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Reduced Antinociception of Opioids in Rats and Mice by Vaccination with Immunogens Containing Oxycodone and Hydrocodone Haptens

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Abstract

Prescription opioids abuse and associated deaths are an emerging concern in the USA.

Vaccination against prescription opioids may provide an alternative to pharmacotherapy. An oxycodone hapten containing a tetraglycine linker at the C6 position (6OXY(Gly)₄OH) conjugated to keyhole limpet hemocyanin (KLH) has shown early proof-of-efficacy in rodents as a candidate immunogen (6OXY(Gly)₄–KLH) for the treatment of oxycodone abuse. In this study, oxycodone-based and hydrocodone-based haptens were conjugated to KLH to generate immunogens that would recognize both oxycodone and hydrocodone. Vaccination with 6OXY(Gly)₄–KLH increased drug binding in serum, reduced drug distribution to brain, and blunted analgesia for both

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Supporting Information Characterization of serum antibody affinity, serum protein binding of oxycodone or hydrocodone in rats, evaluation of immunogen 8 conjugate conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions Marco Pravetoni designed and performed studies and wrote the manuscript. Morgan Le Naour synthesized haptens and assisted with manuscript preparation. Ashli M. Tucker assisted with ELISA and animal studies. Theresa M. Harmon performed analytical chemistry. Tara M. Hawley performed protein binding assays. Philip S. Portoghese assisted with study design, manuscript preparation, and data interpretation. Paul R. Pentel assisted with study design, manuscript preparation, and data interpretation.

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oxycodone and hydrocodone. An analogous C6-linked hydrocodone vaccine blocked hydrocodone effects but less so than 6OXY(Gly)₄-KLH. C8-Linked hydrocodone immunogens had only limited efficacy. Amide conjugation showed higher haptenation ratios and greater efficacy than thioether conjugation to maleimide activated KLH (mKLH). The 6OXY(Gly)₄-KLH vaccine may be used for treatment of prescription opioid abuse.

INTRODUCTION

Prescription opioid abuse is increasing and is now more common than heroin abuse in the USA.¹ Oxycodone, oxymorphone, and hydrocodone are the most commonly abused prescription opioids.^{1a,b,2} Treatment of prescription opioid abuse is complex because addicts may switch or transition between different prescription opioids to overcome lack of availability.³ The prevalence of prescription opioid abuse highlights the need for effective therapies.

Vaccines to treat addiction are a promising alternative to current pharmacotherapies. Addiction vaccines act by stimulating production of drug-specific antibodies that bind and retain the target drug in serum. Vaccination reduces the distribution of drug to the brain and its ability to have central effects, thus attenuating behavioral effects. Proof-of-principle of clinical efficacy has been shown with nicotine and cocaine vaccines.⁴

Several groups have developed immunogens based upon the morphine structure to block the effects of heroin and its active metabolites 6-monoacetylmorphine and morphine.⁵ Heroin/morphine vaccine efficacy has been established in animal models but has not been evaluated in humans. Fewer studies have focused on development of immunogens to target prescription opioids such as oxycodone, oxymorphone, or hydrocodone.^{5d,6}

Evaluation of a recently described oxycodone immunogen, designated as 6OXY(Gly)₄-KLH (**6**, Figure 1), showed that modifying oxycodone at the C6 position and conjugating this hapten to a carrier protein through a tetraglycine linker provided an immunogen that generates a strong immune response in mice and rats.^{5d,6b} The tetraglycine linker was more effective than a shorter succinic acid based linker.^{6b} Vaccination with **6** elicited antibodies that recognize oxycodone and its active metabolite, oxymorphone, and reduced oxycodone distribution to brain and blocked its analgesia.^{6b}

In the current study, we adopted an analogous approach and employed the C6 position for attachment of the tetraglycine linker to generate a candidate hydrocodone immunogen, designated as 6HYDROC(Gly)₄-KLH (**7**) and the C8 position to generate 8HYDROC(Gly)₄-KLH (**9**). Derivatization at the C8 position was thought to mask differences between oxycodone and hydrocodone, thus providing an immunogen that would recognize both drugs. At both positions, the thioether-maleimide conjugation method was compared to amide linkage for coupling haptens [[[17-methyl-4,5-epoxy-14-hydroxy-3-methoxy-morphinan-6-[[[(ylidene)-amino)-oxy]-N-(2-mercaptoethyl)-acetamido]morphinan (**3**) and 17-methyl-4,5-epoxy-6-one-3-methoxy-8-[thio-N-(2-mercaptoethyl)-acetamido]morphinan (**5**) to maleimide-activated KLH (mKLH). The resulting immunogens, 6OXY(S)-mKLH (**8**) and 8HYDROC(S)-mKLH (**10**) were compared to the previously characterized **6**. The effect of vaccination on the distribution of oxycodone and hydrocodone to the brain was determined in rats. Vaccine efficacy in blunting oxycodone and hydrocodone behavioral effects was determined in a test of thermal nociception in mice and rats. This study represents an early screening of oxycodone- and hydrocodone-based immunogens to identify candidate vaccines for further development. Results show that the previously described oxycodone immunogen **6** had efficacy against hydrocodone as well as

oxycodone and that the oxycodone-based haptens **1** and **3** were more effective than the hydrocodone-based haptens 17-methyl-4,5 -epoxy-3-methoxy-6-[[[(ylidene)-amino)-oxy]-*N*-(glycylglycylglycylglycine)-acetamido]morphinan (**2**), 17-methyl-4,5 -epoxy-6-one-3-methoxy-8-[thio-*N*-(glycylglycylglycylglycine)-acetamido]morphinan (**4**), and **5**.

RESULTS

Hapten Design and Synthesis

Critical parameters for the generation of hapten–protein conjugate vaccines are the derivatization site on the hapten to which the linker is attached, linker length, the conjugation method used, and the immunogenicity of the carrier protein. In prior studies, a 12 atom tetraglycine linker was found superior to a shorter linker for an oxycodone hapten.^{6b} For these reasons, this linker was used to generate tetraglycine-containing hydrocodone haptens. In general, the immunogenicity of hapten–protein conjugate vaccines is improved with a higher ratio of haptenation, so this study investigated if thiol-based maleimide conjugation would improve haptenation ratios compared to the carbodiimide method of conjugation. The synthesis of haptens **2** and **3** is shown in Scheme 1.

To generate a hydrocodone vaccine, a close analogue of our lead oxycodone hapten **1** was synthesized using hydrocodone as starting material. Conditions similar to those previously described^{6b} led to intermediate 17-methyl-4,5 -epoxy-3-methoxy-6-[[[(ylidene)-amino)-oxy]acetic acid]morphinan (**11**).⁷ Carboxylic acids **11** and **12** were then coupled to linkers Gly₄OtBu or S-tritylcysteamine⁸ using the dicyclohexylcarbodiimide/hydroxybenzotriazole (DCC/HOBt) procedure. Hydrolysis of the tertbutyl ester protecting group with trifluoroacetic acid (TFA) in dichloromethylene (DCM) produced hapten **2** in good yield (68% overall). The precursor of the sulfhydryl-containing hapten **12** was detritylated in situ by treatment with acetic acid (AcOH) and trifluoroacetic acid in dichloromethane to give the free thiol hapten **3** in a similar yield (77%). To investigate the role of the 6-keto group on the opioid scaffold, these linkers were also attached at the C8 position. Thus, the Michael addition of thioglycolic acid to codeinone⁹ (Scheme 2) allowed modification at the C8 position with retention of the 6-keto-group of the corresponding 8-substituted dihydrocodeinone (**4** and **5**).

Under these conditions, the reaction afforded a mixture of 8 - and 8 - isomers as determined by ¹H NMR. Assignment of the ¹H NMR of the mixture of - and -epimers was made possible as H1 and H5 had a slightly different chemical shift between the two isomers. Integration of the two peaks showed a ratio of 75/25 in favor of the 8 - isomer as reported in similar studies.¹⁰ The identification of the -adduct as the major isomer is consistent with the less hindered 8 position of the morphinan scaffold. As the isomers could not be separated by conventional chromatographic methods, the isomeric mixture was used for conjugation to proteins.

Conjugation of Haptens to Carrier Proteins

Haptenation ratios for the KLH conjugates could not be measured due to the large size of KLH, so conditions were standardized using bovine serum albumin (BSA) as a model carrier protein. Haptenation ratios for **1** and **4** to BSA were comparable and ranged from 17 to 21 moles of hapten per mole of carrier protein as determined by MALDI-TOF. The hapten **2** exhibited molar haptenation ratios of 12–14 bound to BSA.

Activation of BSA resulted in 15–17 maleimide per mole of BSA (mBSA), as determined by MALDI-TOF, similar to activated proteins from commercial sources and in the same range as previous reports by our and other groups.¹¹ Conjugation ratios for hapten **3** bound to

mBSA were ~5–10 and similar to previously described haptens conjugated through maleimide,^{11a} although higher haptenation ratios have been reported with a morphine hapten.^{5e} The haptenation ratio was lower than ~5 for hapten **5** conjugated to mBSA. To minimize concerns of maleimide activation efficacy, hapten **3** conjugated to commercially available mKLH was compared to immunogen **8** containing the in-house mKLH by evaluation of the effect of vaccination on analgesia in mice but was not found to be more effective.

Biological Studies

Immunogenicity and Effect of Vaccination on Oxycodone Distribution after Intravenous Drug Dosing—The immunogenicity of **6**, **8**, **9**, and **10** was first tested in rats. Vaccination elicited higher oxycodone-specific serum antibody titers generated by **6** compared to **9** (Table 1). Oxycodone-specific serum antibody titer generated from the immunogens **8** and **10** were generally lower due to apparent interference in the ELISA with use of the SH linkage. However, serum antibodies directed against immunogen **8** recognized hapten **1** and serum antibodies against immunogen **6** recognized hapten **3** (Table 1). These data suggest that immunogens **6** and **8**, despite their different linkers, generate antibodies with similar specificity. Serum antibodies against immunogens **6** and **8** showed higher affinity (lower IC₅₀) for oxycodone than antibodies directed against immunogen **9** (Supporting Information Table 1) and were the most efficient in blocking oxycodone distribution (Figure 2).

In rats immunized with either **6** or **8** immunogens, the retention of oxycodone in serum was increased compared to the unconjugated KLH control group at 5 min after the oxycodone dose ($p < 0.05$, Figure 2, upper panel). The immunogen **6** was more effective than **8** ($p < 0.05$, see Figure 2, upper panel). The immunogens **9** and **10** did not increase the serum oxycodone concentration. Both immunogens **6** and **8** significantly reduced the distribution of oxycodone to brain ($p < 0.05$, Figure 2, lower panel). The effect of vaccination on oxycodone distribution was further confirmed by the dramatically increased protein binding of oxycodone in serum from 14 ± 10 to $100 \pm 2\%$ (Supporting Information Table 2); the unbound drug concentrations were not different across groups, but there was considerable variability in serum concentrations and a trend toward lower unbound concentration in the group vaccinated with **6**.

Effect of Vaccination on Oxycodone and Hydrocodone Distribution and Antinociception after Subcutaneous Drug Dosing in Rats—Immunogen **6** was more efficient than **9** in blocking oxycodone antinociception and distribution to brain (Figure 3) and also markedly increased oxycodone protein binding in serum from 10 ± 5 to $100 \pm 3\%$ (Supporting Information Table 2). In these rats, no titers are available because trunk blood was collected by euthanasia after drug administration; the presence of oxycodone interfered with measurement of serum antibody titers by ELISA (data not shown).

In this experiment, immunogen **6** elicited serum antibody titers of 142000 ± 103000 (mean $\pm$ SD), which were significantly higher than those elicited by **7** (50000 ± 48000 , $p < 0.05$). The immunogen **6** generated a stronger blockage of hydrocodone analgesia than immunogen **7** ($p < 0.05$; Figure 3C). This finding is consistent with the greater effect of **6** on hydrocodone distribution and protein binding (Figure 3B and Supporting Information Table 2). Rats immunized with **6** showed a stronger reduction in brain oxycodone (74%, $p < 0.05$ compared to KLH control, Figure 3) than brain hydrocodone (54%, $p < 0.05$ compared to KLH control, Figure 3). Vaccination with **6** comparably attenuated both oxycodone and

hydrocodone analgesia in rats, but fentanyl analgesia was preserved (data not shown) at 82 ± 18 (mean $\pm$ SEM) MPE%.

Effect of Vaccination on Oxycodone Antinociception after Subcutaneous Drug Dosing in Mice—To investigate generality across species and using a different adjuvant and route of administration (alum, sc), the effect of vaccination with the **6**, **8**, or **9** immunogens was compared using the hot plate assay in BALB/c mice. Similar to rats, mice immunized with **6** and **8** showed a significant decrease in oxycodone behavioral effects compared to KLH controls ($p < 0.05$, Figure 4). Conjugation conditions for **8** were optimized through changes of hapten to protein ratios and evaluated for their ability to block oxycodone analgesia in mice (Supporting Information Figure 1). Conjugation conditions used a range of hapten to carrier protein ratios previously shown to be effective with a nicotine hapten conjugated through maleimide chemistry.^{11b} The effect of vaccination on blockage of oxycodone analgesia suggested optimal ratios between hapten **3** and mKLH, and the commercially available mKLH was not found superior to the in-house activated mKLH.

DISCUSSION

The main finding of this study was that haptens based on modifications of oxycodone at the C6 position produced more effective immunogens than hydrocodone haptens modified at the C6 or C8 positions for eliciting antibodies against both oxycodone and hydrocodone and in blocking their distribution to brain or their behavioral effects. Use of a (Gly)₄OH linker and amide linkage to KLH resulted in a greater efficacy probably due to higher protein haptenation ratios than thioether linkage to mKLH. These data suggest that the previously characterized immunogen **6** may be useful in treating both oxycodone and hydrocodone abuse.

Linker position and composition of hapten–protein conjugate vaccines are important determinants of their immunogenicity, but the number of haptens attached to each molecule of protein also profoundly affects immunogenicity. Haptenation ratios are not always measured when investigating or comparing conjugate vaccines, so it can be difficult to know whether an immunogen with greater immunogenicity is intrinsically more stimulating to the immune system or simply more efficiently linked to its carrier protein so that it provides a higher density of hapten epitopes. KLH is a commonly used carrier protein for small molecule haptens because of its efficacy and acceptability for human use. As KLH haptenation ratios cannot be measured by mass spectrometry because of its large size, conjugation reactions in the current study were first carried out using BSA as a carrier protein because it allows such measurement as well as optimization of conjugation conditions. We have found previously that conjugation procedures that increase the haptenation and immunogenicity of nicotine–BSA conjugates also increase the immunogenicity of the corresponding nicotine–KLH conjugates.^{11b} Haptenation ratios to BSA were therefore used as a surrogate for a direct measure of KLH haptenation.

The 8-substituted immunogen **9** had only limited efficacy in blocking oxycodone distribution and antinociception despite having a protein haptenation ratio equal to that of the more effective immunogen **6**. This is somewhat surprising because the C8 substitution was expected to generate antibodies that would recognize both oxycodone and hydrocodone by masking their C14 position. Because this immunogen was derived from an unresolvable mixture of 8 - and 8 - epimers, 25/75, respectively, the density of the active isomer on KLH may have been lower. However, it has been shown that specific antibodies can be selectively generated for each isomer of a racemic hapten mixture.¹² The immunogen **9** was also less effective than both C6-linked OXY haptens even though **8** had a substantially

lower haptenation ratio than **9**. This suggests that the 6OXY hapten structure is intrinsically more immunogenic than the 8HYDROC hapten structure and that the absence of substitution at C8 is important for preserving this immunogenicity. In fact, serum antibodies generated from the **6** and **8** immunogens recognized both haptens **1** and **3** despite the different linker composition and linker chemistry. Serum antibodies generated from the immunogens **6** and **8** did not recognize the hydrocodone-based haptens **4** and **5**, which contrasts with the in vivo data showing that the immunogen **6** blocked the distribution to brain and nociception of both oxycodone and hydrocodone. This result was unexpected, since we have previously shown that vaccination with **6** elicits serum antibody displaying high affinity for oxycodone and its metabolite oxymorphone but lower affinity for hydrocodone.^{6b} It is possible that derivatization at the C8 position masked critical binding sites for antibody recognition of hydrocodone or that characterization of vaccine-generated serum antibodies by the ELISA method may not fully predict the effect of vaccination against opioids in vivo. Similar discrepancies between ELISA data and in vivo efficacy have been recently reported for a morphine conjugate vaccine.¹³ Recognition of linker by antibodies is not a likely contributor to antibody efficacy because similar efficacy was found with immunogens utilizing either tetraglycine or the thiol-maleimide linkers. Also, we have previously shown that antibodies raised by immunogen **6** do not recognize the (Gly)₄OH linker.^{6b}

Maleimide activation of BSA resulted in a number of maleimide groups similar to that of mBSA obtained from commercial sources and in the same range as previous reports by our and other groups.^{11a,14} The hapten **3** conjugated to mBSA showed a haptenization ratio similar to previously described haptens conjugated through maleimide,^{11a} although higher haptenization ratios have been reported with a maleimide-containing morphine conjugate.^{5e} Despite a relatively low haptenation ratio, the immunogen **8** elicited an efficient immune response that blocked the distribution and the behavioral effects of oxycodone. These data suggest that if the haptenation were further improved, immunogen **8** could be an alternative to **6**.

Because the previously studied **6** was highly effective in blocking oxycodone effects in rats, the analogous conjugate **7** was anticipated to be similarly immunogenic against hydrocodone and effective in blocking its effects. The immunogen **7** blocked hydrocodone distribution and antinociception but, surprisingly, less so than **6**. The cross-reactivity of antibodies generated by **6** suggests that the presence of substitution at the 14-position, the only structural difference between oxycodone and hydrocodone, is not recognized by the antibodies generated by this immunogen. The lesser immunogenicity of the hydrocodone-based immunogen may have been due to the possibility that hapten **2** is less efficient for haptenation of KLH than hapten **1**. However, this seems less likely given that the haptenation ratio was only 10% lower.

The 12 atom (Gly)₄OH linker was used because it has been found to be suitable and superior to shorter linkers containing 0–2 glycines for an analogous morphine vaccine (not shown). The linker used for the SH immunogens was shorter than (Gly)₄OH, but its effective length is similar given that, like the carbodiimide conjugates, it attaches to mKLH through the ϵ -amino group of lysine residues. In a previous study, **6** was compared with an oxycodone immunogen containing a shorter hemisuccinate-like linker at the C6 position, and the tetraglycine linker was more effective in evoking serum antibody titers.^{6b}

Because the purpose of this study was to identify an effective hydrocodone immunogen, not all immunogens were compared in each assay. However all immunogens were compared to the lead compound **6** and by all measures this immunogen was the most effective. Prescription opioid abuse is a challenging treatment target because abusers may use different

prescription opioids at different times. A vaccine that blocks both oxycodone and hydrocodone, two of the most commonly abused prescription opioids, might be of value. These data suggest that the immunogen **6** is a reasonable candidate for generating polyspecific antibodies against both drugs and for further investigation of the merit of this therapeutic approach.

The structure of fentanyl differs substantially from that of oxycodone and hydrocodone. As expected, rats vaccinated with the conjugate **6** did not show a significant reduction in fentanyl antinociception, indicating that the efficacy of this immunogen does not extend to all opioids. This may be therapeutically useful because it would allow individuals vaccinated with this immunogen to still obtain full effect from fentanyl or other opioids if required for their medical needs. On the other hand, if fentanyl abuse should emerge as a common problem, it may be necessary to combine **6** with an immunogen specifically targeting this drug.

CONCLUSIONS

A previously described oxycodone-based immunogen, **6**, was found to generate polyspecific antibodies with activity against hydrocodone as well as oxycodone, and was more effective in doing so than hydrocodone-based immunogens. The greater efficacy of this immunogen was likely due to both its intrinsic immunogenicity and a high protein haptentation ratio. The ability of **6** to attenuate the distribution of both oxycodone and hydrocodone to brain, as well as their analgesic activity, identifies it as a useful tool for studying the potential use of vaccination to treat prescription opioid abuse.

EXPERIMENTAL SECTION

Drugs and Reagents

Opioids were obtained through the NIDA Drug Supply Program, Sigma (St. Louis, MO), and Mallinckrodt (St. Louis, MO). All drug doses and concentrations are expressed as the weight of the base.

Synthesis of Oxycodone and Hydrocodone Haptens

General Information—NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer (Bruker Daltonics, Billerica, MA). Chemical shifts are in parts per million (ppm). The assignments were made using one-dimensional ^1H and ^{13}C spectra. ESI mass spectra were recorded on a BrukerBioTof II system. Preparative high-performance liquid chromatography (HPLC) was performed using a VERSAPrep system with a Haisil column (100 C-18, 5 mm, 10 mm $\times$ 250 mm). A gradient starting from 90% CH_3CN /10% H_2O /0.1% trifluoroacetic acid (TFA) reaching 70% CH_3CN /30% H_2O /0.1% TFA was used. Flash chromatography on silica gel were performed on EM Science silica gel 60 (230–400 mesh). A gradient starting from 2% methanol/97% dichloromethane (DCM)/1% NH_4OH reaching 10% methanol/89% DCM/1% NH_4OH was used. Purity (%) was determined by reverse phase HPLC, using UV detection (254 nm), and all compounds showed purity greater than 95%. Melting points were determined on a Thomas–Hoover melting point apparatus and are uncorrected. All commercial reagents and solvents were used without further purification.

17-Methyl-4,5 α -epoxy-3-methoxy-6-[[[(ylidene)-amino)-oxy]-acetic acid]morphinan (11**)**—To a solution of hydrocodone base (300 mg, 1 mmol) in methanol (5 mL) were added pyridine (196 μL , 2 mmol) and carboxymethoxylamine hemichloride (262 mg, 1.2 mmol). The reaction was stirred at 80 $^\circ\text{C}$ for 4 h. After the solvent was removed in vacuo, the crude residue was taken up and triturated in an acetone/diethyl ether

mixture to afford **2** (97%) as a white solid. ^1H NMR (DMSO) : 1.13 (m, 1H), 1.85–1.88 (m, 2H), 2.14 (m, 1H), 2.41–2.49 (m, 2H), 2.55 (s, 3H), 2.99–3.01 (m, 2H), 3.22–3.25 (m, 2H), 3.87 (s, 3H, OCH_3), 4.47 (s, 2H), 4.94 (s, 1H), 6.64 (d, 1H, $J_{\text{H1-H2}} = 8.1$ Hz), 6.72 (d, 1H, $J_{\text{H2-H1}} = 8.1$ Hz). ESI-TOF MS calculated for $\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_3$, m/z 372.415, found 373.367 (MH) $^+$.

17-Methyl-4,5 α -epoxy-6-one-3-methoxy-8-[thioacetic acid]-morphinan (13**)**—A solution of codeinone (9 250 mg, 8.4 mmol) and thioglycolic acid (65 μL , 0.92 mmol) in anhydrous tetrahydrofuran (THF, 5 mL) was stirred at room temperature for 20 h. A solid began to form almost immediately. The suspension was filtered, the resulting white solid washed with water and diethyl ether, and dried to afford **13** (87%) as a 25/75 mixture of isomers. ^1H NMR (DMSO) (major isomer): 1.52 (m, 1H), 2.05 (m, 2H), 2.37 (s, 3H, NCH_3), 2.41–2.59 (m, 5H), 2.77 (m, 1H), 2.94 (m, 2H), 3.30 (dd, 2H, $J = 6.4$ Hz, $J = 15.4$ Hz), 3.77 (s, 3H, OCH_3), 4.93 (s, 1H), 6.65 (d, 1H, $J_{\text{H1-H2}} = 8.1$ Hz), 6.73 (d, 1H, $J_{\text{H2-H1}} = 8.2$ Hz). ^1H NMR (DMSO) (minor isomer): 1.52 (m, 1H), 2.05 (m, 2H), 2.37 (s, 3H, NCH_3), 2.41–2.59 (m, 5H), 2.77 (m, 1H), 2.94 (m, 2H), 3.30 (m, 2H), 3.77 (s, 3H, OCH_3), 4.86 (s, 1H), 6.62 (d, 1H, $J_{\text{H1-H2}} = 8.3$ Hz), 6.73 (d, 1H, $J_{\text{H2-H1}} = 8.2$ Hz). ESI-TOF MS calculated for $\text{C}_{20}\text{H}_{23}\text{NO}_5\text{S}$, m/z 389.461, found 390.258 (MH) $^+$.

General Procedure for Coupling and Deprotection of Haptens 2–5

To a solution of carboxylic acid (1 mol equiv) in *N,N*-dimethylformamide (DMF), amine (1.2 mol equiv), dicyclohexylcarbodiimide (DCC, 2.0 mol equiv), hydroxybenzotriazole (HOBt, 2 mol equiv), and catalytic *N,N*-dimethylaminopyridine (DMAP, 0.1 mol equiv) were sequentially added. After stirring at room temperature for 24 h, the reaction was evaporated and the residue was dissolved in DCM and then filtered to remove dicyclohexylurea. The resulting solution was treated with TFA (10 mol equiv) and glacial acetic acid (AcOH 10 mol equiv) when a trityl protecting group was used. After stirring at room temperature for 16 h, the solvent was removed by evaporation and the residue was purified by column chromatography on silica gel or on a reverse-phase HPLC column.

17-Methyl-4,5 α -epoxy-3-methoxy-6-[[[(ylidene)-amino)-oxy]-N-(glycylglycylglycylglycine)-acetamido]morphinan [6HYDROC-(Gly) $_4$ OH] (2**)**—Reacting amine GlyOtBu (180 mg, 0.6 mmol), acid **11** (185 mg, 0.5 mmol), DCC (205 mg, 1.0 mmol), HOBt (134 mg, 1.0 mmol), and DMAP (6 mg, 0.05 mmol), followed by treatment with TFA as reported above, gave the target compound **2**, which was purified by reverse-phase HPLC; product retention time, 9 min; white solid; yield 68%. ^1H NMR (CD_3OD) : 1.28 (m, 1H), 1.61–1.67 (m, 2H), 2.34–3.14 (m, 14H with s, 3H, NCH_3 at 2.67), 3.71 (m, 1H), 3.86 (s, 3H, OCH_3), 4.50 (s, 2H), 5.00 (s, 1H), 6.28 (t, 1H, NH, $J = 8$ Hz), 6.73 (d, 1H, $J_{\text{H1-H2}} = 8.1$ Hz), 6.82 (d, 1H, $J_{\text{H2-H1}} = 8.1$ Hz). ^{13}C NMR (DMSO) 17.82, 21.34, 28.45, 28.51, 31.64, 39.78, 41.34, 42.13, 42.33, 42.56, 42.63, 44.65, 49.37, 59.72, 70.20, 85.74, 116.74, 119.54, 126.70, 136.57, 142.40, 144.46, 156.68, 168.23, 171.0, 171.30, 171.32, 174.61; mp 176 $^\circ\text{C}$ (decomposition). Anal. Calcd for $\text{C}_{28}\text{H}_{36}\text{N}_6\text{O}_9$: C, 55.99; H, 6.04; N, 13.99. Found: C, 55.83; H, 5.97; N, 13.87. ESI-TOF MS calculated for $\text{C}_{28}\text{H}_{36}\text{N}_6\text{O}_9$, m/z 600.620, found 601.548 (MH) $^+$.

[[[17-Methyl-4,5 α -epoxy-14-hydroxy-3-methoxy-morphinan-6-[[[(ylidene)-amino)-oxy]-N-(2-mercaptoethyl)-acetamido]morphinan [6OXY(SH)] (3**)**—Reacting *S*-tritylcysteamine (121 mg, 0.38 mmol), acid **12** (122 mg, 0.31 mmol), DCC (128 mg, 0.62 mmol), HOBt (84 mg, 0.62 mmol), and DMAP (4 mg, 0.03 mmol), followed by treatment with TFA and AcOH as reported above, gave the target compound **3**, which was purified by flash chromatography on silica gel; white solid; yield 77%. ^1H NMR (DMSO) : 1.28 (m, 1H), 1.61–1.67 (m, 2H), 2.34–3.14 (m, 14H with s, 3H, NCH_3 at 2.67), 3.71 (m,

1H), 3.86 (s, 3H), 4.51 (s, 2H), 4.99 (s, 1H), 6.73 (d, 1H, $J_{H1-H2} = 8.1$ Hz), 6.82 (d, 1H, $J_{H2-H1} = 8.1$ Hz). ^{13}C NMR (DMSO) 17.96, 23.25, 26.84, 27.34, 28.17, 28.42, 41.13, 45.26, 46.28, 56.32, 65.14, 69.47, 70.34, 85.27, 115.30, 119.68, 123.56, 128.84, 142.26, 144.46, 155.83, 170.66; mp 184 °C. ESI-TOF MS calculated for $\text{C}_{22}\text{H}_{29}\text{N}_3\text{O}_5\text{S}$, m/z 447.548, found 448.370 (MH)⁺.

17-Methyl-4,5 α -epoxy-6-one-3-methoxy-8-[thio-N-(glycylglycylglycylglycine)-acetamido]morphinan [8HYDROC(Gly)₄OH] (4)—Reacting amine GlyO₄tBu (112 mg, 0.37 mmol), acid **13** (120 mg, 0.31 mmol), DCC (128 mg, 0.62 mmol), HOBt (84 mg, 0.62 mmol), and DMAP (4 mg, 0.03 mmol), followed by treatment with TFA as reported above, gave the target compound **4**, which was purified by reverse-phase HPLC; product retention time, 12 min; slightly brownish solid; yield 56%. ^1H NMR (DMSO) (major isomer): 1.53 (m, 1H), 2.05 (m, 2H), 2.32 (s, 3H, NCH₃), 2.39–2.47 (m, 5H), 2.75 (m, 1H), 2.91 (m, 2H), 3.31–3.35 (m, 10H), 3.79 (s, 3H, OCH₃), 4.95 (s, 1H), 6.62 (d, 1H, $J_{H1-H2} = 8.1$ Hz), 6.72 (d, 1H, $J_{H2-H1} = 8.1$ Hz). ESI-TOF MS calculated for $\text{C}_{28}\text{H}_{35}\text{N}_5\text{O}_9\text{S}$, m/z 617.671, found 618.723 (MH)⁺.

17-Methyl-4,5 α -epoxy-6-one-3-methoxy-8-[thio-N-(2-mercaptoethyl)-acetamido]morphinan [8HYDROC(SH)] (5)—Reacting *S*-tritylcysteamine (83 mg, 0.34 mmol), acid **13** (110 mg, 0.28 mmol), DCC (115 mg, 0.56 mmol), HOBt (76 mg, 0.56 mmol), and DMAP (4 mg, 0.03 mmol), followed by treatment with TFA and AcOH as reported above, gave the target compound **5**, which was purified by reverse-phase HPLC; product retention time, 10 min; slightly brownish solid; yield 56%. ^1H NMR (CD₃OD) (major isomer): 1.53 (m, 1H), 2.07 (m, 2H), 2.35 (s, 3H, NCH₃), 2.40–2.62 (m, 7H), 2.72–2.81 (m, 3H), 2.95 (m, 2H), 3.78 (s, 3H, OCH₃), 4.94 (s, 1H), 6.67 (d, 1H, $J_{H1-H2} = 8.1$ Hz), 6.71 (d, 1H, $J_{H2-H1} = 8.1$ Hz). ESI-TOF MS calculated for $\text{C}_{22}\text{H}_{28}\text{N}_2\text{O}_4\text{S}_2$, m/z 448.599, found 449.641 (MH)⁺.

Conjugation of 6, 7, and 9 Immunogens

Glycine linker containing haptens for use in the vaccines were conjugated to KLH using carbodiimide conjugation chemistry as described previously.^{5d,6b} For use as coating antigen in ELISA assays, haptens were conjugated to bovine serum albumin (BSA). Molar hapten:protein conjugation ratios (moles of hapten conjugated per mole of protein) for the BSA conjugates were quantitated by mass spectrometry (Reflex III, Bruker), as described previously.^{6b}

Conjugation of 8 and 10 Immunogens

Sulfhydryl-containing haptens **3** and **5** were conjugated to maleimide activated BSA (mBSA) and KLH (mKLH) as described before with minor modifications.^{11b} BSA and KLH (Thermo Scientific, Rockford, IL) were activated with sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (maleimide moles 200 $\times$:BSA moles) (sulfo-SMCC, Thermo Scientific) in degassed 50 mM phosphate buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma) for 1 h at room temperature under nitrogen (N₂) purge to avoid oxidation. Maleimide-activated proteins were purified by dialysis in degassed 0.05 mM PBS under N₂ for 4 h and stored at +4 °C. The haptens **3** and **5** were dissolved in 1 mL of degassed 50 mM PBS, 1 mM EDTA containing 15 mM TCEP (Sigma), and added to 2 mg of mBSA or mKLH in a final 2 mL reaction volume. For 6OXY(S)-mBSA, the following hapten to protein molar ratios were used to determine the optimal reaction conditions: 1000 $\times$, 825 $\times$, 496 $\times$, 331 $\times$, and 165 $\times$. For **8**, the following hapten to protein molar ratios were used to determine the optimal reaction conditions: 33500 $\times$, 11200 $\times$, 5600 $\times$, 2800 $\times$, 700 $\times$. Reactions were stirred for 2 h at room temperature in glass vials sealed with N₂, followed by dialysis in degassed 0.05 mM PBS under N₂ for 6 h

and stored at +4 °C. All **8** conjugation conditions were replicated using commercially available mKLH (Thermo Scientific) to verify the immunogenicity of in-house mKLH. Conjugation of **10** to BSA and KLH was based upon optimal conditions employed for the preparation of **8**.

Vaccination

Male Holtzman rats and BALB/c mice (Harlan Laboratories, Madison, WI) were housed with a 12/12 h standard light/dark cycle. In rats, conjugates or unconjugated KLH control were injected ip in a final volume of 0.4 mL in complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for two subsequent booster injections at 3 and 6 weeks as described.^{6b} Mice were vaccinated on days 0, 14, and 28 with 25 µg of immunogen or unconjugated protein control; vaccine was administered sc in a final volume of 0.2 mL containing alum adjuvant as described.^{5d} Blood was obtained on day 35 for serum antibody titer measurement by facial vein sampling. All experiments were conducted 7–10 days after the third immunization.

Antibody

ELISA plates were coated with 5 ng/well of BSA conjugates or unconjugated protein control in carbonate buffer at pH 9.6 and blocked with 1% gelatin. Primary antibodies were incubated with goat antirat IgG antibodies conjugated to horseradish peroxidase or rabbit antimouse IgG antibodies to measure immunized rat and mouse sera, as described previously.^{5d} Competitive binding ELISA to determine IC₅₀ was performed as described previously.^{5d}

Protein Binding of Oxycodone and Hydrocodone in Serum

Serum protein binding of oxycodone and hydrocodone were measured as described previously.^{6b} Protein binding of oxycodone was measured in five rats randomly chosen in each vaccination group receiving oxycodone sc.

Oxycodone and Hydrocodone Assay

Serum and brain drug concentrations were measured by gas chromatography coupled to mass spectrometry as previously described.^{6b} The reported drug concentrations represent the total drug (protein or antibody-bound as well as free) in each sample.

Statistical Analysis

Group differences were analyzed by one-way analysis of variance followed by Bonferroni post hoc test using Prism 5.0 (Graph Pad, La Jolla Ca).

Effects of Vaccination on Oxycodone Distribution after iv Dosing in Rats

The effects of immunization with 100 µg of the **6**, **8**, **9**, **10**, or unconjugated KLH control on oxycodone distribution were first measured in rats ($n = 5$ per group) receiving 0.1 mg/kg oxycodone administered iv. One week after the final vaccine dose, rats were anesthetized with ketamine/xylazine and an indwelling catheter was placed in their right external jugular vein. Blood was withdrawn for ELISA assays and oxycodone administered as a 10 s infusion. Rats were decapitated 5 min later and trunk blood and brain collected. The oxycodone dose was chosen to compare effects with previous reports from our laboratory.^{5d,6b}

Effect of Vaccination on Oxycodone and Hydrocodone Distribution and Antinociception after sc Dosing in Rats

The effects of immunization with 100 µg of the **6**, **9**, or KLH control on oxycodone distribution and antinociception were measured at 30 min in rats ($n = 10$ per group) receiving 2.25 mg/kg oxycodone administered sc. This dose was previously shown to elicit a near-maximal analgesic effect that was blocked by vaccination with **6**.^{6b} Hot plate antinociception tests were performed at 54 °C with 60 s cutoffs to prevent thermal damage; hind paw licks and jumps were the predetermined behavioral end points to calculate % maximal possible effect (MPE).^{6b}

In rats immunized with either 25 µg of the **6**, **7**, or KLH control ($n = 7-12$ per group), hydrocodone antinociception was assessed after administration of 6.75 mg/kg hydrocodone sc; this dose was chosen through pilot studies in naïve rats to elicit a near-maximal response. In previous studies, serum antibody titers elicited from immunogen doses of 25 and 100 µg of the **6** immunogen did not differ in rats.^{6b} After completion of the hot plate behavioral end point, trunk blood and brain were collected to measure hydrocodone concentration.

As a specificity control for the hot plate test, fentanyl antinociception was tested using a dose of 0.3 mg/kg sc, in a separate cohort of rats vaccinated with **6** or unconjugated KLH.

Evaluation of Vaccination on Oxycodone Antinociception after sc Dosing in Mice

The effects of immunization with 25 µg of either the **6**, **8**, **9**, or KLH control on oxycodone distribution, and its effect on the hot plate assay was measured in BALB/c mice ($n = 5-8$ per group) receiving 2.25 mg/kg oxycodone administered sc, the same dose as used for rats. Antinociception tests in mice were performed as with rats at 30 min. Hind paw lifts, hind paw licks and jumps were the predetermined behavioral end points to calculate the % MPE. The effect of immunization on oxycodone antinociception with the immunogen **6** was compared to several immunogen **8** conjugates to determine the most efficient sulfhydryl–maleimide conjugation conditions in BALB/c mice ($n = 5-7$ per group). Mice received 2.25 mg/kg oxycodone sc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

KLH	keyhole limpet hemocyanin
BSA	bovine serum albumin

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Figure 1.
Oxycodone, hydrocodone, and derivatized haptens.

Figure 2.

Effect of immunization on oxycodone distribution after intravenous administration in rats. Vaccine effects on (A) serum and (B) brain oxycodone after administration of 0.1 mg/kg oxycodone iv. The % values above columns indicate decreases relative to the KLH control group. Data are the mean $\pm$ SD; * $p < 0.05$ compared to KLH control; # $p < 0.05$ compared to other vaccines.

Figure 3.

Effect of immunization on oxycodone or hydrocodone distribution and antinociception after subcutaneous drug administration in rats. Vaccine effects on (A) serum concentrations, (B) brain concentrations, and (C) antinociception after administration of 2.25 mg/kg oxycodone sc or 6.75 mg/kg hydrocodone sc. The % values above columns indicate decreases relative to the KLH control group. Distribution data are the mean $\pm$ SD, while behavioral data are mean $\pm$ SEM; * $p < 0.05$ compared to KLH control; # $p < 0.05$ compared to other vaccines.

Figure 4.

Comparison of immunogens on oxycodone antinociception in mice. Vaccine effects on oxycodone analgesia, after administration of 2.25 mg/kg oxycodone sc in BALB/c mice immunized with alum adjuvant sc. The % values above columns indicate decreases relative to the KLH control group. Data are mean $\pm$ SEM; * $p < 0.05$ compared to KLH control.

Scheme 1. Synthesis of Haptens Derivatized at the C6 Position

Reactants. (a) *O*-(carboxymethyl) hydroxylamine hemichloride, pyridine, MeOH, 80 °C, 4 h; (b) (i) Gly₄OtBu, DCC/HOBt, DMAP, anh. DMF, rt, 24 h, (ii) TFA/DCM, rt, overnight; (c) (i) *S*-tritylcysteamine, DCC/HOBt, DMAP, anhyd DMF, rt, 24 h, (ii) TFA, AcOH, DCM, rt, overnight. Synthesis yields are reported as % of starting material for both haptens.

Scheme 2. Synthesis of Haptens Derivatized at the C8 Position

Reactants. (a) thioglycolic acid, THF, rt, 8 h, 87%; (b) (i) Gly₄OtBu, DCC/HOBt, DMAP, anhyd DMF, rt, 24 h, (ii) TFA/DCM, rt, overnight; (c) (i) *S*-tritylcysteamine, DCC/HOBt, DMAP, anhyd DMF, rt, 24 h, (ii) TFA, AcOH, DCM, rt, overnight. Synthesis yields are reported as % of starting material for both haptens.

Table 1Characterization of Serum Antibody Titers ($\times 10^3$) from Vaccinated Rats^a

ELISA coating immunogen	immunogen used for vaccination			
	6OXY(Gly) ₄ -KLH 6	8HYDROC(Gly) ₄ -KLH 9	6OXY(S)-mKLH 8	8HYDROC(S)-mKLH10
6OXY(Gly) ₄ -BSA	186 $\pm$ 71	77 $\pm$ 92	82 $\pm$ 6	1.0 $\pm$ 1.4
8HYDROC(Gly) ₄ -BSA	3 $\pm$ 1	76 $\pm$ 48	23 $\pm$ 31	5 $\pm$ 4
6OXY(S)-mBSA	144 $\pm$ 72	32 $\pm$ 11	18 $\pm$ 5	1.0 $\pm$ 0.4
8HYDROC(S)-mBSA	15 $\pm$ 11	2 $\pm$ 3	20 $\pm$ 21	1.5 $\pm$ 1

^aData are expressed as mean $\pm$ SD and represent serum antibody titers obtained by ELISA. Serum antibody titers from rats vaccinated with each individual immunogen (**6**, **9**, **8**, and **10**) were measured against each respective hapten bound to bovine serum albumin (BSA) or maleimide activated BSA (mBSA) as coating immunogen in the ELISA assay. Sera were measured against other conjugates to determine cross-reactivity across haptens.