucker, C.; Hua, D. H. Studies with Trialkylsilyltriflates—New Syntheses and Applications. *Tetrahedron Lett.* **1981**, *22* (36), 3455−3458.

(17) Wolfe, J P.; Åhman, J.; Sadighi, J. P.; Singer, R. A.; Buchwald, S. L. An Ammonia Equivalent for the Palladium-Catalyzed Amination of Aryl Halides and Triflates. *Tetrahedron Lett.* **1997**, *38* (36), 6367−6370.

(18) Oriyama, T.; Oda, M.; Gono, J.; Koga, G. A One-Step and Chemoselective Conversion of Silyl-Protected Alcohols into the Corresponding Acetates. *Tetrahedron Lett.* **1994**, *35* (13), 2027−2030.

(19) Waibler, Z.; Anzaghe, M.; Konur, A.; Akira, S.; Muller, W.; Kalinke, U. Excessive CpG 1668 stimulation triggers IL-10 production by cDC that inhibits IFN-alpha responses by pDC. *Eur. J. Immunol.* **2008**, *38* (11), 3127−3137.

(20) (a) Tudor, D.; Yu, H.; Maupetit, J.; Drillet, A. S.; Bouceba, T.; Schwartz-Cornil, I.; Lopalco, L.; Tuffery, P.; Bomsel, M. Isotype modulates epitope specificity, affinity, and antiviral activities of anti-HIV-1 human broadly neutralizing 2F5 antibody. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (31), 12680−12685. (b) McLean, G. R.; Torres, M.; Elguezabal, N.; Nakouzi, A.; Casadevall, A. Isotype can affect the fine specificity of an antibody for a polysaccharide antigen. *J. Immunol.* **2002**, *169* (3), 1379−1386. (c) Cooper, L. J.; Shikhman, A. R.; Glass, D. D.; Kangisser, D.; Cunningham, M. W.; Greenspan, N. S. Role of heavy chain constant domains in antibody−antigen interaction. Apparent specificity differences among streptococcal IgG antibodies expressing identical variable domains. *J. Immunol.* **1993**, *150* (6), 2231−2242. (d) Casadevall, A.; Janda, A. Immunoglobulin isotype influences affinity and specificity. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (31), 12272−12273.

(21) (a) Hosokawa, M.; Furihata, T.; Yaginuma, Y.; Yamamoto, N.; Koyano, N.; Fujii, A.; Nagahara, Y.; Satoh, T.; Chiba, K. Genomic structure and transcriptional regulation of the rat, mouse, and human carboxylesterase genes. *Drug Metab. Rev.* **2007**, *39* (1), 1−15. (b) Satoh, T.; Hosokawa, M. The mammalian carboxylesterases: from molecules to functions. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 257−288.

(22) (a) Oldendorf, W. H.; Hyman, S.; Braun, L.; Oldendorf, S. Z. Blood−brain barrier: penetration of morphine, codeine, heroin, and methadone after carotid injection. *Science* **1972**, *178* (4064), 984−986. (b) Andersen, J. M.; Ripel, A.; Boix, F.; Normann, P. T.; Morland, J. Increased locomotor activity induced by heroin in mice: pharmacokinetic demonstration of heroin acting as a prodrug for the mediator 6-monoacetylmorphine in vivo. *J. Pharmacol. Exp. Ther.* **2009**, *331* (1), 153−161.

(23) (a) Jiang, J. B.; Hanson, R. N.; Portoghese, P. S.; Takemori, A. E. Stereochemical studies on medicinal agents. 23. Synthesis and biological evaluation of 6-amino derivatives of naloxone and naltrexone. *J. Medi. Chem.* **1977**, *20* (8), 1100−1102. (b) Fang, S.; Takemori, A. E.; Portoghese, P. S. Activities of morphinone and *N*-(cyclopropylmethyl)normorphinone at opioid receptors. *J. Med. Chem.* **1984**, *27* (10), 1361−1363. (c) Uchida, K.; Yokoshima, S.; Kan, T.; Fukuyama, T. Total synthesis of (±)-morphine. *Org. Lett.* **2006**, *8* (23), 5311−5313. (d) Majumdar, S.; Subrath, J.; Le Rouzic, V.; Polikar, L.; Burgman, M.; Nagakura, K.; Ocampo, J.; Haselton, N.; Pasternak, A. R.; Grinnell, S.; Pan, Y. X.; Pasternak, G. W. Synthesis and Evaluation of Aryl-Naloxamide Opiate Analgesics Targeting Truncated Exon 11-Associated mu Opioid Receptor (MOR-1) Splice Variants. *J. Med. Chem.* **2012**, *55* (14), 6352−6362.

(24) Since the submission of our manuscript, another example of using a CpG ODN adjuvant (1668) was reported: Chen, X.; Pravetoni, M.; Bhayana, B.; Pentel, P. R.; Wu, M. X. High immunogenicity of nicotine vaccines obtained by intradermal delivery with safe adjuvants. *Vaccine* **2012**, epub ahead of print.